การศึกษาลักษณะทางชีวเคมีและการก่อภูมิแพ้ของสารก่อภูมิแพ้ไรฝุ่น ชนิด Der p 13 ที่ผลิตในยีสต์ *Pichia pastoris*

นางสาวภัทราภรณ์ สถิตย์สุขเสนาะ

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Biochemical and immunological characterization of the house dust mite Der p 13 produced in *Pichia pastoris*

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ภัทราภรณ์ สถิตย์สุขเสนาะ : การศึกษาลักษณะทางชีวเคมีและการก่อภูมิแพ้ของสารก่อภูมิแพ้ไรฝุ่น ชนิด Der p 13 ที่ผลิตในยีสต์ *Pichia pastoris* (Biochemical and immunological characterization of the house dust mite Der p 13 produced in *Pichia pastoris*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. พญ. ณัฏฐิยา หิรัญกาญจน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. อลัน แจ็คเควท, 146 หน้า.

สารก่อภูมิแพ้ไรฝุ่นชนิด 13 อาจเป็นโปรตีนที่จับกับไขมัน ซึ่งสามารถกระตุ้นการส่งสัญญาณของระบบภูมิคุ้มกันที่ติดตัวมาแต่กำเนิด ให้เริ่มการตอบสนองของภูมิแพ้ในระยะเริ่มต้น ในการวิจัยครั้งนี้ได้ทำการศึกษาอัตราการแพ้ของสารก่อภูมิแพ้ไรฝุ่นชนิด 13 ในรูปแบบรีคอมบิเน้นท์โปรตีน รวมไปถึงความสามารถของสารก่อภูมิแพ้ชนิดนี้ในการจับกับไขมัน และความสามารถของสารก่อภูมิแพ้นี้ในการกระตุ้นเซลล์ทางเดินหายใจของมนุษย์ ลักษณะของรีคอมบิเน้นท์โปรตีนบริสุทธิ์ชนิดที่ 13 ได้นำไปศึกษาด้วยเทคนิคในการวิเคราะห์ผลการวัดสัดส่วนมวลต่อประจุ (mass spectrometry),การศึกษาโครงสร้างทุติยภูมิ (circular dichorism), เทคนิควิเคราะห์การจับกับไขมันด้วยฟลูออเรสเซนต์ (fluorescence-based lipid binding assays), และ การทำนายโครงสร้างผ่านโปรแกรมทางคอมพิวเตอร์ ลักษณะการแพ้ที่มีการสร้าง IgE และลักษณะอาการแพ้ที่อาจเกิดขึ้นต่อสารก่อภูมิแพ้ไรฝุ่นชนิด 13 ได้ถูกตรวจสอบโดยเทคนิคตรวจหาความเฉพาะเจาะจงของสารก่อภูมิแพ้โดยใช้แอนติบอดี้ (ELISA), การหลั่งของเซลล์basophil, และการกระตุ้นเซลล์ทางเดินหายใจในห้องทดลอง ซึ่งผลการทดลองจากโครงสร้างของโปรตีนและการวิเคราะห์ทางชีวฟิสิกส์ของโปรตีน ได้แสดงว่าสารก่อภูมิแพ้ไรฝุ่นชนิด 13 นี้มีโครงสร้างแบบ β barrel ประกอบกับ apolar pocket ขนาดใหญ่ ซึ่งเป็นบริเวณที่จับกับลิแกน เทคนิควิเคราะห์การจับกับไขมันด้วยฟลูออเรสเซนต์ได้สนับสนุนว่าโปรตีนมีลักษณะจำเพาะในการเลือกจับกับลิแกน ที่เป็น fatty acid ที่มีส่วนเกี่ยวข้องในโปรตีนขนส่งไขมันทั่วไป นอกจากนี้ยังพบว่าความถี่ของการแพ้ที่มีการสร้าง IgE อยู่ในระดับต่ำ (7%, n= 224) ในผู้ป่วยคนไทยที่เป็นภูมิแพ้ไรฝุ่น พอๆกับความสามารถที่จำกัดในการกระตุ้นเซลล์เบโซฟิล ส่งผลให้สารก่อภูมิแพ้ไรฝุ่นชนิด 13 ถูกจำแนกอยู่ในกลุ่มสารก่อภูมิแพ้ในระดับต่ำ แต่อย่างไรก็ตาม โปรตีนที่อาจจะมีความเกี่ยวของกับไขมันสามารถกระตุ้นการสร้างของไซโตไคน์ IL-8 และ GM-CSF ในเซลล์ทางเดินหายใจผ่านทาง TLR2, MyD88-, NF-kB- and MAPK-dependent signaling pathway ถึงแม้ว่าสารก่อภูมิแพ้ไรฝุ่นชนิด 13 จะอยู่ในกลุ่มสารก่อภูมิแพ้ในระดับต่ำ แต่ทว่าความสามารถของสารก่อภูมิแพ้ต่อการจับของไขมัน มีบทบาทสำคัญในกระบวนการเริ่มต้นของอาการแพ้ไรฝุ่นผ่านทาง TLR2.

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The house dust mite (HDM) allergen Der p 13 could be a lipid-binding protein able to stimulate the airway epithelium through activation of key innate signaling pathways involved in the initiation of the allergic response. We investigated the IgE reactivity of recombinant Der p 13 (rDer p 13), its lipid binding activities and its capacity to stimulate airway epithelium cells. Purified rDer p 13 was characterized by mass spectrometry, circular dichroism, fluorescence-based lipid binding assays and *in-silico* structural prediction. IgE binding activity and allergenic potential of Der p 13 were examined by ELISA, basophil degranulation assays and *in-vitro* airway epithelial cell activation assays. The results from protein modeling and biophysical analysis indicated that Der p 13 adopts a β barrel structure forming a predominately apolar pocket representing a potential binding site for hydrophobic ligands. Fluorescent lipid binding assays confirmed that the protein is highly selective for ligands and that it binds a fatty acid with a dissociation constant typical of lipid transporter proteins. The low IgE binding frequency (7%, n= 224) in Thai HDM-allergic patients as well as the limited propensity to activate basophil degranulation classifies Der p 13 as a minor HDM allergen. Nevertheless, the protein with its presumptively associated lipid(s) triggered the production of IL-8 and GM-CSF in respiratory epithelial cells through a TLR2-, MyD88-, NF-kB- and MAPK-dependent signaling pathway. Although a minor allergen, Der p 13 may, through its lipid binding capacity, play a role in the initiation of the HDM allergic response through TLR2 activation.

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Student's Signature .....
Advisor's Signature .....
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LIST OF ABBREVIATIONS

AA	amino acid
APCs	Antigens Presenting Cells
Al (OH3)	Aluminium hydroxide
ANS	1-anilinonapthalene-8 sulfonate
ASM	airway smooth muscle
BALB/C	Bagg Albino laboratory mouse strain
BCIP	5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt
BEAS-2B	bronchial epithelial cell line
bis-ANS	4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid
BMGY	Buffered Glycerol-Complex Medium
BMMY	Buffered Methanol-Complex Medium
Bodipy® FL C16	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3 hexadecanoic acid
BSA	Bovine Serum Albumin
CaCl2	Calcium Chloride
CD	Circular Dichroism
cDNA	complementary DNA
CLRs	C-type Lectin Receptors
cm	centimeter
CO2	Carbon Dioxide
cPnA	cis-parinaric acid
ºC	Celsius
DACA	dansyl-DL-α-aminocaprylic acid
DAUDA	11-([5-dimethylaminonaphthalene-1 sulfonylamino]) undecanoic acid

DCs	Dendritic cells
DHE	dihydroergosterol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECM	extracellular matrix
FABP	Fatty acid binding protein
Fc**ε**RI	Fc epsilon receptor 1
FP	Fecal pellet
GM-CSF	Granulocyte macrophage colony stimulating factor
hr	hour
HCl	Hydrochloric acid
HDM	House Dust Mite
Hz	Hertz
IgE	Immunoglobulin E
IL	Interleukin
ILC	Innate Lymphoid Cell
IRB	Institutional Review Board
KCl	Potassium Chloride
Kd	Dissociate Constant
kDa	kilo Dalton
LPS	lipopolysaccharide
M	Molarity
MB	Mite Bodies
MD-2	Myeloid differentiation-2
m/z	Mass to-charge ratio
mg	milligram
MgCl2	Magnesium Chloride

MgSO4	Magnesium Sulfate
min	minute
mL	milliliter
mM	millimolar
NaCl	Sodium Chloride
NBT	Nitrotetrazolium Blue chloride
ng	nanogram
NLRs	NOD-like receptors
NMR	Nuclear magnetic resonance
OD	Optical Density
PAGE	Polyacrylarmide Gel Electrophoresis
PAMPs	Pathogen-Associated Molecular Patterns
PB	polymyxin B
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDB	Protein Databank
Phe	Phenylalanine
pg	pictogram
RBL SX-38	Rat basophil leukemia cells expressing humanFc**ε**R1 receptor
RLRs	RIG-like receptors
SDS	Sodium Dodecyl Sulfate
TH1	T-Helper 1
TH2	T-Helper 2
TLRs	Toll-like receptors
TSLP	Thymic Stromal Lymphopoietin
U	Units
µg	microgram
µL	microliter

YPD	Yeast Peptone Dextrose
3D	three dimensional

CHAPTER I

INTRODUCTION

House dust mites (HDM; *Dermatophagoides sp.*) are one of the commonest sources of airborne allergens worldwide (1-4). HDM sensitizations affect more than 20% of the population from industrialized countries and such level of allergic response is also measured in Thailand (5-7). The exposure to HDM allergens occurs through the airways as well as the skin, following contacts with mite bodies and fecal particles adsorbed onto dust particles (8-12). The HDM allergic response is a strong inflammatory response orchestrated by the HDM allergen-specific Th2 cells leading to the typical clinical manifestations as allergic rhinitis and asthma together with atopic dermatitis (AD) (13).

In recent years, numerous studies clearly highlighted that the activation of innate immune signaling pathways at the level of the airway epithelium represents the key event for the initiation of the Th2-biased HDM allergic responses (14). In the household dust, the allergens from house dust mite (HDM) are always in association with the microbial compounds from the commonly associated bacteria, fungi, molds such as lipopolysaccharides (LPS), β-glucans and chitin (15-21). Such microbial components represent a large variety of Pathogen Associated Molecular Patterns (PAMPS) which activate the innate immunity for the initiation of the HDM allergic response.

To date, we can consider that more than thirty different allergens which elicited specific immunoglobulin E (IgE) antibodies responses in sensitized patients were identified in two most common dust mite species, *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farin*ae (Der f) (22, 23). According to their respective biological activity, HDM allergens could be roughly classified into three main types of proteins: the proteases, proteins displaying affinities for lipids/fatty acids, and non-proteolytic enzymes and chitin binding proteins (24-30).

The typical HDM sensitizations are characterized by the production of allergen specific IgE in atopic patients and the IgE binding frequency, measured for each allergen in any defined allergic population, can classify the allergens as major (>50%), intermediate (20-50%) and minor (<20%) allergens. The two most potent HDM allergens are Der p 1 and Der p 2. Indeed, not only, the IgE reactivities to these two allergens are very high (commonly from 60 % to 80 %) but key pro-Th2 innate signaling pathways are triggered by their biological activities (cysteine protease for Der p 1 and LPS-binding protein for Der p 2) (25-29). Together with Der p 1 and Der p 2, the newly discovered allergen, Der p 23 was also as the measured IgE reactivity to Der p 23 reached 60 % to 70 % in selected HDM allergic cohorts (31, 32). In contrast, minor HDM allergens that induce low level of specific IgE remains poorly studied up to now.

A typical example is the group 13 mite allergen. The measured IgE binding frequencies against Der p 13, Blo t 13, Tyr p 13, Lep d 13 and Aca s 13 in HDM allergic patients worldwide were low, ranging from 6 to 20% (33-38). However, such HDM allergen group, considered as a minor one based uniquely on the IgE binding frequency, could play an important role for the elicitation of HDM sensitizations. Indeed, the HDM allergen from group 13 displays sequence homologies with the

cytosolic fatty acid binding proteins (FABP), a protein family involved in the transporting of lipids into the cells (39). Consequently, group 13 mite allergens, through their potential propensity to transport lipid ligands from their environment, could play an important function in the initiation of the HDM allergic through the activation of innate signaling pathways. Up to now, only one study clearly evidenced the lipid binding activity of Blo t 13 (40), the stimulation of innate immunity through activation of specific Pathogen Recognition Receptor(s) (PRR) remains to be fully demonstrated.

Research hypothesis

Although Der p 13 could represent a minor HDM allergen, Der p 13, through its capacity to transport microbial lipid ligands, is a key activator of TLR2 from the airway epithelium. Such TLR2 activation stimulates the production of proinflammatory cytokines essential for the initiation of the HDM allergic response.

Research question

Could the HDM allergen Der p 13 be considered as the fatty acid binding protein allergen which triggers the innate immunity?

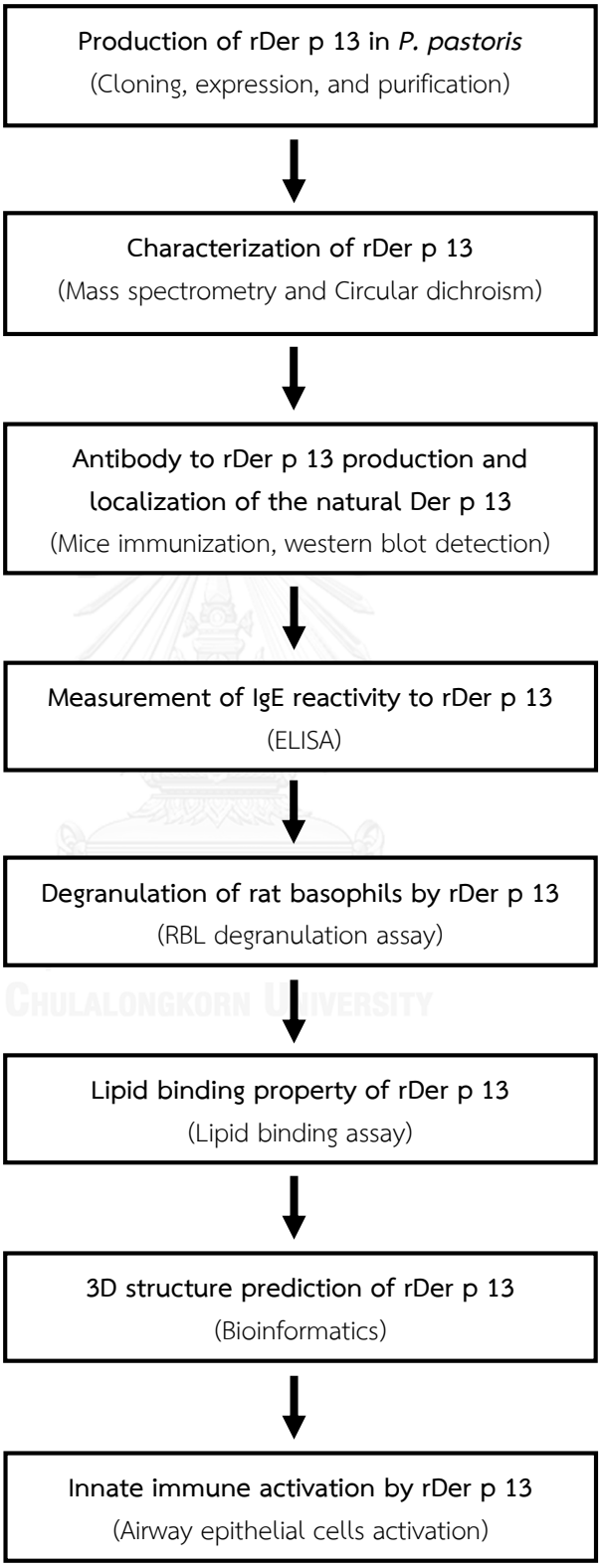
Study Design

Experimental study

Objectives

- To produce a recombinant form of Der p 13 using the Pichia pastoris yeast expression system
- To demonstrate whether Der p 13 is allergenic and whether Der p 13 is a minor allergen in thai allergic patients
- To demonstrate that rDer p 13 is a fatty acid binding protein
- To characterize the innate signaling pathways triggered by rDer p 13

Conceptual framework



CHAPTER II

LITERATURE REVIEW

2.1 Generalities on house dust mite and HDM allergy

House dust mites (HDM) represent a prominent perennial allergenic source responsible of inflammatory diseases in atopic patients such as atopic asthma (AA), atopic rhinitis (AR) and atopic dermatitis (AD) (2). House dust mites are arachnids from the Pyroglyphidae family living in human habitats such house dust but also carpets and bedding (especially in pillows). The two most common HDM species that induce allergic sensitizations are *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f) (4, 13) (Figure 2.1). The HDM allergic responses are mediated by the indoor mite allergens present into the fecal pellets together with the mite bodies (41).

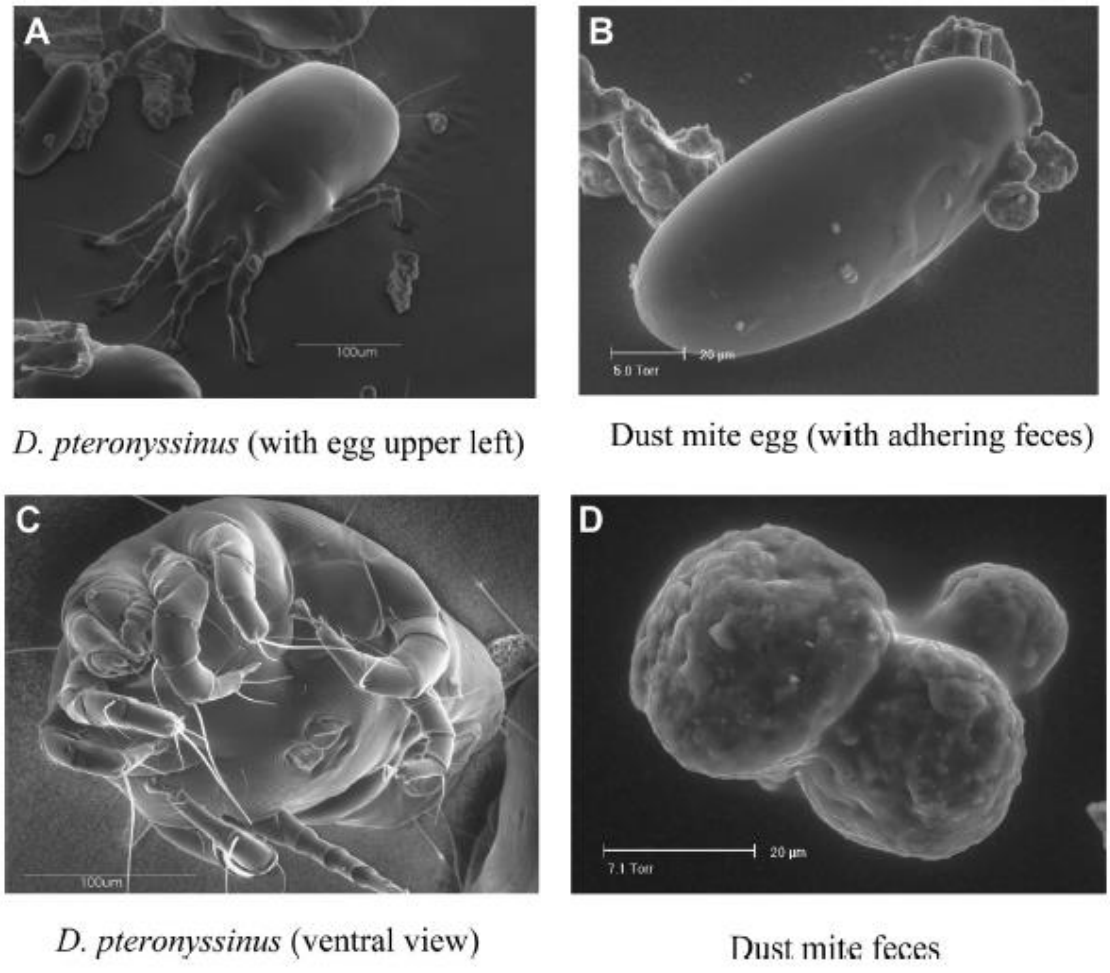


Figure 2. 1 The HDM D. pteronyssinus.

HDM (upper view, panel A), mite egg with adherent feces (panel B), HDM (ventral view, panel C), and dust mite feces (panel D) under scanning electron (SEM) microscope [derived from (41)].

It is now well accepted that three main factors interplay together to trigger HDM allergy; the HDM allergens (allergen source) the environmental factors (including pollution, microbial co-exposures) as well as a genetic predisposition (atopy) (13, 42).

HDM allergens can be classified as airborne allergens and consequently, can be easily inhaled to be directly in contact with the human airways, leading to the initiation of allergic reactions. Although a large volume of literatures evidenced that

the HDM allergic response is a strong inflammatory response orchestrated by the HDM allergen-specific T_H2 cells (43), the detailed mechanism of the development of such exacerbated inflammatory response remains to be fully elucidated.

The environmental factors influencing HDM sensitizations are related notably to our changes of indoor living conditions, such as temperature and humidity, that control the proliferation of mites together with the composition of microorganisms commonly present into the house dust and in the air (gram+/gram- bacteria, fungi and mold, viruses) (44-48), but also related to other environmental agents such as air pollution; tobacco, forest fires, and pollutants from factories (Figure 2.2, panel A). These environmental agents clearly participate to the development of the HDM allergic response through co-inhalations together with the HDM allergens

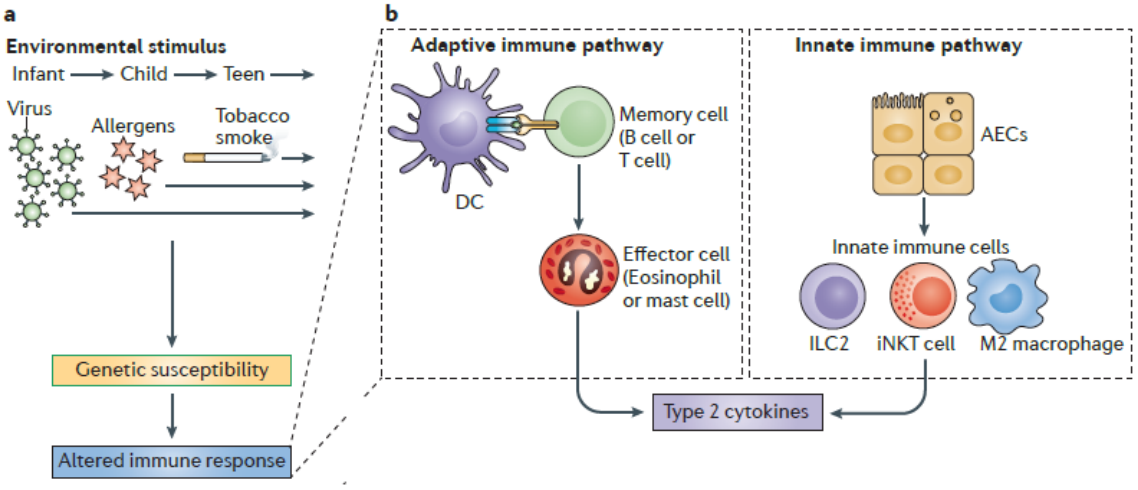
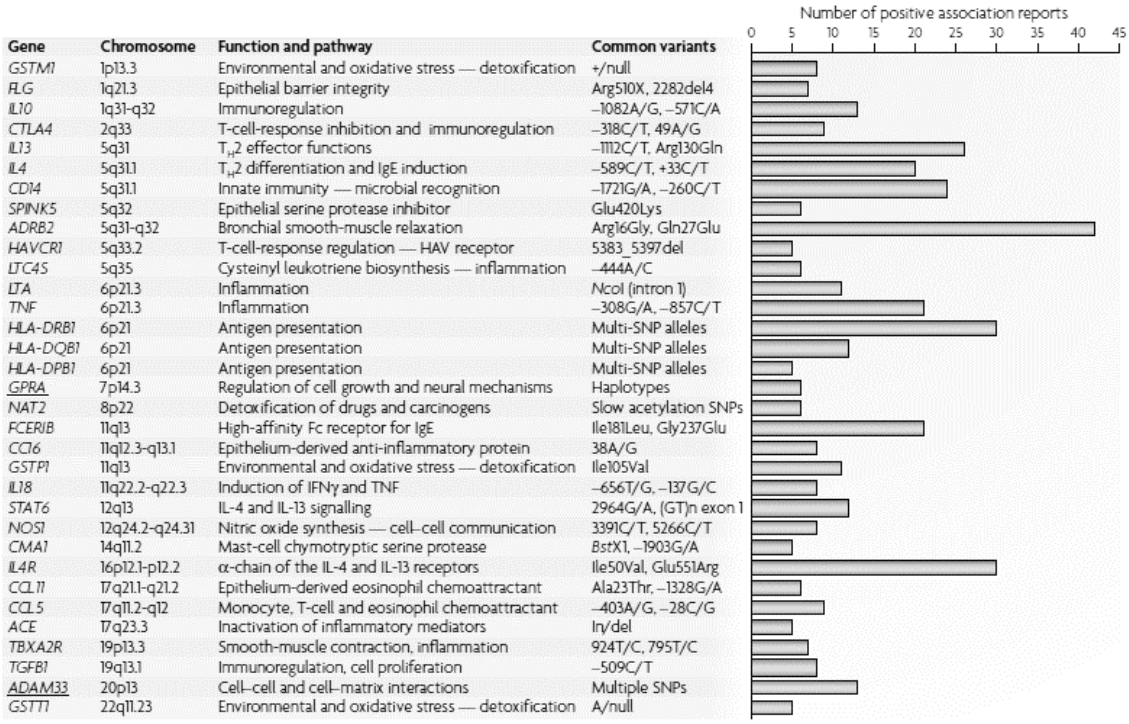


Figure 2. 2 Factors triggering HDM allergy.

environmental stimuli — such as respiratory viruses, allergens and/or tobacco smoke that may act synergistically on genetically susceptible individuals to induce chronic inflammatory diseases through altered, adaptive and innate immune responses [modified from (42)].

In the context of HDM allergy, the term “genetic predisposition” or “genetic susceptibility” refers to genetic background leading to atopy, involving the “control” of the immune system to produce allergen-specific Immunoglobulin E (IgE) antibodies when exposed to allergens. Many asthma-associated genes have been summarized in Figure 2.3.



Gene	Chromosome	Function and pathway	Common variants
GSTM1	1p13.3	Environmental and oxidative stress — detoxification	+/null
FLG	1q21.3	Epithelial barrier integrity	Arg510X, 2282del4
IL10	1q31-q32	Immunoregulation	–1082A/G, –571C/A
CTLA4	2q33	T-cell-response inhibition and immunoregulation	–318C/T, 49A/G
IL13	5q31	T_H2 effector functions	–1112C/T, Arg130Gln
IL4	5q31.1	T_H2 differentiation and IgE induction	–589C/T, +33C/T
CD14	5q31.1	Innate immunity — microbial recognition	–1721G/A, –260C/T
SPINK5	5q32	Epithelial serine protease inhibitor	Glu420Lys
ADRB2	5q31-q32	Bronchial smooth-muscle relaxation	Arg16Gly, Gln27Glu
HAVCR1	5q33.2	T-cell-response regulation — HAV receptor	5383_5397del
LTC4S	5q35	Cysteinyl leukotriene biosynthesis — inflammation	–444A/C
LTA	6p21.3	Inflammation	*Nco*I (intron 1)
TNF	6p21.3	Inflammation	–308G/A, –857C/T
HLA-DRB1	6p21	Antigen presentation	Multi-SNP alleles
HLA-DQB1	6p21	Antigen presentation	Multi-SNP alleles
HLA-DPB1	6p21	Antigen presentation	Multi-SNP alleles
GPRA	7p14.3	Regulation of cell growth and neural mechanisms	Haplotypes
NAT2	8p22	Detoxification of drugs and carcinogens	Slow acetylation SNPs
FCERIB	11q13	High-affinity Fc receptor for IgE	Ile181Leu, Gly237Glu
CC16	11q12.3-q13.1	Epithelium-derived anti-inflammatory protein	38A/G
GSTP1	11q13	Environmental and oxidative stress — detoxification	Ile105Val
IL18	11q22.2-q22.3	Induction of IFNγ and TNF	–656T/G, –137G/C
STAT6	12q13	IL-4 and IL-13 signalling	2964G/A, (GT)n exon 1
NOS1	12q24.2-q24.31	Nitric oxide synthesis — cell–cell communication	3391C/T, 5266C/T
CMA1	14q11.2	Mast-cell chymotryptic serine protease	*Bst*X1, –1903G/A
IL4R	16p12.1-p12.2	α-chain of the IL-4 and IL-13 receptors	Ile50Val, Glu551Arg
CCL11	17q21.1-q21.2	Epithelium-derived eosinophil chemoattractant	Ala23Thr, –1328G/A
CCL5	17q11.2-q12	Monocyte, T-cell and eosinophil chemoattractant	–403A/G, –28C/G
ACE	17q23.3	Inactivation of inflammatory mediators	In/del
TBXA2R	19p13.3	Smooth-muscle contraction, inflammation	924T/C, 795T/C
TGFB1	19q13.1	Immunoregulation, cell proliferation	–509C/T
ADAM33	20p13	Cell–cell and cell–matrix interactions	Multiple SNPs
GSTT1	22q11.23	Environmental and oxidative stress — detoxification	A/null

Figure 2. 3 Genes associate to asthma.

Genes were identified by searching the public databases using the keyword ‘association’ together with each of the following terms: asthma, bronchial hyper-responsiveness, atopic dermatitis or IgE [derived from (49)].

For example, polymorphisms of the IL-4 receptor gene (IL-4R) induces the high expression of IL-4 cytokines which then up-regulating the production of IgE, especially in patients suffering from mild asthma and atopic dermatitis (50-53). Single nucleotide polymorphisms (SNPs) in the IL-13 gene such as IL13-1112CT (rs1800925), IL13+2044GA

(rs20541) or IL13-1512AC (rs1881457) were also associated with high IgE levels in asthmatic populations (49).

2.2 Epidemiology of HDM allergy

The published data during the last decades showed the continuous increase of HDM-sensitized patients worldwide. Nowadays, the prevalence of HDM allergy reached around 700 million people around the world and distributed into the 6 main continents (Figure 2.4).

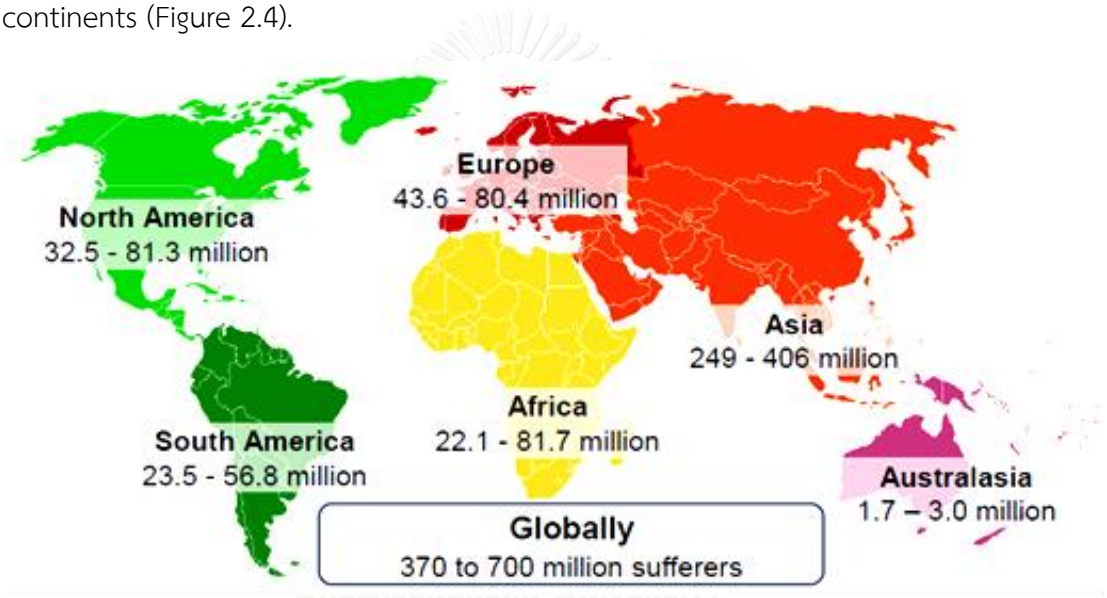


Figure 2. 4 Epidemiology of house dust mite allergy in the world.
[modified data from (54-58)].

Over the past 10 years, HDM was the most common cause of respiratory allergy worldwide, affecting approximately 20 % to 30 % in industrialized countries. 50 % of European patients with AA, AR, AD were HDM-allergic. (59). In America, the studies in 2015 showed the prevalence of HDM allergy reached 15% in a cohort of atopy and asthmatic patients (60, 61). The study from South Africa in 2015, revealed 52% of asthma and allergic rhinitis patients sensitized to HDM (62). The recent study in

Australia, showed 20% to 60% in atopic dermatitis children were HDM-allergic (63). The trend of HDM allergy in Asia countries, revealed 45%, 38%, and more than 50% in atopic patients were mainly sensitized by HDM in Taiwan, Singapore, Malaysia, respectively (64-66).

Finally, the prevalence of HDM in Thailand has also been studied over the past three decades. The first study in 1987, reported 94 % of atopic patient cases were skin prick tested positive to house dust mite allergens (67). The SPT result in 2002, revealed more than 50% and 60% of atopic adults and children were HDM-sensitized (68). The following studies reported *Dermatophagoides* species were the most common inducers of HDM sensitizations among Thai adult asthmatics (frequency of 40%-60%) (69-71). During the 2004-2009 period, the asthma severity rate was correlated with the number of HDM sensitization cases Thailand (72). The most recent data published in 2016 showed that more than 50% of Thai patients suffering from allergic rhinitis and allergic asthma were sensitized to HDM (7).

2.3 Mechanism of the allergic response

The mechanism of allergic response consists mainly into two main steps. The initiation of the allergic response, also called "sensitization", occurs when atopic patients are in contact for the first time with the allergenic sources. The development of allergenic sensitizations is followed by a series of events triggered by the allergen re-exposures. The resulting inflammatory response, commonly associated with the clinical symptoms of HDM allergy such as AA, AR, AD, can be subdivided into 3 phases: the immediate, late and chronic allergic phases (73).

The initiation of the allergic response occurs through the activation of innate immune signaling pathways leading to the production of a large panel of pro-Th2 inflammatory cytokines (notably IL-25, IL-33, and TSLP) to prepare an appropriate immunological milieu to bias the allergen-specific adaptive immune response towards T_H2 (Figure 2.5).

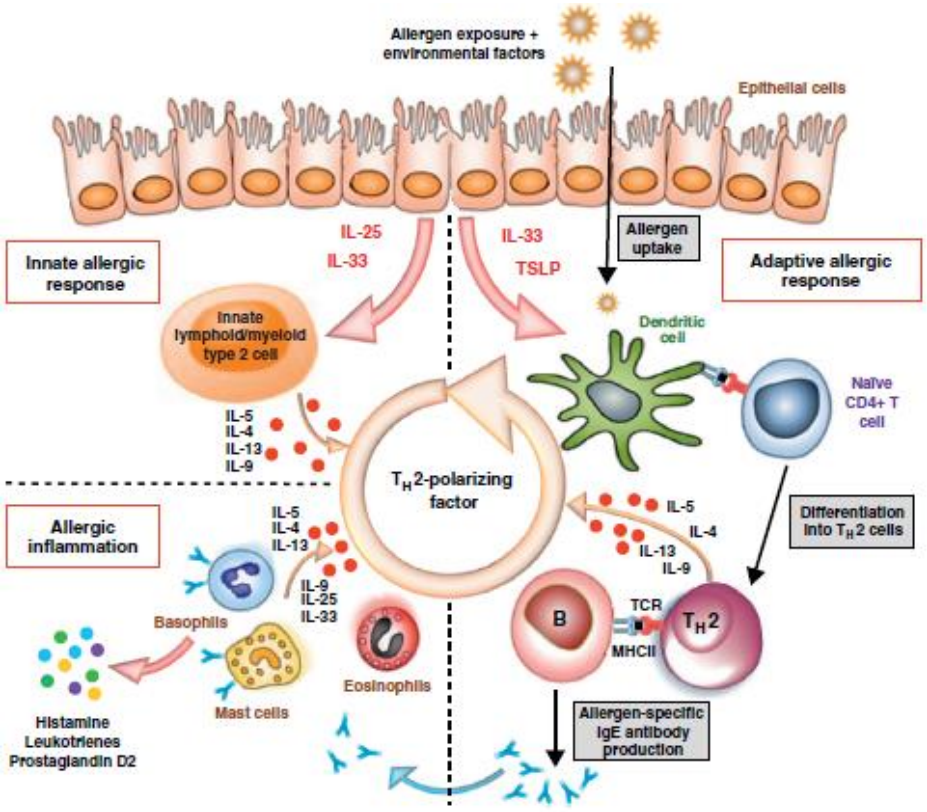


Figure 2. 5 Cross-talk between airway epithelium, innate and adaptive immune system to initiate the allergic response.

The hallmark of Type I allergic immune response is production of allergen specific IgE and activation of mast cells, basophils and eosinophils. Allergen exposure together with environmental factors induced the cytokines production. In innate allergic response, ILC-2 cells were activated and lead to TH2 cytokines production. In adaptive allergic response, allergens were recognized by DCs and presented to naïve CD4+ T

cells to initiate the TH2 polarizing factor. The immunoglobulin class switching occurred from IgM to IgE in B-cells [derived from (43)].

During that initial event, several pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptor (NLRs) and PAR receptors present on the airway epithelial cells (Figure 2.6), are stimulated by pathogen-associated molecular patterns (PAMPs) and damaged-associated molecular patterns (DAMPs). These PAMPs and DAMPs consist of LPS from gram-negative bacteria, chitin from the mite/yeast/fungi, and β-glucans from molds/fungi which could be transported by HDM allergens or just co-inhaled with the allergenic proteins (Figure 2.7) (74, 75). Many studies have demonstrated that the LPS, chitin, and β-glucan by themselves could activate the immune responses. For LPS, the study revealed that the level of LPS exposure can determine the type of inflammatory response; low LPS dose promotes T_H2 response, whereas, high LPS dose induced TH1 responses through TLR4 (15). The direct airway epithelial cells by LPS via TLR4 drives the Th2 polarization in HDM allergy (76) and triggers IL-1β expression in airway epithelium, leading to the releasing of GM-CSF, and IL-33 (77). In TLR4 or MyD88 deficiency mice model, the allergic response is reduced after HDM sensitization, confirm that the presence of contaminated LPS regulate the HDM allergic response (78). For chitin, it is a common polysaccharide found in nature and involved in innate immunity (20). The airway epithelial cells secrete CCL2 in response to chitin and CCR2 signaling mediates chitin-induced alternative activation of macrophages and allergic inflammation *in vivo* (79). The effects of size-dependence chitin could induce innate eosinophilia in vivo (19). The recent study demonstrated that HDM-derived chitin enhances airway hypersensitivity via the TLR2-dependent pathway, and develops of T_H2 cell response

to inhaled allergens in mice model (21). For β-glucan, the study showed both β-glucan-induced TLR2 activation and LPS-induced TLR4 activation generated DUOX2-induced ROS (80). Innate immune responses of airway epithelium to house dust mite are mediated through β-glucan-dependent pathways (18). Moreover, glycan structure from HDM extracts stimulate the cysteinyl leukotriene production in DC through Dectin-2 and is critical for the HDM allergic response (81).

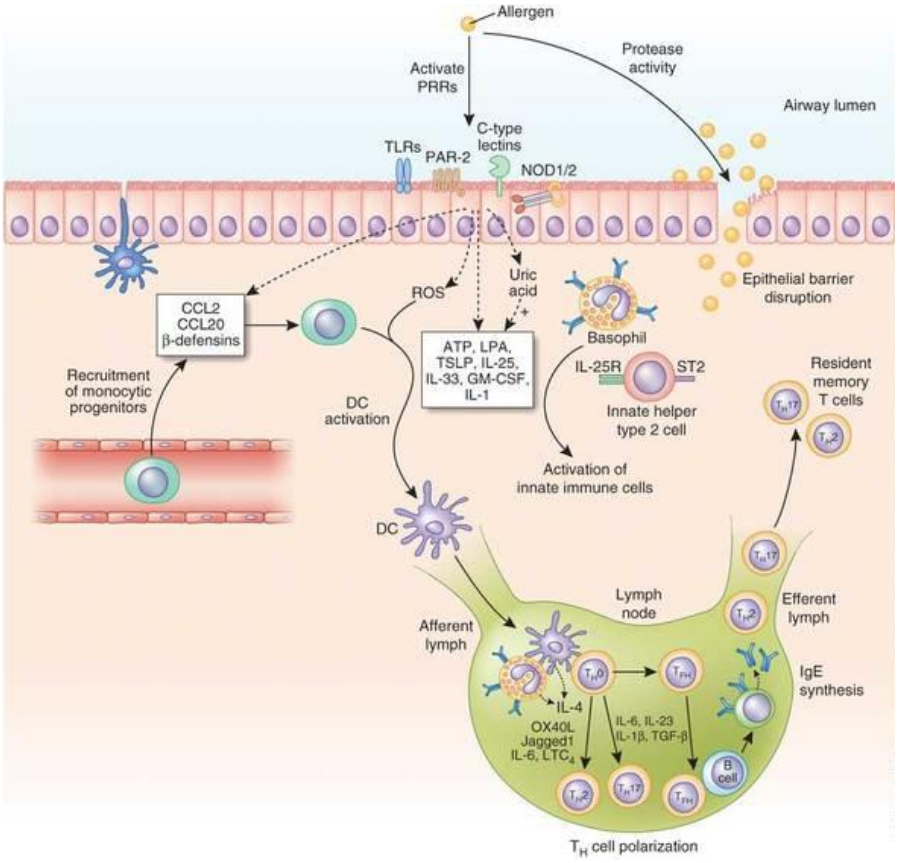


Figure 2. 6 Airway epithelium activation during the HDM sensitization.

Inhaled allergens from HDM can activate PRRs; including TLRs, CLRs, NLRs and PAR receptors, are presence on airway epithelial cells. Some allergens can cleave epithelial tight junctions, gaining access to the DCs network, whereas, some allergens activate DCs and induce the production of the cytokines; TSLP, GM-CSF, and IL-1β. Airway epithelial cells also produce CCL2 and CCL20, which attract more DC progenitors, such

as monocytes, to the lung. Under the influence of these signals, DCs migrate to the T cell area of the draining lymph nodes, where they interact with naive T cells and induce TH2, TH17 and T follicular helper (TFH) differentiation. TFH cells can then promote B cell differentiation and IgE synthesis. Basophils are an important source of IL-4, which further supports T_H2 development initiated by DCs and promotes IgE synthesis by B cells [derived from (82)].

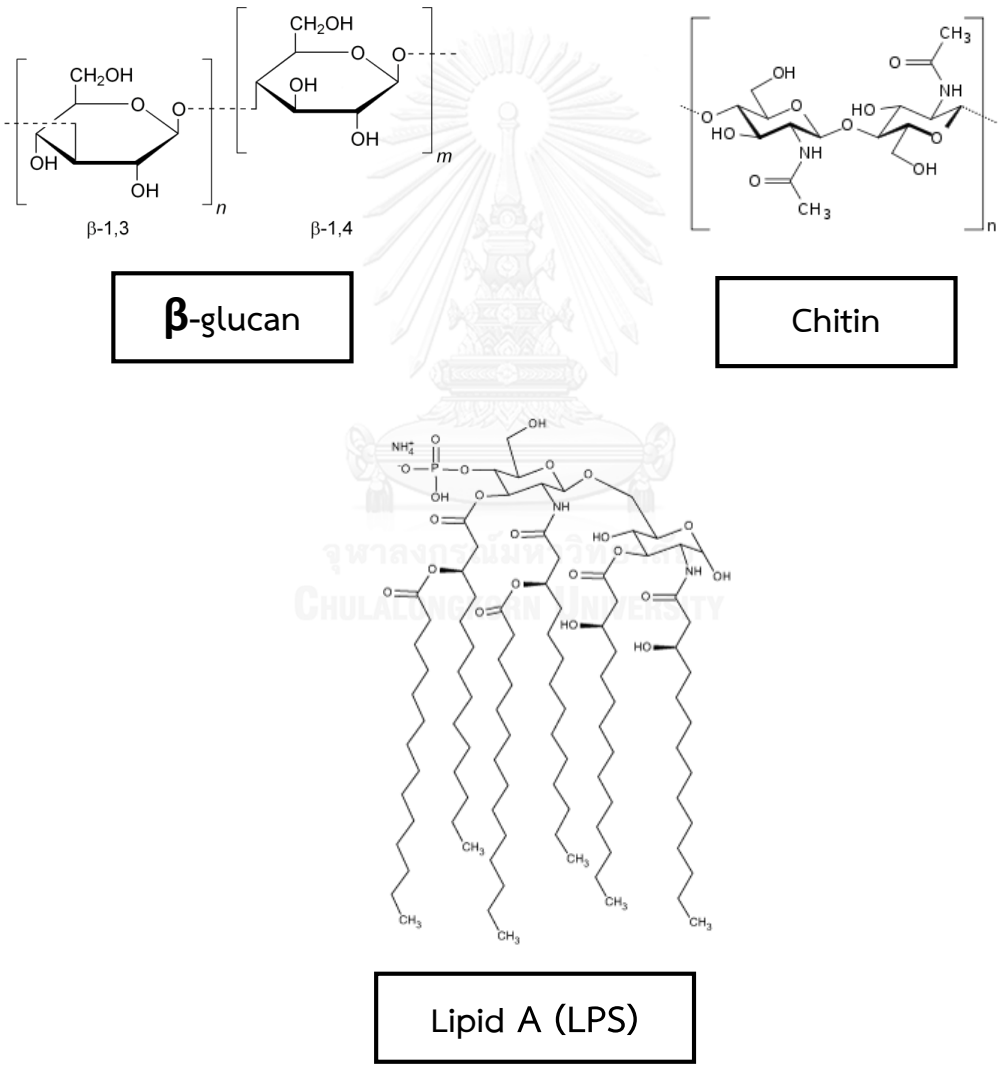


Figure 2. 7 Structure of LPS, Chitin and β-glucan.

These components are presences in HDM, fecal pellets or dust, which can activate the immune system to initiate an inflammatory response [derived from (5)].

The release of IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) by activated epithelial cells are, in turn, able to stimulate a class of innate immune cells called type 2 innate lymphoid cells (ILC2) and their activation will result in production of "T_H2-type" cytokines (i.e., IL-5, IL-9, and IL-13). These other T_H2 type cytokines as IL-5, IL-9, and GM-CSF is involved in stimulation and recruitment of eosinophils, whereas, IL-9, IL-13 is involved in inflammatory tissue reactions (83).

Dendritic cells (DCs) play an important role in bridging the innate to adaptive immunity by presenting the allergens to naïve T_H2 cells and to participate to the T_H2 polarization as well. GM-CSF, IL-1α/β, IL-25, IL-33, CCL2, CCL20, TSLP, released from activated-airway epithelial cells, drive the maturation of immature DCs to conventional DC capable of presenting the allergen to naïve T cells and to initiate the Th2 differentiation. In addition, secretion of IL-13 induces mucus production, smooth muscle contractility, and alternative activation of alveolar macrophages, which leads to amplification of IL-33 production.

The T_H2 type cytokines including IL-4 and IL-13 trigger IgM to IgE class switching at the level of the B cell (Figure 2.8). (84). The increased production of allergen specific IgE is followed by their binding to high affinity IgE receptors (FCεR1) on the surface of mast cells and basophils (85-87).

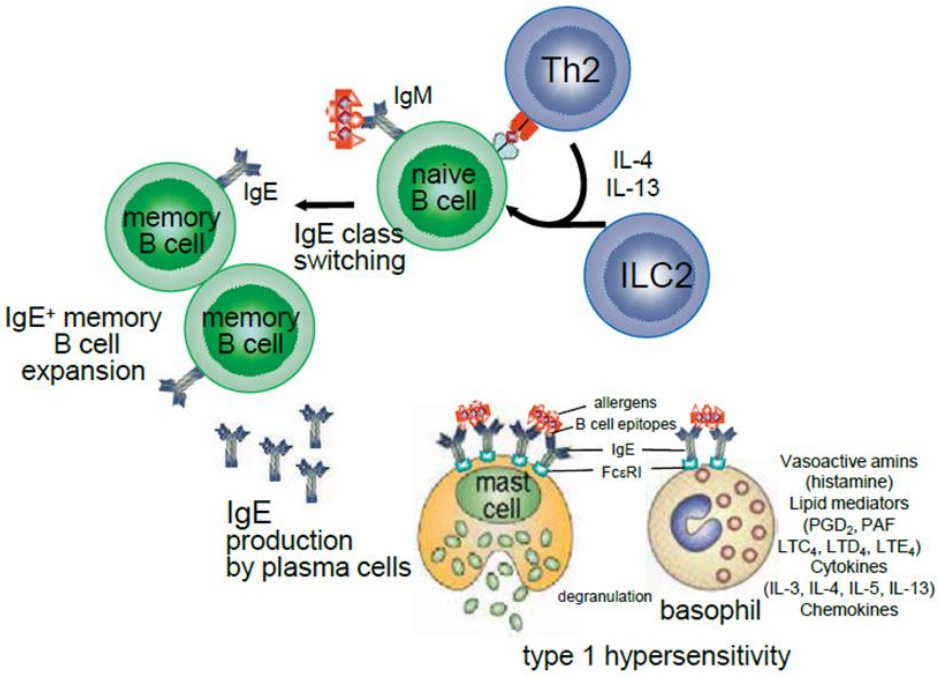


Figure 2. 8 IgE class switching.

Naïve B cell stimulated with T_H2 type cytokines (IL-4 and IL-13) from allergen-specific T_H2 or ILC2 cells to become IgE-producing B cells. Allergen specific IgE then binds to high affinity IgE receptors (FC**ε**R1) in mast cells, basophils, and eosinophils [Figure source: Global atlas of allergy (88)].

The clinical manifestations of HDM allergy, absent during the initial phase of the allergic response are clearly apparent following HDM allergen re-exposures. The inflammatory response following allergen re-exposure can contain 3 phases; immediate-phase, late-phase, and chronic allergic phase (Figure 2.9).

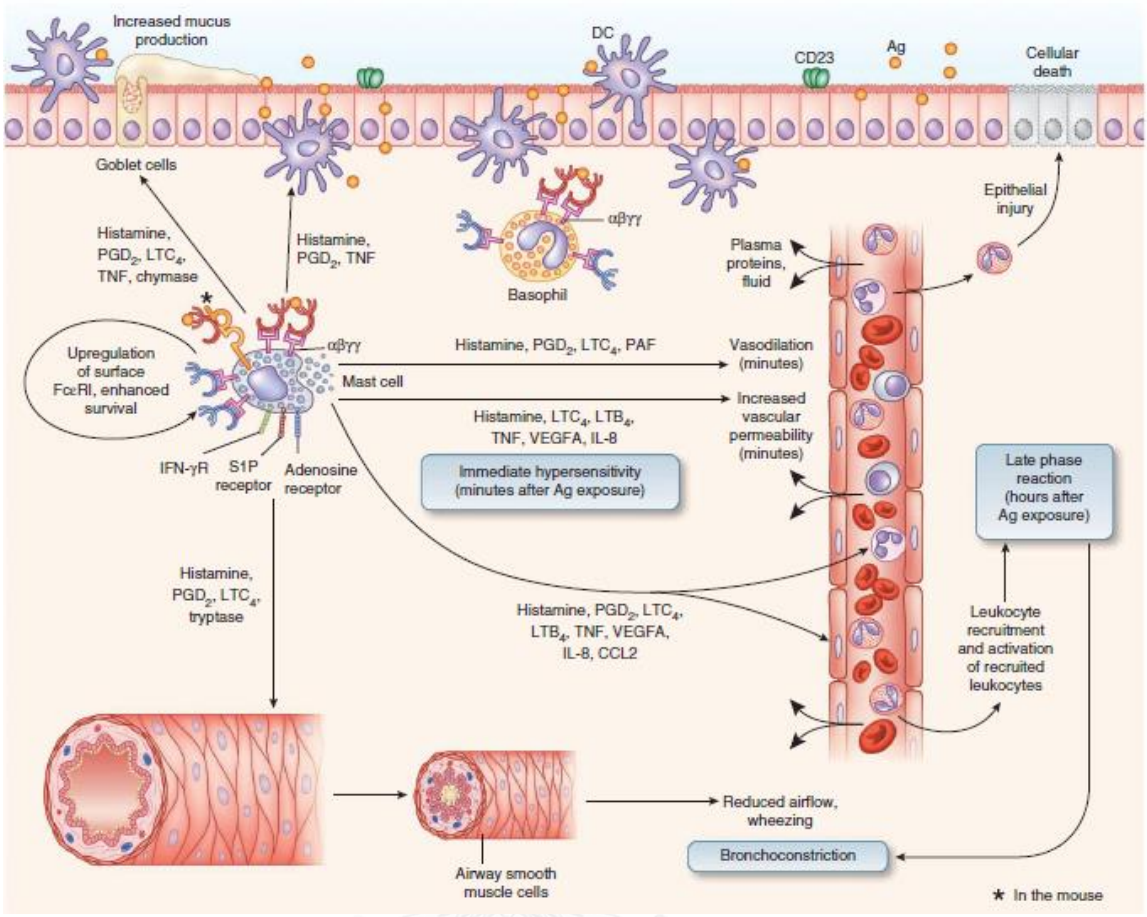


Figure 2. 9 The early, immediate hypersensitivity, phase of antigen-induced airway inflammation.

The individual IgE molecules that are bound to FcεRI molecules on a single mast cell can be specific for different antigens. Some IgE molecules can enhance cytokine production (IL-4, IL-8) to initiate an immediate hypersensitivity response. Within minutes of exposure, the rapidly secreted mediators lead to bronchoconstriction, vasodilation, increased vascular permeability and increased mucus production. Mast cell mediators produced rapidly after antigen challenge can also promote dendritic cell migration, maturation and function and can contribute to the transition to the late phase reaction [derived from (85)].

Immediate-phase reactions

The immediate phase reaction classified commonly as type I immediate hypersensitivity takes place within just minutes following allergen re-exposures and induced the degranulation of mast cells/ basophils. The released mediators from the cell granules include histamine or serotonin, heparin, kinins, proteases, cytokines (IL-4, IL-8), prostaglandins, and leukotrienes.

The allergic symptoms during the immediate phase also include 1) vasodilation; producing erythema (reddening) of the skin or conjunctiva, 2) increased vascular permeability that leads to tissue swelling in the eyes, tear formation, contraction of bronchial smooth muscle which producing airflow obstruction and wheezing, 3) increased secretion of mucus that exacerbating airflow obstruction in the lower airways and producing a runny nose. Such mediators can also stimulate in sneezing, itching or coughing. When such mediators are released locally, an early-phase reaction is presence. In contrast, the rapid and systemic release of such mediators, from mast cells and basophils, accounts for much of the pathology associated with anaphylaxis (89).

Late-phase reactions

Late-phase reactions are long-term consequences of the mediators released by activated mast cells during early-phase reactions, including antigen-stimulated T cells. Late-phase reactions develop in 2–6 hr after the exposure to allergen, and often peak after 6–9 hr. The differences between early-phase and late-phase are not well-understood but the clinical features of late-phase reactions reflect the activities of both resident cells and circulating leukocytes. For example, calcitonin-gene-related peptide (CGRP), which is produced by epithelial cells, T cells, monocyte–macrophage

lineage cells and possibly other sources, may contribute to the vasodilation that is associated with late-phase reactions.

Chronic allergic reactions

The continuous or repetitive of allergen exposure to the allergic affected sites can leads to the persistent inflammation. The patients with chronic asthma can develop profound change in cell structures at all of the layers of the airway wall. It refers to “airway remodeling” and typically is associated with: changes in the epithelium, including an increased number of goblet cells which produce mucus, increased production of cytokines such as IL-4, IL-5, IL-9, and IL-13, and chemokines such as IL-8 and MPC-1, by epithelial cells, as well as areas of epithelial injury and repair; substantial inflammation of the sub-mucosa, including the development of increased deposition of extracellular-matrix molecules in the lamina reticularis (beneath the epithelial basement membrane); changes in fibroblasts, increased development of myofibroblasts and increased vascularity; and increased thickness of the muscular layer of the airways, with increased size, number and function of smooth muscle cells (Figure 2.10).

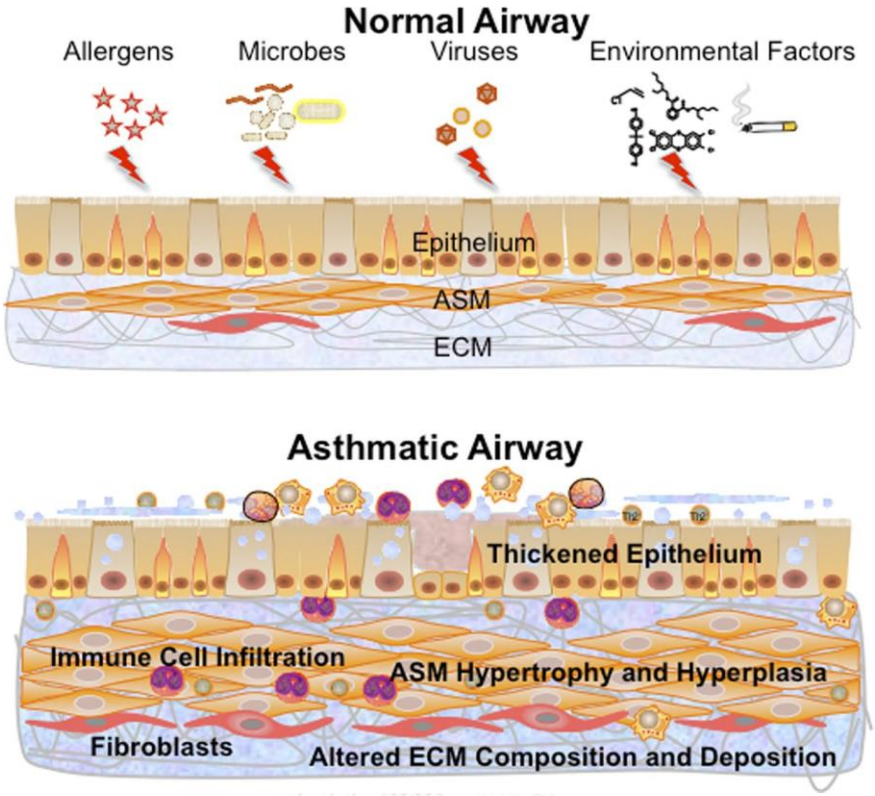


Figure 2. 10 Airway reactivity and remodeling.

The exposure of the normal airway to allergens, microbes, viruses or environmental factors (pollutants, tobacco smoke, or nanoparticles) results in changes throughout the epithelium, airway smooth muscle (ASM), and extracellular matrix (ECM). The asthmatic airway involves infiltration of immune cells, a thickened epithelium with goblet cell hyperplasia, increased mucus, a thickened, more fibrotic ASM layer with increased cell size (hypertrophy) and numbers (hyperplasia), along with altered ECM composition [derived from (90)].

2.4 HDM allergens and innate immunity

Using different molecular biology methods such as cloning, transcriptomics, proteomics, more than thirty different allergen groups which elicit specific IgE

responses in sensitized patients were identified in the *D. farina*e and *D. Pteronyssinus* mite species.

However, it is interesting to note that, all the natural HDM allergens are not detected in allergen extracts from mite cultures. Moreover, the localization of HDM allergens were quite variable: some HDM allergens being detected only in mite bodies or in the fecal pellets or on both, (Figure 2.11).

Allergen group	Isoallergen	Avg. mass (kDa)	Whole extracts		Bodies		Feces	
			Coverage (%)	#Peptides	Coverage (%)	#Peptides	Coverage (%)	#Peptides
1	Der p 1	36	48	53	42	23	50	38
2	Der p 2	16	64	23	61	13	71	17
3	Der p 3	28	62	24	53	12	62	15
4	Der p 4	57	77	29	43	14	36	13
5	Der p 5	17	12	1	n.a.	n.d.	n.a.	n.d.
6	Der p 6	30	72	39	54	14	71	21
7	Der p 7	24	40	25	5	1	5	1
8	Der p 8	26	18	4	13	3	n.a.	n.d.
9	Der p 9	29	34	14	30	7	31	9
10	Der p 10	33	18	5	28	11	n.a.	n.d.
11	Der p 11	102	n.a.	n.d.	23	18	n.a.	n.d.
14	Der p 14	191	7	9	23	36	n.a.	n.d.
15	Der p 15	61	47	21	n.a.	n.d.	n.a.	n.d.
18	Der p 18	52	42	16	11	3	26	6
20	Der p 20	40	25	7	39	13	n.a.	n.d.
21	Der p 21	17	18	2	n.a.	n.d.	n.a.	n.d.
23	Der p 23	10	44	8	21	2	57	6

Avg. mass, theoretical average molecular mass of the allergen; coverage, allergen amino acid sequence coverage; n.a., not applicable; n.d., not detected; #peptides, number of peptides identified.

Figure 2. 11 MS/MS identification of HDM allergens from extracts from whole mite cultures, purified bodies and mite feces.
some HDM allergens could not be identified at all [data from (61)].

The WHO/International Union of Immunological Societies nomenclature identifies allergens by using the first three letters of the genus, then the first letter of the species (i.e., Der p and Der f), followed by the group number of different allergens which display a specific biological activity (Table 1.1), (91).

Table 1. 1 Nomenclature and biological activity of HDM allergens. Nomenclature and biological activity of HDM allergens.

The corresponding biological activities of HDM allergens were determined mainly through amino acid sequences homologies.

allergen group	Identified allergens	Biological activity	References
1	Der p 1, Der f 1, Blo t 1	Cysteine protease	(27, 29, 92-108)
2	Der p 2, Der f 2, Lep d 2, Tyr p 2, Aca s 2, Blo t 2	MD-2–like lipid-binding protein	(109-116)
3	Der p 3, Der f 3, Blo t 3, Lep d 3	Trypsin-like serine protease	(27, 117-120)
4	Der p 4, Der f 4, Blo t 4	Amylase	(121, 122)
5	Der p 5, Blo t 5, Der f 5, Lep d 5	Lipid-binding protein	(29, 110, 123, 124)
6	Der p 6, Der f 6, Blo t 6	Chymotrypsin-like serine protease	(27)
7	Der p 7, Der f 7, Lep d 7, Blo t 7	Lipid-binding protein	(110)
8	Der p 8, Lep d 8, Blo t 8	Glutathione-S-transferase	(27)
9	Der p 9, Der f 9, Blo t 9	Collagenolytic-like serine protease	(125)
10	Der p 10, Der f 10, Blo t 10, Lep d 10, Tyr p 10	Tropomyosin	(126, 127)
11	Der p 11, Der f 11, Blo t 11,	Paramyosin	(128, 129)
12	Blo t 12, Lep d 12	Chitinase	(130)
13	Der f 13, Blo t 13, Lep d 13, Aca s 13, Try p 13,	Cytosolic Fatty Acid binding protein (FABP)	(35-38, 61)
14	Der p 14, Der f 14, Blo t 14	Vitellogenin/apolipophorin-like	(131)
15	Der p 15, Der f 15	Chitinase	(132)
16	Der f 16	Gelsolin	(133)

allergen group	Identified allergens	Biological activity	References
18	Der p 18, Der f 18, Blo t 18	Chitinase	(132, 134)
20	Der p 20, Der f 20	Arginine kinase	(135)
21	Der p 21, Der f 21, Blo t 21	Lipid-binding protein	(136, 137)
22	Der f 22	Lipid-binding protein	(138)
23	Der p 23	Chitin-binding protein	(31, 32, 139, 140)
24	Der f 24	Ubiquinol- cytochrome C reductase–binding protein homolog	(138)
27	Der f 27	Serpin	(141)
29	Der f 29	Cyclophilin	(142)
33	Der f 33	α-tubulin	(143)

Up to now, with the exception of natural Der p –f 1 and Der p –f 2, the isolation of the other natural HDM allergens from mite cultures is quite impossible according to their presence into very tiny amount. Consequently, the characterization of the HDM allergens was performed through recombinant protein productions, mainly using the *E.coli* and *P.pastoris* expression systems (144-147). A brief description for some important HDM allergen groups, mainly focused on their capacity to activate innate immune responses in the following paragraphs.

Group 1 HDM allergens

The structures of Der p 1 and Der f 1 have been extensively studied by X-ray crystallography (103, 148) (Figure 2.12). The studies showed that Der p 1 adopt a structure characteristic of a papain-like cysteine protease (103).

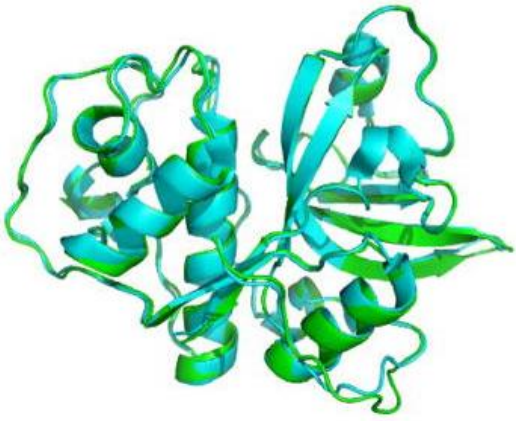


Figure 2. 12 Superposition of the group 1 HDM crystal structures.

Der f 1 (PDB code: 3D6S; green) and Der p 1 (PDB code: 2AS8; cyan), [data form (103)].

As Der p 1 was shown to be present into the mite feces, we speculate that Der p 1 is present into the gastrointestinal gut of the mite and plays digestive function (27, 29, 96, 106). The group 1 allergens stimulate the expression of interleukin-8 in human airway epithelial cells via a proteinase-activated receptor-2-independent mechanism (99). Not only that, Der p 1 has down-regulated defense of the lung by inactivating elastase inhibitors (94). Der p 1 cleaves human cell surface molecules, the low-affinity IgE receptor (CD23/Fc**ε**RII), the alpha-subunit of the IL-2 receptor (CD25), and a protease inhibitor alpha1-antitrypsin, suggested its proteolytic activity involving in the pathogenesis of allergy (98). Der p 1 cleaves tight junction proteins in the airway epithelial barriers to facilitate the accessibility of HDM allergens to the immune system (149-151). Moreover, Der p 1 is evidenced to be the primary activator of other HDM allergen Der p 3, Der p 6 and Der p 9 (27).

Group 2 HDM allergens

Der f 2 and Der p 2 can bind lipopolysaccharide (LPS) between the two large β-sheets at high affinity with structural changes similar to those occurring for its MD-2 homologue which is a co-receptor of TLR4 and it consists of β-sheets folded like β-barrel form with the internal lipid-binding cavity in the center of the protein (152-154) (Figure 2.13).

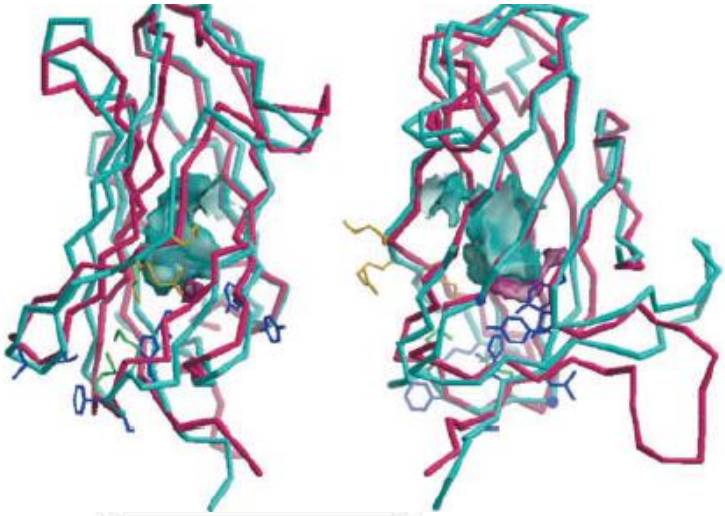


Figure 2. 13 Superposition of Der f 2 and Der p 2.

Inner cavities analysis of Der f 2 (magenta) and Der p 2 models (blue) [data from (155)].

The studies defined that Der p 2 and Der f 2 have structural homology with MD-2 or the LPS-binding component of the Toll-like receptor (TLR) 4 signaling complex which could induce airway inflammation *in vivo* (111, 153, 156). In human bronchial epithelial cell (BEAS-2B), findings indicated that Der p 2 suppressed cell growth and triggered apoptosis, which might be essential for the induction of both intrinsic and extrinsic pathways via TLR2 and p38/JNK signaling (112). Moreover, Der p 2 significantly elevated level of the tight junction protein claudin-2 and increased expression and

nuclear translocation of β-catenin in lung alveolar cell (A549 cells), suggesting that Der p 2 altered epithelial junction (114). In BEAS-2B cells Der p 2 induced dose-dependent up-regulation of GM-CSF, IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-3a (MIP-3a) through the NF-kB and MAPK signaling pathways (115, 157).

Groups 5, 7, 21 HDM allergens

The elucidation of the Der p 5 crystallographic structure clearly showed that the formation of a Der p 5 dimer produced a large hydrophobic cavity at the interface which could represents a ligand-binding site for hydrophobic ligands. Such putative lipid cargo could in turn stimulate innate signaling pathways. However, the nature of the lipid cargo transported by natural Der p 5 together with the capacity of this allergen to stimulate innate immune signaling remain to be evidenced (124, 158, 159). The Der p 7 structure also revealed similarities with LPS-binding protein (LBP), notably at the level of the presence of hydrophobic pocket which, as for group 2 mite allergens, could transport lipid compounds. In contrast with Der p 2, Der p 7 could bind weakly to bacteria lipopeptide polymyxin B (PB), instead of LPS (159). Der p 21 and Der p 5 share overall alpha helix structure. rDer p 21 could trigger IL-8 production in airway epithelial cells through TLR2-dependent signaling (136).

Group 14 HDM allergens

Complete cDNA of Der f 14 was homologous to the large lipid transfer protein (LLTP) family which could involve in the innate immune activation via TLR2 signaling pathway (160).

Group 23 HDM allergens

rDer p 23 represents a new major *D. pteronyssinus* allergen with displays sequence homologies with the chitin binding protein (31, 161). Such Der p 23 chitin complex could play a role in the initiation of the HDM allergic response as chitin is involved in T_H2 biased HDM-specific adaptive immunity (21). However, two recent papers using recombinant and natural forms of this allergen could not demonstrate any chitin binding activities for Der p 23 (32, 139).

2.5 Diagnosis of HDM allergy

The standard diagnostic techniques to diagnose the HDM allergen sensitizations consist in skin prick test, basophil activation assay, and solid phase immunoassay, as ImmunoCAP, which detects the presence of HDM-specific IgE into the serum. The skin prick test is a technique which directly puts in contact the HDM allergen extracts with the skin of the allergic patient and evaluate the growth of wheal, a small swelling of the skin. For the basophil activation assay, the method evaluates the releasing of histamine after the allergen incubation but needs the purification of such cells and in-vitro culture. Mostly the reliable value to diagnose usually based on the solid phase immunoassay as ImmunoCAP value. This ImmunoCAP Specific IgE detects IgE antibodies into the serum of individuals within a range from 0 to 100 kU_A /L, which the result is reported quantitatively (Table 1.2). In clinical practice, 0.35 kU_A / L has commonly been used as a cut-off.

Table 1. 2 Classification of results of allergen specific IgE assay

Specific IgE class	KU_A/L	Specific IgE level
0	Below 0.35	Absent or undetectable
1	0.35-0.69	Low
2	0.7-3.49	Moderate
3	3.5-17.49	High
4	17.5-49	Very high
5	50-99	Very high
6	100 or more	Very high

(Table source: http://www.phadia.com/ImmunoCAP-Assays)

It must be pointed that classically all these above assays are based on the use of defined total HDM allergen extracts. The HDM allergen extracts are made from the mixture of mite bodies, excrement and other emanations of mites. For safety reasons, extracts are standardized by their ability to produce skin prick test reactions in allergic volunteers rather than by the allergen content (162, 163). Unfortunately, such methods cannot allow the identification of sensitizations to any individual HDM allergens, according notably to the limitations mentioned in 2.4: too tiny amounts for the assays, no presence of some allergens into the extracts. Thanks to the isolation of natural Der p-f 1, Der p-f 2 together with the homogenous production and purification of recombinant HDM allergens, the IgE reactivity assays for any single HDM allergen is made possible (Table 1.3). The diagnosis of the HDM allergy is consequently refined but allows the possibility to produce tailor-made immunotherapeutic treatments selecting only the HDM allergens responsible for the sensitizations (164-167).

Table 1. 3 IgE binding frequencies for the different HDM allergen groups

allergen group	IgE binding frequency	References
1	70 % to 80 %	(61, 135, 168-171)
2	70 % to 80 %	(61, 110, 135, 168, 171)
3	16 % to 50 %	(117, 119)
4	40 % to 46 %	(61, 122)
5	50 % to 70 %	(61, 110, 123, 172, 173)
6	30 % to 40 %	(174)
7	50 % to 60 %	(61, 110, 175, 176)
8	20 % to 40 %	(177)
10	30 % to 55 %	(61, 126, 178, 179)
11	60 % to 80 %	(128)
12	40 % to 50 %	(130)
13	6 % to 20 %	(35-38, 61)
14	60 % to 90 %	(131)
15	60 % to 70 %	(61, 132)
16	40 % to 50 %	(180)
17	35 % to 40 %	(180)
18	40 % to 55 %	(132)
20	6 % to 10 %	(61, 135)
21	20 % to 40 %	(136, 137)
23	50 % to 70 %	(61, 139, 140)
24	40 % to 50 %	(138)
29	10 % to 25 %	(142)
27	30 % to 40 %	(141)
33	15 % to 25 %	(143)

2.6 Group 13 HDM allergens

Although several HDM group 13 allergens were cloned from dust mites including *D.pteronyssinus* (Der p 13), *D.farina*e (Der f 13), *B.tropicalis* (Blo t 13), *L.destructor* (Lep d 13), *A.siro* (Aca s 13) and *T.putrescentiae* (Tyr p 13), very few studies focused on the characterization of these proteins.

A recombinant form of Der f 13 has been characterized in terms of IgE reactivity and structure. By using nuclear magnetic resonance (NMR), the elucidation of Der f 13 structure mapped some unique charged residues involved in some key IgE binding epitopes and the mapping of the IgE binding region (181). Previous studies reported IgE binding frequencies against recombinant forms of Der p 13, Blo t 13, Tyr p 13, Lep d 13, and Aca s 13, ranging from 6 % -20 % (35-38, 61).

Protein sequence homology analysis highlighted that group 13 mite allergens displays homologies with human cytosolic fatty acid-binding proteins (FABP family). Consequently, these putative fatty acid/lipid binding proteins could trigger cell activation through interactions between their lipid cargo and TLR2 and/or TLR4 during the HDM sensitization. However, the lipid binding capacity of this mite allergen group was only evidenced for a recombinant form of Blo t 13 using fluorescent lipid probes (40).

CHAPTER III

MATERIALS AND METHODS

3.1 Isolation, cloning and sequencing of Der p 13 cDNA

The full-length cDNA sequence encoding mature Der p 13 (AA 2-131 was amplified from the total cDNA library of *D. pteronyssinus* by Polymerase Chain Reaction (PCR) as the followings;

3.1.1 PCR amplification

Gotag® Flexi PCR reagent (Promega, USA) was selected as the PCR reagents. The PCR was performed in a 50 µl reaction mixtures; consist of final concentration of 1X GoTag buffer, 2 mM magnesium chloride ($MgCl_2$), 0.2 mM deoxy nucleotide triphopastes (dNTPs), 0.2 µM of each forward and reverse primer, and 5 Units of Taq polymerase. In details, the forward and reverse primers were designed and shown in figure 3.1. Template (1 µg of HDM total cDNA) was added into PCR reagents mixture prior to the running of PCR. The PCR thermal cycler profile was set to include 5 min of pre-denaturation at 95°C, 30 seconds for annealing step at 52°C, and 30 seconds of elongation at 65°C, lastly ended with 5 min of final elongation at 72°C and hold at 4°C. The PCR reaction was carried out for 35 cycles in Veriti® thermal cycler (Applied Biosystems, USA).

Forward primer:

5’ GGCTCGAGAAAAGAGAGGCTGAAGCTGCAAGTATCGAAGGCAAATATAAATTG 3’

(Xho I site in green highlighted),

Reverse primer:

5’ GCGGCCGCTTAAATGCGTTTGTAGGTTCGAACCGAAG-3’

(Not I site, in green highlighted).

Figure 3. 1 A set of Der p 13 specific forward and reverse primers.

Green highlighted indicated the both restriction enzymes sites (XhoI and NotI), whereas, yellow highlighted indicated the stretch of α-factor signal sequence (Biodesign, Thailand).

The coding sequence of Der p 13 was corresponded to the GenBank accession number HM560018.1 (Figure 3.2).

Dermatophagoides pteronyssinus Der p 13 allergen mRNA, complete cds

GenBank: HM560018.1

FASTA Graphics

Go to:

```
LOCUS       HM560018                396 bp    mRNA    linear   INV 04-AUG-2010
DEFINITION  Dermatophagoides pteronyssinus Der p 13 allergen mRNA, complete
            cds.
ACCESSION   HM560018
VERSION     HM560018.1  GI:302035349
KEYWORDS    .
SOURCE      Dermatophagoides pteronyssinus (European house dust mite)
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  ORGANISM  Dermatophagoides pteronyssinus
            Eukaryota; Metazoa; Ecdysozoa; Arthropoda; Chelicerata; Arachnida;
            Acari; Acariformes; Sarcoptiformes; Astigmata; Psoroptidia;
            Analgoidea; Pyroglyphidae; Dermatophagoidinae; Dermatophagoides.
REFERENCE   1  (bases 1 to 396)
  AUTHORS   Heinrich,T., Smith,W.-A. and Thomas,W.R.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-JUN-2010) Molecular Biotechnology, Telethon Institute
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      121 gaagtggcca aggaaaatga ccaatatgtt ttccgatcgt tgagcacatt caaaaatacc
      181 gaaatcaaat tcaaattggg cgaagaattt gaagaagatc gtgccgatgg taaacgagtc
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      361 ggtgtcactt cggttcgaac ctacaaacgc atttaa
//
```
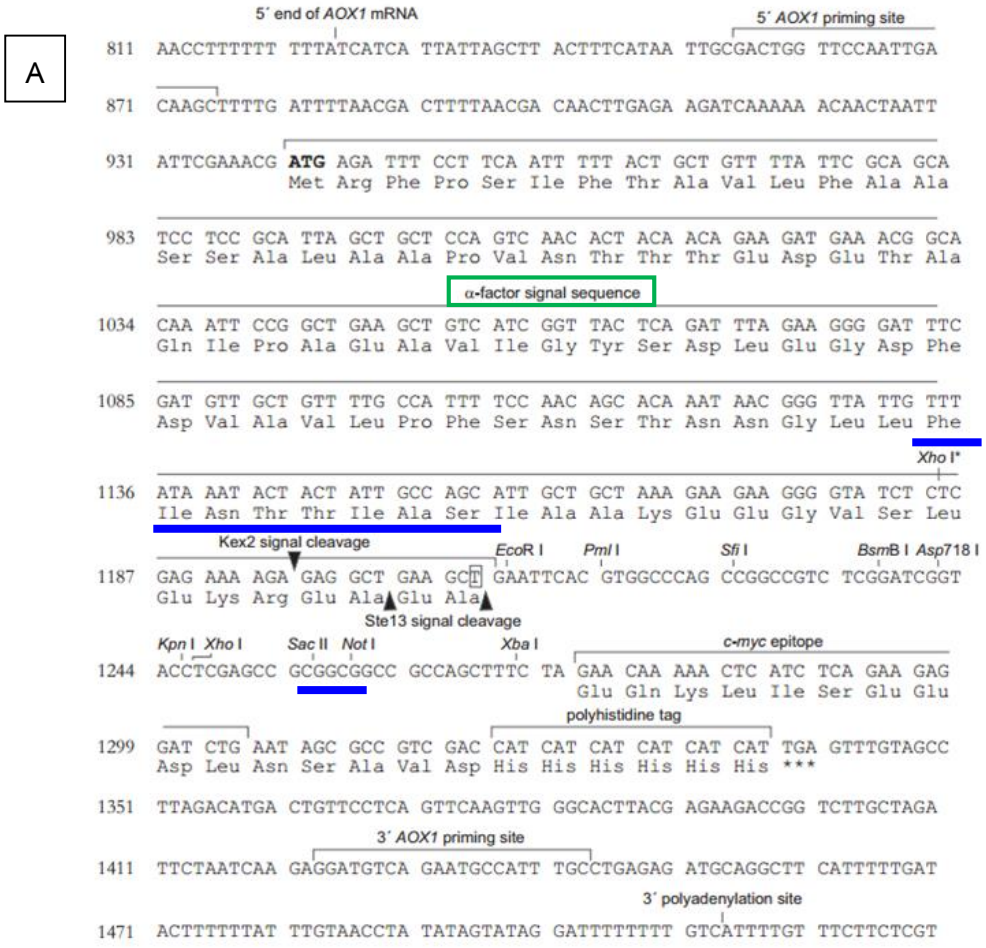

Figure 3. 2 Der p 13 GenBank accession number (HM560018.1)

Information from NCBI (data from http://www.ncbi.nlm.nih.gov/nuccore/HM560018.1).

The Der p 13 PCR product was then cloned into the *P. pastoris* expression vector pPICZαA (Invitrogen, USA) directly downstream to the sequence encoding the alpha mating factor leader sequence (Figure 3.3, panel A). Consequently, the following forward (5') and reverse (3') primers were designed to amplify only the mature Der p 13 coding sequence with the incorporation of restriction enzymes digestion site at the 5' and 3' end of amplicon. The Der p 13 cloning strategy was shown in pPICZαA

expression vector map (Figure 3.3, panel B), illustrating 5’ end of the amplicon consists of *XhoI* enzyme restriction site with the following of yeast secretion leader sequence, whereas, 3’ end consists of *NotI* enzyme restriction site. Therefore, the most important objective of this cloning strategy is to prevent any addition of extra amino acid into the target protein, including the c-Myc and the His tag at the c-terminus.



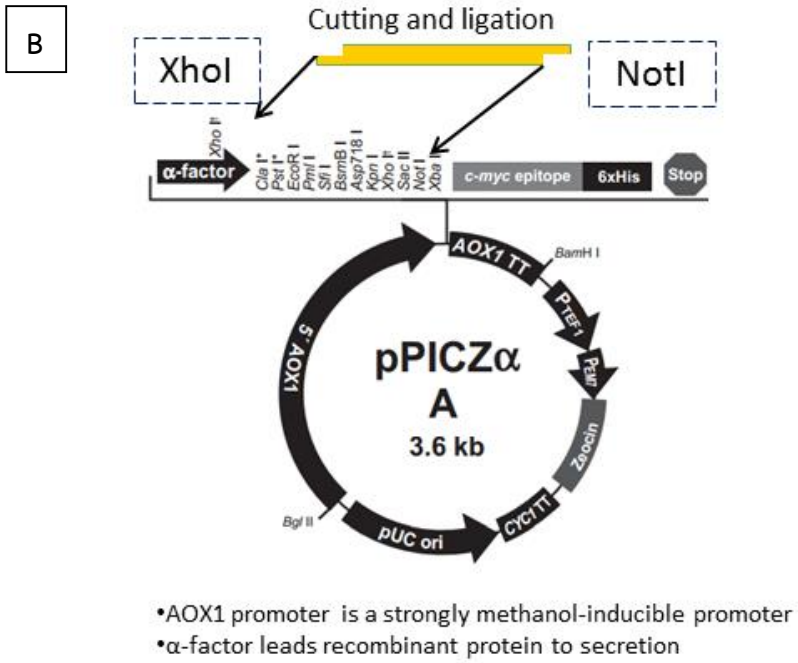


Figure 3. 3 Multiple cloning sites for pPICZαA yeast expression vector.

A) Amino acid sequence of the alpha mating factor leader sequence indicated in square box and restriction sites (XhoI & NotI) in blue underlined.

B) pPICZαA expression vector map, including AOX1 promoters and zeocin resistance gene, arrows indicated the restriction sites (derived from the pPICZαA expression vector manual, Invitrogen, USA).

3.1.2 agarose gel-electrophoresis

Der p 13 PCR product was mixed with loading buffer (20 % Ficoll 400, 0.1 M Na_2EDTA, pH 8, 1.0 % sodium dodecyl sulfate, 0.25 % bromphenol blue, 0.25 % xylene cyanol) and run on a 1.5 % agarose gel electrophoresis using TAE as migrating buffer. 0.7 µg Ready load 1 kb DNA ladder (Invitrogen, USA) was run as a standard. Der p 13 PCR products was checked by agarose gel-electrophoresis and visualized under UV light exposure by using Gel-Doc XR system (Bio-rad, USA).

3.1.3 Gel extraction

QIAquick Gel Extraction Kit (Qiagen, Germany) was used to extract any DNA products from agarose gel. The DNA fragment from the agarose gel was excised with a clean and sharp scalpel. The DNA extraction was prepared according to the manufacturer's protocol. The purified DNA was quantified at A_{260nm} by Nanodrop® ND-1000 UV-Vid spectrophotometer (ThermoFisher Scientific, USA) and kept at -20°C.

3.1.4 TA cloning

pGEM®-T Easy vector system (Promega, USA) was used in the cloning of purified Der p 13 amplicon. The cloning was performed with an insert to vector ligation in 10 µl ligation reaction; consisting in 2X rapid ligation buffer, 50 ng of pGEM-T Easy vector, 10 ng of purified Der p 13 DNA insert and T4 ligase (Promega, USA). The reaction was mixed at the room temperature for 1 hr and then carried on overnight at 16°C.

3.1.5 E.coli competent cell preparation

To start preparing *E.coli* DH5α competent cell, the *E.coli* glycerol stock were streaked on LB agar plate (1 % NaCl, 1 % tryptone, 0.5 % Yeast Extract, 2 % agar) and incubated for 16-20 hr at 37°C. Ten colonies were picked and inoculated into 250 mL of SOB medium (2 % trytone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) and incubated at 18 °C under shaking conditions at 200rpm. Cell growth was measured at OD_{600} nm. After, the cell density reached 0.6; the cells were pre-chilled on the ice for 10 min and pelleted at 3000 rpm for 10 min at 4°C. Then, the cell pellet was washed with ice-cold transformation buffer (TB: 10 mM Pipes, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$, pH 6.7) and resuspended in 20 mL of TB containing 7 % final concentration of DMSO. The cell suspension was kept (150 µl per

tube containing approximately $1X10^9$ cells) and frozen immediately under liquid nitrogen and subsequently stored at -80°C.

3.1.6 Transformation into *E. coli* competent cell

The frozen *E. coli* competent cells ($1 X 10^9$ cells/ tube) were placed and thawed on ice approximately for 5 min. The 10 µl of the overnight ligation was carefully incubated with the competent cells on ice for 30 min followed by a heat shock carried out at 42°C for 40 seconds and followed by incubation on ice for 2 min. Next, 0.85 mL of SOC medium were added and incubated at 37°C under shaking conditions at 200 rpm for 50 min. The cells were then pelleted at 13,000 rpm for 5 min. Supernatant of 500 µL was discarded and the cell pellet was suspended with remaining broth. Later, 100µL of each transformation culture was plated onto LB/ampicillin/IPTG/X-Gal plates (LB + 0.1 mM IPTG + 0.2 mg/mL of X-Gal) for 16 hr at 37°C for the selection of positive clones.

3.1.7 Colony PCR

Colony PCR was performed with Der p 13 specific primers to confirm the presence of the Der p 13 cDNA in the randomly selected colonies. Briefly, the selected colonies were picked with sterile pipette tips and directly transferred into PCR reaction mix. The PCR products were then analyzed on a 1.5% agarose gel electrophoresis and visualized under UV light exposure by using Gel-Doc XR system (Bio-rad, USA).

3.1.8 Plasmid extraction

The plasmid pGEMT-Der p 13 was purified from colony PCR-positive clones using the high-speed plasmid Mini Kit (Geneaid, Taiwan). 5 mL of culture bacteria cells was harvested and prepared according to the manufacturer's protocol. The purity of

plasmid was checked in the 1.5 % agarose gel electrophoresis. The DNA sequence of the Der p 13 was checked by DNA sequencing company (U2Bio, Thailand).

3.2 Cloning strategy of Der p13 into *P. pastoris* KM71

3.2.1 DNA double digestion

20 µg of purified pGEM T-Der p 13 plasmid was double restricted by using *XhoI* and *NotI* (NEB, USA). In parallel, 4 µg of pPICZαA expression vector (Invitrogen, USA) were also digested with the same restriction enzymes. The efficacy of the double digestions was checked by 1.5 % agarose gel electrophoresis. DNA pieces corresponding to the restricted Der p 13 cDNA and linearized pPICZαA were gel purified by QIAquick Gel Extraction Kit (Qiagen, Germany). The DNA fragment from the agarose gel was excised with a clean and sharp scalpel. The gel extraction containing pGEM T-Der p 13 plasmid was prepared according to the manufacturer's protocol. The purified plasmid was quantified at A_{260nm} by Nanodrop® ND-1000 UV-Vid spectrophotometer (ThermoFisher Scientific, USA) and kept at -20°C. For the nanodrop read-out (1 unit A260nm) corresponded to 500 ng DNA/mL.

3.2.2 DNA ligation

After Der p 13 fragments and the linearized pPICZαA was purified. Both of the products were ligated as the pPICZαA –Der p 13 plasmids. Ligafast rapid DNA ligation system (Promaga, USA) was used in DNA ligation step. The DNA ligation was carried out according to the manufacture's protocol at 16°C for overnight. After that, the ligation was transformed by heat shock technique into DH5α in order to increase to plasmid yield. The LB agar plate with the additional of 50 µg/mL Zeocin was selected to screen

the positive clones as the pPICZαA –Der p 13 plasmids contained Zeocin resistant gene. The positive clones were screened and selected for the inoculation. Next, designed plasmid was then gel purified and sent for DNA sequencing (U2Bio, Thailand)

3.2.3. *P. pastoris* competent cell preparation

P. pastoris KM71 strain was grown in YPD (1 % yeast extract, 2 % peptone, 2 % dextrose (glucose)) at 30°C for overnight. The 500 mL of fresh medium was inoculated in a 2 L flask with 0.1–0.5 mL of the overnight-culture. When the density of culture reached OD_{600} nm = 1.3-1.5, then the culture was centrifuged at 1500 × g for 5 min at 4°C. The supernatant was discarded but the cell pellet was resuspended in 500 mL of ice-cold (0°C), sterile water. The resuspension was then centrifuged for 2 times and resuspended in 20 mL of ice-cold (0°C) 1 M sorbitol. The cell pellet was resuspended again in 1 mL of ice-cold 1 M sorbitol for a final volume of approximately 1.5 mL. The cells were kept on ice and continued with the transformation. At this step, the cells were only for 1 time use and must be prepared freshly.

3.2.4 *P. pastoris* KM71 strain transformation by electroporation

The transformation of purified pPICZαA-Der p 13 plasmids into *P. pastoris* KM71 strain started from the linearization with *Sac I* restriction enzyme (NEB, USA). The small aliquot of digestion was checked in 1 % agarose gel electrophoresis for complete linearization. Once the vector was completely linearized, heat inactivated to stop the reaction. 5 μg of linearized pPICZαA-Der p 13 was transferred to an ice-cold (0°C) 1 mM electroporation cuvette and incubated the cuvette with the cells on ice for 5 min. Then Gene PulserXcell™ (Bio-rad, USA) was used to transform the plasmid at 1,500 V, 200 Ω in 1 mM electrode cuvette. The products were spread in YPDS agar plate (1 %

yeast extract, 2 % peptone, 2 % dextrose, 1 M sorbitol, 2 % agar) containing 100 μg/mL Zeocin (Invitrogen, USA) and incubated at 30°C for a couple days. Approximately 10–20 colonies were picked to perform colony PCR. The positive clones were confirmed and prepared for the initiation of protein expression.

3.3 rDer p 13 Expression

According to the Invitrogen protocol of protein expression in *P. pastoris*, the positive Zeocin-resistant colonies were selected and inoculated overnight at 30°C under shaking conditions in YPD starter culture medium (1 % yeast extract, 2 % peptone, 2 % dextrose). Then, the overnight cultures were transferred into secondary cultures using the 500 mL of BMGY medium (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate, pH 6.0, 1.34 % YNB, 4×10^{-5} % biotin, 1 % glycerol) under the same culture conditions. Once the cell density reached an OD_{600nm} of 5, the cultures were pelleted and the cells were resuspended at a final OD_{600nm} of 10 in 250 mL of BMMY medium (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate, pH 6.0, 1.34 % YNB, 4×10^{-5} % biotin) containing from 0.5 to 5 % methanol to trigger Der p 13 expression. Every 24hrs, the culture medium was sampled and methanol was added to maintain the inducer concentration. After selection of the best Der p 13 producing clone and the optimization of the culture and methanol induction conditions, large scale cultures were initiated in order to collect the supernatant containing rDer p 13 for the allergen purification. The high speed centrifugation at 13,000 rpm for 10 min was performed to separate the secreted rDer p 13 in supernatant from the cell pellets.

3.4 Purification of rDer p 13

The supernatants containing secreted rDer p 13 were purified by using different strategies to get the maximum yield and highest purity. We observed difficulty in purifying the rDer p 13. So, we came up with different methods in purification of rDer p 13.

3.4.1 Cation-exchange chromatography

The supernatants containing secreted rDer p 13 were diluted ten times with MilliQ water and the pH was adjusted to pH 4. This material was applied onto a SP sepharose Ion Exchange Chromatography column (GE Healthcare Life sciences, UK) or S Ceramic HyperD® F Ion Exchange Chromatography Sorbents column (PALL, USA), (1.6cm in diameter, 3 cm tall) that connected with AKTA prime purification system (GE Healthcare Life sciences, UK). For the SP sepharose Ion Exchange Chromatography column, it was equilibrated with 20 mM sodium acetate pH 4.0 at the flow rate of 15 mL/min. After washing extensively, the unbound proteins from the column with the equilibrating buffer until the A_{280nm} reached the baseline, a first elution step was performed using 20 mM sodium acetate pH 4.0 containing different concentration as 100 mM, 200 mM, 300 mM, 500 mM, and 1000 mM NaCl. For S Ceramic HyperD® F Ion Exchange Chromatography Sorbents column, a 1^{st} elution step was performed using 20 mM sodium acetate pH 4.0 containing 1000 mM NaCl to remove contaminants and followed by a 2^{nd} elution step with 20mM Tris-HCl pH 9.0. The purified fractions were then analyzed onto the SDS-PAGE.

3.4.2 Anion-exchange chromatography

The supernatants containing secreted rDer p 13 were diluted ten times with MilliQ water and the pH was adjusted to pH 9. This material was applied onto a Q sepharose Ion Exchange Chromatography column (1.6cm in diameter, 3 cm tall) (GE Healthcare Life sciences, UK) that connected with AKTA prime purification system (GE Healthcare Life sciences, UK). The column was equilibrated with 20 mM Tris-HCl pH 9.0 at the flowrate of 15 mL/min. After washing extensively, the unbound proteins from the column with the equilibrating buffer until the A_{280nm} reached to baseline, a first elution step was performed using 20 mM Tris-HCl pH 9.0 containing different concentration as 100 mM, 200 mM, 300 mM, 500 mM, and 1000 mM NaCl. The purified fractions were then analyzed onto the SDS-PAGE.

3.4.3 Hydrophobic interaction chromatography

The supernatants containing secreted rDer p 13 were diluted 1 to the third with saturated 4M $(NH_4)_2SO_4$ and adjusted to pH 7. This material was applied onto a Phenyl sepharose Chromatography column (1.6cm in diameter, 3 cm tall) (GE Healthcare Life sciences, UK) that connected with AKTA prime purification system (GE Healthcare Life sciences, UK). The column was equilibrated with 1000 mM $(NH_4)_2SO_4$ in PBS pH 7.0 at the flowrate of 15 mL/min. After washing extensively, the unbound proteins from the column with the equilibrating buffer until the A_{280nm} reached to baseline, a first elution step was performed using PBS pH 7.0 containing different concentration as 200 mM, 400 mM, 600 mM, 800 mM and 1000 mM 1000 mM $(NH_4)_2SO_4$. The rDer p 13 was subsequently eluted with MilliQ water. The purified fractions were then analyzed onto the SDS-PAGE.

3.4.4 Mixed-Mode chromatography

The supernatants containing secreted rDer p 13 were diluted 1 to the third with saturated 4M $(NH_4)_2SO_4$ and adjusted to pH 7. This material was applied onto a pre-pack 1 mL of Mep-Hypercel™ Sorbent column (Pall, USA) manually. The column was equilibrated with MilliQ water manually. After washing extensively, the unbound proteins from the column with the equilibrating buffer, a first elution step was performed using MilliQ water for 5 column volumes and subsequently eluted with and 20 mM sodium acetate, pH 4.0 for 5 column volumes as well. The purified fractions were then analyzed onto the SDS-PAGE.

3.4.5 Size exclusion chromatography

For the second step purification, the purified fractions of rDer p 13 from S Ceramic HyperD® F Ion Exchange Chromatography were pooled together and concentrated to the minimum using 3kDa cut-off membrane (Pall, USA). The allergen purification was achieved by a gel filtration step onto a Superdex 75 HR column 10/30 (GE Healthcare Lifesciences, UK), at a flow rate of 0.5 mL/min in PBS pH 7.4

After that, the purified rDer p 13 was filtered with 0.22 μM membrane (Millipore, USA) to minimize the contamination. The purity of rDer p 13 was also analyzed again onto the SDS-PAGE.

3.5 SDS-PAGE

12% SDS-PAGE (final concentration) gel was prepared with the combination of resolving gel (1.5 M Tris-HCl, pH 8.8, 40% acrylamide (ratio19:1), 10% SDS, 10% APS and TEMED (Bio-rad, USA)) and stacking gel (1.0 M Tris-HCl, pH 6.8, 40% acrylamide

(ratio19:1), 10% SDS, 10% APS and TEMED (Bio-rad, USA)), together with the SDS-PAGE electrophoresis running buffer (1XSDS; 25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH8.3). SDS sample loading buffer with reducing agent (4X; 240 mM Tris-HCl, pH 6.8, 40% Glycerol, 8% SDS, 5% of 2-β-mercaptoethanol, and 0.04% bromophenol blue) or SDS sample loading buffer without reducing agent (4X; 240 mM Tris-HCl, pH 6.8, 40% Glycerol, 8% SDS, and 0.04% bromophenol blue) was added to the protein sample (ratio 1:3) and heated at 95ºC in heat box before the run. PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (ThermoFisher Scientific, USA) was loaded as the control protein marker. After the protein finished migrating, the SDS-PAGE was stained with commassie blue R-250 (Bio-Rad, USA) for a suitable period (1 hr or overnight). The SDS-PAGE was then de-stained using destaining solution (10% (v/v) acetic acid, 40% (v/v) Methanol, 50% (v/v) distilled water).

3.6 Protein quantity measurement

The fractions containing purified rDer p 13 were pooled and the protein concentration was assay by using the microBCA protein assay kit (Pierce, USA). A protein standard calibration curve was made using 1 mg/mL of bovine serum albumin (BSA) stock.

3.7 Mass spectrometry

rDer p 13 samples were analyzed using nano UHPLC (nanoRSLC Ultimate 3000, ThermoFisher Scientific, USA). Briefly, 20 ng of intact rDer p 13 or 2 µg of the allergen digested in solution with trypsin (0.1 µg) were loaded on an Accucore C18 column

(C18, 2.6 µm, 150 Å, 15 cm x 75 µm i.d., ThermoFisher Scientific, USA) with a flow rate of 450 µL/min and eluted with a gradient of acetonitrile. MS analysis was performed using an Impact HD mass spectrometer (Bruker Daltonics, USA) equipped with a Captive Spray source (Bruker Daltonics, USA). Acquisitions were performed in positive mode with end plate offset and capillary voltages set at -500 and 1300 V, respectively. MS spectra were acquired over the m/z range 110-2000 with a scan rate of 2 Hz. MS/MS spectra were acquired using the Intensity Dependent Acquisition Speed (IDAS) mode with a MS-MS/MS cycle time fixed at 2 s, a MS/MS trigger threshold set at 4.3 x 10^3 counts (cts) and a MS/MS acquisition speed defined as follow: 2 Hz for precursor intensity below 2.5 x 10^3 cts and 8 Hz for precursor intensity above 25 x 10^3 cts. Active exclusion list was enabled and precursor ions were excluded after one spectrum for 0.35 s.

3.8 Circular dichroism (CD) analysis

The secondary structure content of purified rDer p 13 was determined by far-UV circular dichroism (CD) with a Jasco J-815 CD spectrometer kindly analyzed by Assoc.Prof. Dr. Surapon Piboonpocanun, Institute of Molecular Biosciences, Mahidol University. The recombinant protein at a 0.2 mg/mL concentration was previously dialyzed against 10 mM NaH_2PO_4 buffer pH 4.8. The spectra were acquired using a 1 mM path length quartz cuvette from 190 to 280 nm with 1 nm resolution at speed 50 nm/min for 5 cycles. Variable temperature measurements were also used to evaluate the melting point (stability) of rDer p 13. A certain wavelength was monitored as the temperature changes with the increment of 25°C (from 25°C until 100°C). The effect

of 1,4-Dithiothreitol (DTT, Sigma-Aldrich, USA) was examined with the rDer p 13 at 50 fold. The percentage of the different secondary structures were converted to mean residue weight ellipticity (θ) mrw (degrees cm^2 $dmol^{-1}$) and then analyzed with CDPro software.

3.9 Murine rDer p 13-specific polyclonal antibody production

Polyclonal antibodies were produced following mice immunizations with purified rDer p 13 and the mixture of Al (OH_3) as an adjuvant. Five BALB/C female mice (6 weeks old) from the National Laboratory Animal Center, Mahidol University, Nakhornpathom, Thailand) were immunized by three intraperitoneal injections at two weeks interval. Such immunization program was approved by the Chulalongkorn University Animal Center and Use Committee in IRB no. 11/2557. The antigen was formulated with Al (OH_3) (Sigma–Aldrich, USA) at the ratio of 1/100 (allergen/adjuvant) in normal saline buffer under agitating conditions at the room temperature. Before the injection, pre-immune serum (200 µL of blood/mouse) was collected from the facial vein of each mouse. As the area of blood collection was from the facial vein, the anesthetization by Isoflurane aerosol (Terrell, USA) wasn't necessary. The amount of 10 µg of rDer p 13 (total amount 300 µL/mouse) was then injected in the peritoneal cavity of each mouse per immunization. Two weeks after the last immunization, immune sera (200µL of blood/mouse) were collected from the facial vein of each mouse. The following sera were centrifuged at 300 X g for 3 min. The red blood cells were discarded and the sera were stored in -20ºC.

3.10 Specificity of anti-rDer p 13 antibodies

3.10.1 Direct ELISA analysis

The production of anti-rDer p 13 antibodies was determined in a direct ELISA assay. rDer p 13 (500 ng/well) was directly coated into Nunc Maxisorp® ELISA plates at 100 µL/ well (ThermoFisher Scientific, USA) using 0.1 M sodium carbonate pH 9.5 at 4°C overnight. To compare the specificity of anti-rDer p 13 antibodies, the negative control recombinant proteins including rDer p 2, rDer p 5, rDer p 7, rDer p 21, and rDer p 23 (500 ng/well of each protein) were also coated with 0.1 M sodium carbonate pH 9.5 at 4°C overnight in the same ELISA plates (total amount 100 µL/well). These negative control recombinant proteins were previouly produced in our laboratory. The plates were washed five times with washing buffer (1X PBS- Tween 20, 0.05 %, PBS-T). The blocking step was performed by addition of 150 µL/well of PBS-Tween 20, 0.05 % + 1 % BSA (PBS-T B) for 1 hr at 37°C. Pre-immune and immune sera serially diluted (dilution range from 10^2-10^8) in PBS-T-B were then added into wells for 1 hr at 37°C. After a washing step with PBS-T, the plates were incubated with 1:5000 dilution of goat-anti mouse antibodies conjugated with horseradish peroxidase (KPL, USA) at 37°C for 1 hr. The plates were than washed again and TMB substrate (BD Biosciences, USA) were used to detect the allergen-antibody complexes. 50 µL/ well of 0.5 M sulfuric acid were used to stop the reaction and A_{450nm} was subsequently read using iMark microplate reader (Bio-Rad, USA).

3.10.2 Colorimetric western bolt analysis

Colorimetric western blot was selected for the basic detection of rDer p 13. Following 12% SDS-PAGE and protein transfer onto nitrocellulose membrane (Bio-rad,

USA), the membrane was then blocked with TBS, 1 % (w/v) BSA, 0.05 % (v/v) Tween 20, at 4°C, for overnight. Next, the membrane was incubated with mouse anti-rDer p 13 polyclonal serum at 1:5000 dilution. As a control, the membrane was also incubated with pre-immune serum). The membrane was washed with TBS-T (1X TBS-Tween 20, 0.05 %) and further incubated with 1:3000 rabbit-anti-mouse antibodies conjugated with Alkaline Phosphatase (KPL, USA). The immunoreactive bands were detected using the NBT/BCIP substrate solution (0.03 % NBT and 0.02 % BCIP, Bio-Rad, USA) and the reaction was stopped with distilled water.

3.11 Detection of natural Der p 13 (nDer p 13) in HDM extracts

3.11.1 HDM extracts preparation

Two HDM allergen extracts were selected for the detection of nDer p 13; enriched mite bodies extract and enriched HDM feces extract.

2 mg of commercially-enriched mite bodies extract (Greer, USA) were resuspended in 1 mL of PBS endotoxin free to get the final concentration of 2 mg/mL and kept at -20°C for further analysis.

Whereas, 36 mg of Purified *D. pteronyssinus* feces (Stallergenes-Greer, France) were extracted in 200 µL of PBS endotoxin free at 4°C for overnight under mild agitation. The soluble extract was collected by centrifugation at 13,000 rpm for 5 min. The extract was kept at -20°C for further analysis.

For the purpose of IgE detection, the specific polyclonal antibodies to rDer p 13 was purified using immobilized rDer p 13 beads. For the matrix preparation, 0.5 mg

of rDer p 13 was dialyzed against the coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) overnight at 4°C. The prepacked HiTrap™ NHS-activated HP column was washed with ice-cold, 1 mM HCl and rDer p 13 was immediately injected onto the column for the protein coupling. The rDer p 13 was recirculated by gently pumping the solution back and forward for 15–30 min. Then the column was let to stand for 15–30 min at 4°C for 4 hr. Later, the column was washed to deactivate any residual active groups by 0.5 M Tris-HCl, 0.5 M NaCl, pH 8.3. Any non-conjugated rDer 13 was removed by successive washing steps using 0.5 M Tris-HCl, 0.5 M NaCl, pH 8.3 followed by the application of (0.1 M sodium acetate, 0.5 M NaCl, pH 4.0). For the purification of anti-rDer p 13 mice antibodies, immobilized rDer p 13 column was equilibrated in 20mM Phosphate Na pH 7.4, 150mM NaCl at a flow rate of 1 mL/min. A pooled of immune-sera was directly injected onto the immobilized rDer p 13 column. The column was then washed with the equilibrating buffer until the A280nm reaches the baseline. The specific antibodies were eluted using 100 mM Glycine-HCl pH 2.8. The eluted fractions were immediately neutralized by addition of 100 µl of 1 M Tris-HCl pH 9). Each fraction was then analyzed onto SDS-PAGE and stored at -20°C.

For the nDer p 13 detection, the specific polyclonal antibodies to rDer p 13 were directly used. Both mite bodies extract (Greer, USA) and feces extract (Stallergenes-Greer, France) were loaded onto 15 % SDS-PAGE and transferred onto nitrocellulose membrane (Bio-rad, USA), the membrane was then blocked with PBS, 1 % (w/v) BSA, 0.05 % (v/v) Tween 20, at 4°C, for overnight. Next, the membrane was incubated with mouse anti-rDer p 13 polyclonal serum at 1:5000 dilution. As a control, the membrane was also incubated with pre-immune serum. The membrane was washed with PBS-T and further incubated with 1:5000 goat-anti-mouse antibodies

conjugated with horseradish peroxidase (KPL, USA). The immunoreactive bands were detected using the chemiluminescent HRP substrate (Millipore, Germany) and following X-ray film exposition (Kodak, USA).

3.12 Spectrofluorometric lipid binding assay

Ligand binding by rDer p13 was investigated spectrofluorometrically using a Perkin Elmer LS50 Spectrometer (Perkin Elmer, USA) at room temperature and a set a fluorescent lipid probes: the naturally fluorescent fatty acid cis-parinaric acid (cPnA, Invitrogen, USA), the fluorophore-tagged fatty acids 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3 hexadecanoic acid (Bodipy® FL C16, Invitrogen, USA), 11-([5-dimethylaminonaphthalene-1 sulfonylamino]) undecanoic acid (DAUDA, Invitrogen, USA), and dansyl-DL-**α**-aminocaprylic acid (DACA, Sigma-Aldrich, USA), the fluorescent hydrophobic probe 1-anilinonapthalene-8 sulfonate (ANS, Sigma-Aldrich, USA), 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS, Sigma Aldrich, USA), the naturally fluorescent sterol dihydroergosterol (DHE, Sigma-Aldrich, USA), and dansyl-glycine (Sigma Aldrich, USA) used as a control for binding by the dansly group on its own. The excitation wavelengths for cPnA, Bodipy® FL C16, ANS, bis-ANS, DHE, DAUDA, DACA, and dansylglycine were 319, 488, 390, 395, 325, 345,345, and 345 nm, respectively. The different lipid probes were stored as stock solutions of 10 mM in ethanol, in the dark at -20°C, and freshly diluted in phosphate buffered saline pH 7.4 (PBS) to 1 mM before use in the fluorescence experiments. For competition binding experiments, oleic acid (Sigma-Aldrich, USA) was prepared in ethanol and diluted to concentrations of 7.9 μM, 79μM and 790 μM in PBS for use in the assays. Raman scattering by solvent

water was corrected for when necessary using appropriate blank solutions. Proteins used as positive controls were bovine serum albumin (BSA, Sigma-Aldrich, USA) and bovine β-lactoglobulin (Sigma–Aldrich, USA), which were prepared as stock solutions at 10 mg/mL in PBS. The titration was carried out by incremental addition of 7.4 μM rDer p13 directly to 2 mL cuvette containing fluorescent cPnA (3 μM) in PBS. Fluorescence emission intensities (recorded at 417 nm) were corrected for dilution and fluorescence of free ligand, and fitted using a standard nonlinear regression equation (Microcal ORIGIN software) to a single noncompetitive binding model to give estimates of the dissociation constant (K_d).

3.13 Modeling of the Der p 13 tertiary structure and its complex with cis-parinaric acid

MODELLER 9.14 program was used to predict the 3D structure of Der p 13 by comparative modeling. As templates, we selected the Der f 13 NMR structure (apo form, 95% sequence identity, PDB code 2A0A) as well as the crystal structure of myelin P2 protein from equine spinal cord associate to a lipid ligand (holo form, 32% sequence identity, (PDB code 1YIV). This second template was selected by searching in all the Protein Databank (PDB) for a structure that is similar to Der f 13 (PDB code 2A0A) and that corresponds to a holo form. The PDBeFold structure superimposition server was used for that purpose. This second template was thus selected on the basis of criteria of structural similarity with Der f 13. The amino acid sequence alignment was performed using EMBOSS Stretcher on the Mobyle portal (http://mobyle.pasteur.fr). We computed a population of 5 models with each template and evaluated their quality with the normalized DOPE score implemented in MODELLER. We selected the

best model according to this score for further analysis. The cavities that could potentially accommodate a lipid in the two Der p 13 models were identified with the CASTp server. In order to model the structure of a complex between Der p 13 and a fatty acid, protein-lipid docking simulations were performed by AutodockVina (www.autodock.scripps.edu) using the two 3D models of Der p 13 and cis-parinaric acid (cPnA) as a lipid ligand. The cPNA 3D structure was modeled by the Corina program (www.molecularnetworks.com /online_demos/ corina_demo) and its flexibility was set by Autodock tool to mimic the appropriate docking conditions. The AutodockVina output contained the nine best poses according to the computed binding energy.

3.14 IgE reactivity to rDer p 13

Two hundred and twenty-four *D. pteronyssinus* ImmunoCap Class 3-6 (>3.5 to >100 kU/L) sera from patients with HDM-associated allergic rhinitis or asthma were obtained from the King Chulalongkorn Memorial (n= 76), Children (n = 81), Ramathibodi (n= 57) and Phramongkutklao (n= 10) hospitals, respectively. Written informed consent was obtained for each case. As negative controls, sera from non-allergic subjects were also collected (ImmunoCap Class 0, <0.35kU/L, n = 67). The study was approved by the Ethic Committees from the Faculty of Medicine, Chulalongkorn University (IRB 023/55), Children hospital (IRB 195/2556), Faculty of Medicine, Ramathibodi hospital, Mahidol University (IRB 03-5634) and Phramongkutklao College of Medicine (IRB S039Q/57_EXP). For the IgE binding assays, ELISA microplates were coated with goat anti-human IgE (1:1000 dilution, KPL, USA) at 4°C for overnight (total amount 100 µL/well). The plates were then washed with PBS-Tween 20, 0.05 % (PBS-T) and blocked

with PBS-T containing 1 % BSA (PBS-T-BSA) for 1 h at 37°C. Serum samples were diluted at 1:8 in PBS-T-BSA and incubated at 37°C for 1 h. The plates were then washed again with PBS-T and further incubated with rDer p13 (500 ng/mL) at 37°C for 1 h. Following another typical washing step, the wells were incubated with purified anti-rDer p 13 mouse polyclonal antibodies (3.5 ng/well) at 37 C for 1h. Next, the plates were washed with PBS-T and incubated with goat HRP-conjugated anti-mouse 1:5000 dilution (KPL, USA) for 1 hr at room temperature. The allergen-antibody complex was detected with TMB substrate (BD Biosciences, USA) and the reaction was stopped with 0.5 M sulfuric acid. Optical density (OD) was determined at 450 nm using iMark microplate reader (Bio-Rad, USA).

The IgE reactivity of rDer p 2 was selected as a control for IgE reactivity of rDer p 13 using the same cohort of HDM allergic patient sera. The strategy of IgE binding assay in Der p 2 was different from Der p 13. ELISA microplates were coated with rDer p 2 (500ng/well) 4°C for overnight (total amount 100 µL/well). The plates were then washed with PBS-Tween 20, 0.05 % (PBS-T) and blocked with PBS-T containing 1 % BSA (PBS-T-BSA) for 1 hr at 37°C. Serum samples were diluted at 1:8 in PBS-T-BSA and incubated at 37°C for 1 hr. The plates were then washed again with PBS-T and further incubated with goat biotinylated conjugated anti-human IgE 1:5000 dilution (KPL, USA) for 1 hr at 37°C. After the washing step, the ELISA microplates were incubated with streptavidin conjugated-HRP (KPL, USA) for 1 hr at 37°C (total amount 100 µL/well). The allergen-antibody complex was detected with TMB substrate (BD Biosciences, USA) and the reaction was stopped with 0.5 M sulfuric acid. OD was determined at 450 nm using iMark microplate reader (Bio-Rad, USA). A patient serum was considered as

positive when the measured OD value was higher than the cut off value, established as the mean OD values of negative control sera plus 2 standard deviations.

3.15 Rat basophil degranulation (RBL) assay

Genetically modified Rat basophil leukemia cells expressing humanFc**ε**R1 receptor (RBL SX-38) were selected for the basophil degranulation assay. The cells were cultured using RPMI 1640 supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin at 37ºC, 5% CO_2 (Gibco, USA). When the cell densities reached 80% confluency, the amount of $1.5x10^5$ cells/ well were seeded into 96-well culture plate. The cells were incubated at the culture conditions for 3 hr. The attachment of cells was observed under the microscope. Cells were primed with positive IgE against rDer p 13 human sera at 1:10 dilution for overnight. Cells were then washed with PBS and incubated with RPMI 1640 phenol red free (Gibco, USA) containing 1 mg/mL BSA for 15 min. The serial dilutions of rDer p 13 (0.00001 µg/mL -1 µg/mL) were added into each well for 30 min. Final concentration of 5% (v/v) Triton X-100 was used as maximum released of β-hexosaminidase. After the cells were incubated with serial dilution of rDer p 13 for 30 min, the 50 µL of supernatant were carefully aspired and transferred to the plate containging 50 µL/well of 2.5 mM p-nitrophenyl N-acetyl-β-D-glucosaminide and further incubated for 3 hr. Finally, the release of β-hexosaminidase reaction was stop with 150 µl of 1 M Tris-HCl, pH 9 and directly read the absorbance at 415 nm by using iMark microplate absorbance reader (Bio-Rad, USA).

In order to compare the result from basophil degranulation, rDer p 2 was also selected as the protein control, whereas, polyclonal anti-human IgE (0.1 μg/mL, KPL, USA) was performed as the positive control. Cells were primed with positive IgE against rDer p 2 human sera at 1:10 dilution for overnight and followed by the same protocol as rDer p 13. The release of β-hexosaminidase was evaluated at the end of assay.

3.16 Human bronchial epithelial cell activation

The BEAS-2B human bronchial airway epithelial cells (ATCC, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C, 5 % CO_2 (Gibco, USA). When the cell densities reached 80 % confluency, the amount of $2.0x10^5$ cells/ well were seeded into 24-well culture plate.

3.16.1 Direct airway epithelial cells activation

The cells in 24 well-plates were incubated at the culture condition for 24 hr. The attachment of cells and cell morphology was observed under the microscope. Subsequently, the cells were incubated in serum-free DMEM for a further 24 hr. The cell stimulation began with the additional of fresh serum-free DMEM media with different concentration of rDer p 13 (1 μg, 5 μg, 10 μg, and 20 μg/mL) in triplicates for 16 hr. In order to compare the results of direct airway epithelial cells activation, rDer p 23 and rProDer p 1 were also selected as the protein controls. The cell stimulations with both proteins were performed using the same concentration as rDer p 13 (1 μg-20 μg/mL). The rDer p 23 is the chitin binding protein which we previously

demonstrated the ability to activate airway epithelial cells, whereas, rProDer p 1 is the pro form of Der p 1 which might not display the ability of cell activation and we assumed no cytokines would be detected in our experiments. As well as, a wild-type KM71 *P. pastoris* BMMY culture medium fractionated using the Der p13 purification protocol as a control. For the positive control, the Pam_3CSK_4 TLR2 ligand (100 ng/mL, Invivogen, USA) was always presence in every cell activation experiment. The different cell supernatants were collected for the detection of IL-8 and GM-CSF by ELISA assays (BD Biosciences, USA).

In order to investigate the important of the of TLR2 in rDer p 13 stimulation, the next experimental conditions were involved with the blockade of TLR2. In the 24 well-plates, BEAS-2B cells were pre-incubated with anti-human TLR2 blocking antibody (Mab TLR2.5, 10 µg/mL, eBiosciences, USA) or an isotype control at 37ºC for 1 hr. Subsequently, the cells were stimulated with the additional of fresh serum-free DMEM media together with of 10 µg/mL of rDer p 13 for 16 hr. The different cell supernatants were collected for the cytokines detection.

For inhibition of intracellular signaling, cells were transfected with plasmid expressing dominant negative version of human MyD88 (pDeNy-hMyD88, Invivogen, USA) or with pCDNA control vector (Invitrogen, USA). The Lipofectamine®3000 (Invitrogen, USA) were used to co-transfect with the plasmid DNA. According to the manufacture's protocol, Lipofactamine®3000 reagent was diluted with OptiMEM® Medium (Invitrogen, USA), as well as, 2 µg of pDeNy-hMyD88 and 2 µg of pCDNA. Both plasmids were prepared as DNA-lipid complex and directly added to the cells for 48 hr at the culture condition. The transfected cells were visualized under the microscope before the cell stimulation. 10 µg/mL of rDer p 13 were then activated the transfected

cells for 16 hr. The different cell supernatants were collected for the cytokines detection.

In the next experiments, we decided to work with intracellular signaling inhibitors including mitogen-activated protein kinase (MAPK) (U0126, 20 μM; SB203580, 1 μM; SP6001125, 20 μM) or inhibitors specific for NF-**K**B (MG132, 10 μM; BAY-11–7082, 10 μM) (Invivogen, USA). All the inhibitors were dissolved in 100% DMSO as the inhibitor stocks and stored in dark at -20°C. Before the cell stimulation, the inhibitors were diluted with DMSO to the appropriated amounts and pre-treated to the cells at the culture condition for 1 hr. The 20 μM of DMSO only was also pre-treated to the cells as the control. The cells were then stimulated with 10 μg/mL of rDer p 13 for 16 hr and supernatants were collected for the cytokines detection. After the collection of cell supernatants, the cell viability was examined in Trypan Blue Exclusion Test.

Finally, the digested form of rDer p 13 was also investigated in cell activation. In order to prepare digested Der p 13, the recombinant allergen was treated at pH 7 with trypsin (1:20 of enzyme/substrate ratio; Gibco, USA) for 1 hr at 37 °C. As controls, rDer p 13 or trypsin alone were incubated under the same conditions. Prior to cell activation, the protein solutions were treated HiTrap Benzamidine FF beads (GE Healthcare Lifesciences, USA) to remove trypsin.

3.16.2 Detection of human IL-8 and GM-CSF

The different cell supernatants were collected for the detection of IL-8 and GM-CSF by ELISA assays (BD Biosciences, USA). According to the manufacture's protocol, the capture antibodies of human IL-8 (Anti-Human IL-8 monoclonal antibody) or human GM-CSF (Anti-Human GM-CSF monoclonal antibody) were coated in 96 well-

plates at 4°C for overnight. The human IL-8 (100 ng/mL) or human GM-CSF (250 ng/mL) were serial diluted in 1 % BSA PBS-T for the ELISA standard curve, together with the incubation of cells supernatants into 96 well-plates at 37°C for 1 hr. The plates were washed with PBS-T extensively and further incubated with detection antibody (Biotinylated Anti-Human IL-8 monoclonal antibody or Biotinylated Anti-Human GM-CSF monoclonal antibody) and enzyme reagent (Streptavidin-horseradish peroxidase conjugate (SAv-HRP)) at 37°C for 1 hr. TMB substrate (BD Biosciences, USA) were used to detect the cytokines production.

3.17 Statistical analysis

Student's t-test was used to compare significant different between treatments in duplicates from the cell activation experiments. P-values of <0.05 was considered as statistically significant. Prism 5.0 (GraphPad software Inc., USA) was used to calculate the significant values.

CHAPTER IV

RESULTS

4.1 Cloning of Der p 13 cDNA

The amplification of the cDNA encoding the mature form of Der p 13 (AA 2-131) was performed using two specific primers which were designed from the sequence HM560018.1 (GenBank accession number) and a *D. pteronyssinus* cDNA library. The Der p 13 cDNA was successfully amplified, as judged by the agarose gel electrophoresis analysis showing the correct size of the PCR product (396 bps) (Figure 4.1.1). The PCR product was cloned into pGEM T easy vector for sequencing and then prepared for the DNA to be restricted. The nucleotidic sequence of the PCR amplicon was aligned by using Emboss stretcher. and matched perfectly (perfectly = 100%) with the one from the GenBank database (HM560018.1).

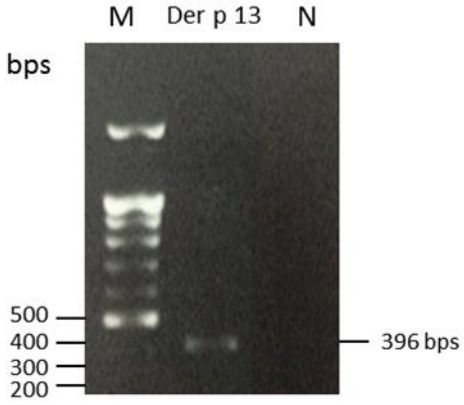


Figure 4.1. 1 PCR amplification of Der p 13 cDNA.

The PCR product was analyzed onto 1% agarose gel electrophoresis. M: molecular weight marker (1 kb hyperladder), N: negative control (PCR master mix without DNA template).

The mature Der p 13 cDNA was then cloned into the pPICZ**α**A expression vector. Following transformation of the P. *pastoris* strain KM71 with the recombinant pPICZaA-Der p 13, clones were successfully selected on agar plates containing 100 µg/mL zeocin. Randomly selected resistant colonies were screened by colony PCR to confirm of the presence of Der p 13 cDNA. Our results indicated that, out of 14 zeocin-resistant colonies, most of them (with the exception of colonies 7 and 8) were indeed recombinant and contained the Der p 13 cDNA (Figure 4.1.2).

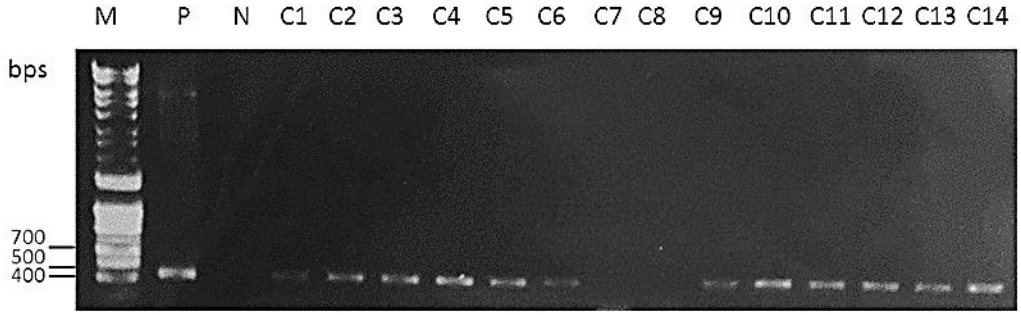


Figure 4.1. 2 Detection of Der p 13 cDNA in zeocin-resistant colonies by colony PCR.

PCR products were analyzed by 1% agarose gel electrophoresis. M: molecular weight marker (1 kb hyperladder), P: positive control (amplification using purified pPICZα A – Der p 13 plasmid), N: negative control (PCR master mix without DNA template), C1-C14: randomly selected clones.

4.2 rDer p 13 expression assay

Five selected colony PCR-positive clones were first cultured under shaking condition at 30ºC in YPD medium until OD_{600nm} reached 2-6. Such precultures were used inoculated 5 ml of BMGY medium until the OD_{600nm} reached 5-10. The cultured cells were centrifuged to remove any trace of BMGY medium and transferred into BMMY medium to get a final OD of 1. The presence of 0.5 % methanol (MetOH) into BMMY triggered the Der p 13 expression. The supernatant was collected directly after 24 or 48 hr of MetOH induction. As shown in Figure 4.2.1, recombinant KM71 clones

were able to express and secrete rDer p 13 which migrated onto 12 % SDS–PAGE as a 15 kDa band. The overall expression level was comparable from clones to clones. The higher expression could be observed at 24 hr than at 48 hr.

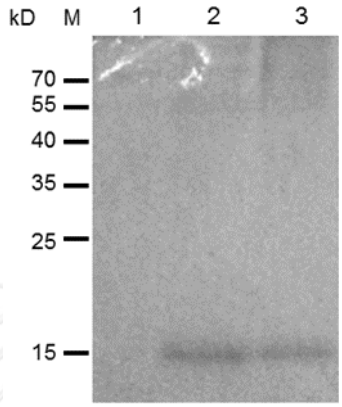


Figure 4.2. 1 rDer p 13 expression in the P. pastoris culture supernatant following MetOH induction.

The secretion of rDer p 13 was analyzed onto 12 % SDS-PAGE and stained with coomassie blue R-250. Lane 1: supernatant from non-induced yeast culture, Lane 2: supernatant after 24h of MetOH induction, Lane 3: supernatant after 48 h of0.5 % MetOH induction.

The MetOH concentration was further optimized through the evaluation of rDer p 13 expression level when the induction was performed with 0.5, 1, 3 and 5% MetOH for 24 hr. The result showed that the rDer p 13 was expressed in a methanol

concentration dependent manner, the highest expression was observed with 5% methanol (Figure 4.2.2). Consequently, these optimized inducing conditions were commonly used for the subsequent large scale production of this allergen.

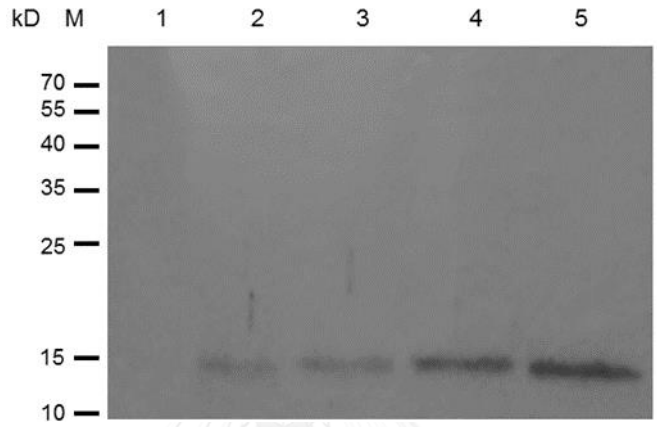


Figure 4.2. 2 MetOH dependence of rDer p 13 expression.

The expression of rDer p 13 was induced with different MetOH concentrations (0.5%, 1%, 3% and 5%) for 24 h. NI: non induced, Lane 1: 0.5% MetOH induction, Lane 2: 1% MetOH induction, Lane 3: 3% MetOH induction, Lane 4: 5% MetOH induction. The secretion of expressed rDer p 13 was monitored by 12% SDS-PAGE and coomassie blue R-250 staining.

4.3 rDer p 13 purification

Large scale *P. pastoris* culture supernatants containing rDer p 13 were prepared (250 mL/2L shake flask), following the optimized culture and induction conditions

described in 4.2.2. As the theoretical pI of rDer p 13 was estimated to 7.98 (from Expasy), we evaluated the use of cation exchange chromatography (IEX) as a first rDer p 13 purification step.

In this first approach, we selected the cation exchanger SP-sepharose beads (GE Healthcare, Lifesciences) to purify the rDer p 13, the pH of supernatants containing secreted rDer p 13 was adjusted to pH4. Our results showed that rDer p 13 could bind totally on the SP beads but, however, could only be tiny eluted under high salt concentrations (NaCl 1M in acetate buffer pH 4) (Figure 4.3.1, panel A). As we estimated the elution yield to only 20%, an explaination would be that rDer p 13 is sticky to the SP sepharose beads. Through the SDS PAGE analysis of the SP beads, we clearly found that a large amount of rDer p 13 was trapped into the beads (Figure 4.3.1, panel B).

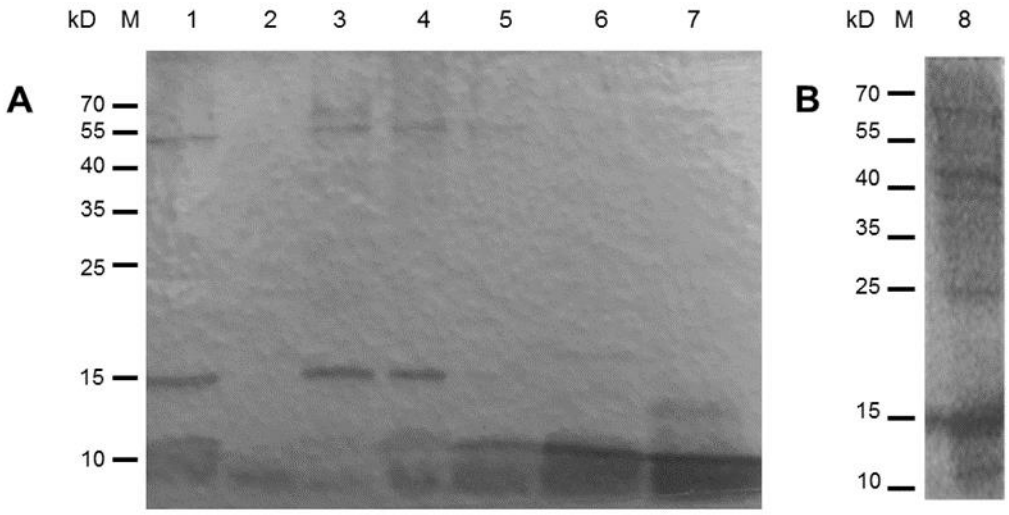


Figure 4.3. 1 Purification of rDer p 13 using SP-sepharose beads.

Lane 1: Starting material, Lane 2: flowthrough, Lanes 3-7: fractions from elutions using100, 200, 300, 500 and 1000 mM NaCl respectively (panel A). Lane 8: an aliquot of SP-sepharose beads after elution with 1000mM NaCl (panel B)

We next purified rDer p 13 using the same yeast culture supernatants but using an anion exchanger: Q-sepharose beads (GE Healthcare, Lifesciences,), at pH9 (According to the theoretical pI, rDer p 13 must be negatively charged at pH9 and consequently can bind to the Q sepahrose beads). The result showed that, under our experimental conditions, rDer p 13 could not bind totally onto the anion exchanger. Moreover, the protein could be partly eluted with an eluting buffer containing 500 mM NaCl (Figure 4.3.2, panel A). The eluting yield was roughly estimated to 70%. We observed also a similar “sticky protein” issue: a large amount of rDer p 13 remained

onto the Q-sepharose beads after a 1000 mM NaCl elution step. (Figure 4.3.2, panel B).

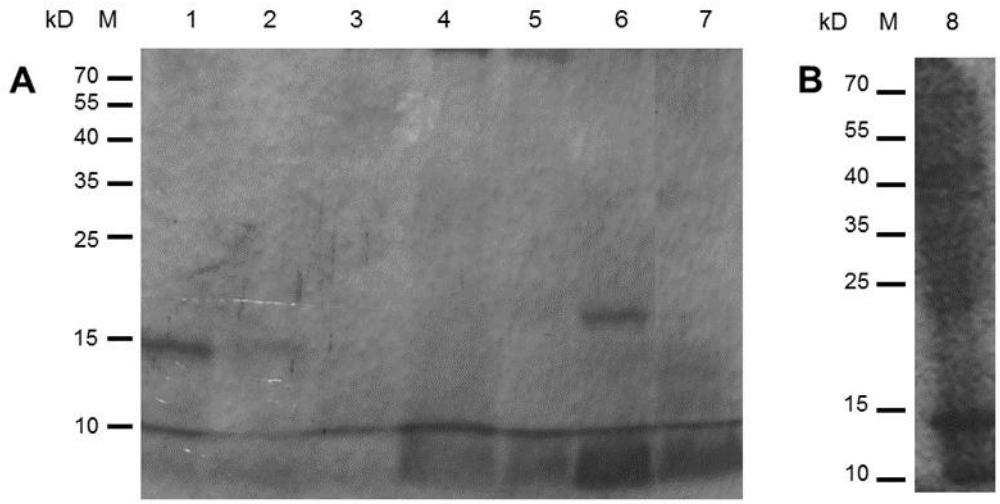


Figure 4.3. 2 Purification of rDer p 13 by anion exchange chromatography.

Lane 1: Supernatant, Lane 2: flowthrough, Lane 3-7: fractions from elutions using100, 200, 300, 500 and 1000 mM NaCl respectively, Lane 8: an aliquot of Q-sepharose beads (panel B).

According to the results generated with the two SP and Q sepharose chromatographies, we decided to change the purification strategy through the use of Phenyl sepharose beads (GE Healthcare, Life sciences) to purify rDer p 13 by hydrophobic interaction chromatography, the ionic strength of the supernatants containing secreted rDer p 13 was adjusted to reach the one of PBS+ 1M$(NH_4)_2SO_4$, pH7.0. The result showed that rDer p 13 could bind poorly to the hydrophobic matrix

under these conditions as a large proportion of rDer p 13 was present in the flowthrough (Figure 4.3.3, panel A). Moreover, not only the bound rDer p 13 was eluted partially at any elution steps but rDer p 13 could be detected also on the phenyl sepharose beads after these different elutions (Figure 4.3.3, panel B).

Altogether, our results suggested that any sepharose (agarose)-based beads were not appropriate for the rDer p 13 purification.

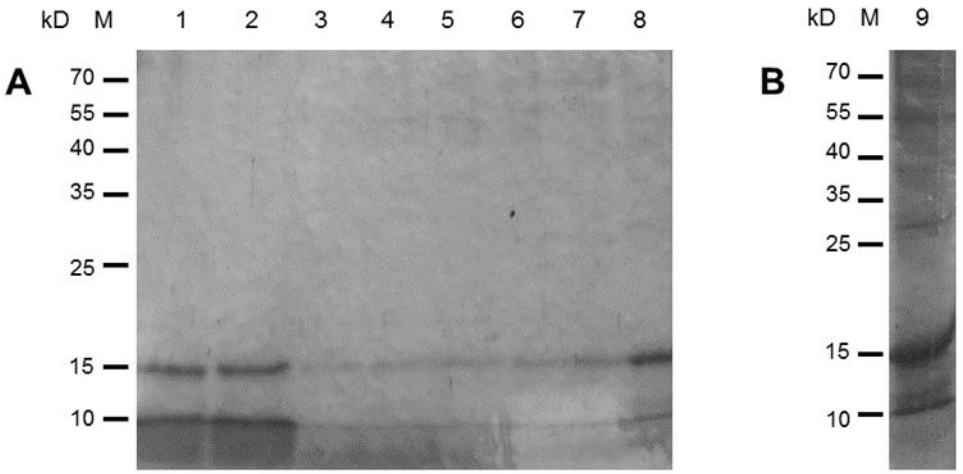


Figure 4.3. 3 Purification of rDer p 13 using Phenyl-sepharose beads.

Lane 1: Starting material, Lane 2: flowthrough, Lanes 3-7: elution with 800, 600, 400, 200 and 0 mM $(NH_4)_2SO_4$ in PBS pH 7.0, lane 8: elution with H_2O (panel A), Lane 9: an aliquot of phenyl-sepharose beads after the different elutions (panel B).

A Mep-Hypercel™Mixed-Mode Chromatography Sorbent (Pall, USA) was next selected to purify rDer p 13. This MEP HyperCel sorbent operates by a mixed-mode or multi-mode mechanism also described as Hydrophobic Charge Induction Chromatography (HCIC). The matrix composed of a proprietary rigid cellulose matrix to which 4-Mercapto-Ethyl-Pyridine (4-MEP) was linked. The structure of the 4-MEP ligand was shown in figure 4.3.4. The elution of bound proteins is induced by decreasing the pH of the eluting buffer to trigger electrostatic charge repulsion.

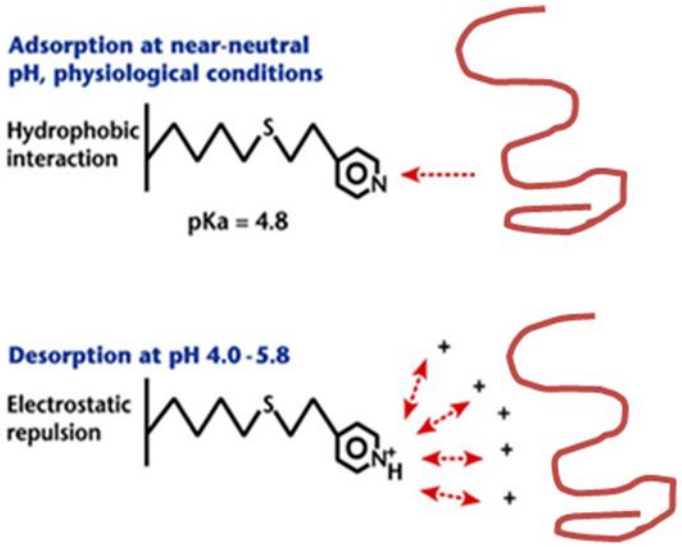


Figure 4.3. 4 Structure of Mep-HyperCel™ sorbents chromatography.

The matrix composed of a proprietary rigid cellulose matrix to which 4-Mercapto-Ethyl-Pyridine (4-MEP) was linked. The 4-MEP ligand has a pKa of 4.8, and contains a hydrophobic tail and an ionizable headgroup (data from http://www.pall.com/).

In order to purify rDer p 13 with Mep-HyperCel™ sorbents, the supernatants containing secreted rDer p 13 were prepared in high salt conditions at the final concentration of 1M saturated $(NH_4)_2SO_4$ and adjust to pH7.0. The purification was performed using 2 eluting buffers; MilliQ (H_2O) and 20 mM sodium acetate, pH 4.0. The obtained results were similar to those obtained using the phenyl sepharose beads: rDer p 13 could not bind completely to the column, as evidenced by the presence of rDer p 13 in the flowthrough (Figure 4.3.5) and the bound proteins were partially eluted, even at any steps of elution.

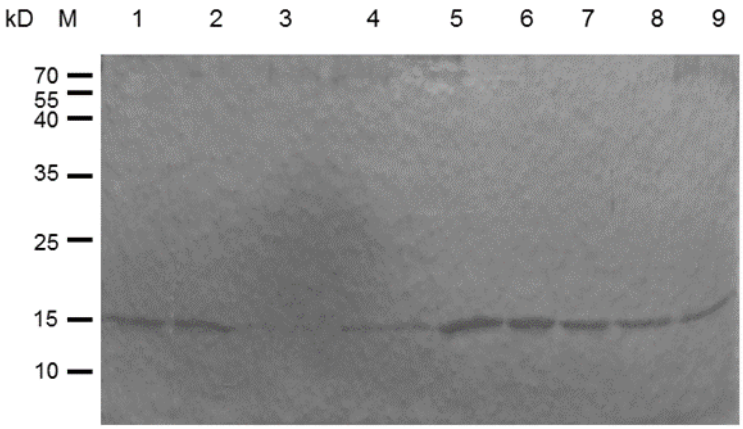


Figure 4.3. 5 Purification of rDer p 13 by mixed mode chromatography using Mep-HyperCel™pre-packed column.

Lane 1: Starting material, Lane 2: flowthrough, Lanes 3-5: elution with MilliQ (H_2O), lane 6-9: elution with 20 mM sodium acetate pH 4.0.

Finally, we purified rDer p 13 using a ceramic-based cation exchanger, the S-ceramic matrix (Pall, USA). Using this chromatographic support, we optimized the elution conditions through a two steps elution protocol: 1000mM NaCl elution step at pH4 followed by elution using a buffer at pH 9.

The result showed the rDer p 13 fully interacted with the S ceramic matrix and could be eluted completely to the column following a pH 9-based elution step. It must be pointed that no trace of rDer p 13 was detected into the matrix after the elution steps (Figure 4.3.6).

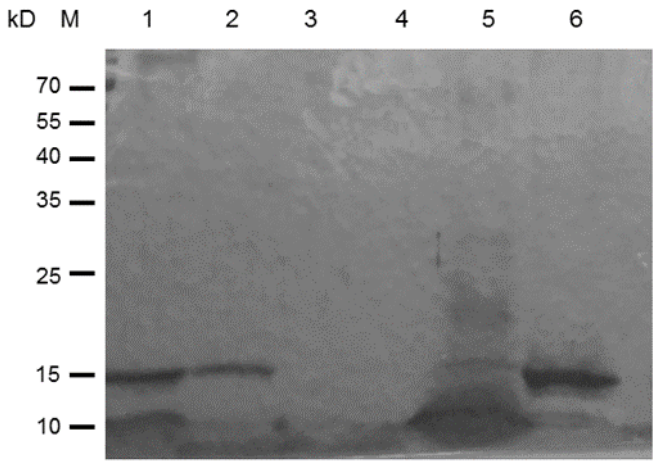


Figure 4.3. 6 Purification of rDer p 13 using S-ceramic based beads.

Lane 1: Supernatant, Lane 2: Supernatant with 10 times dilution and adjust pH to 4.0, Lane 3: flowthrough, Lane 4: an aliquot of S-ceramic based beads after the purification,

Lane 5: 20 mM sodium acetate, pH 4.0 +1000 mM NaCl pH 4.0, Lane 6: 20 mM Tris-HCl pH 9.0.

The purification of rDer p 13 was achieved by gel filtration chromatograpy. For that second purification step, the fractions containing rDer p 13 from the S-ceramic cation exchange chromatography were pooled and concentrated by ultrafiltration using regenerated cellulose membrane with a 3 kDa cut-off (Pall, USA). The concentrated proteins were applied onto a Superdex 75 HR pre-packed column equilibrated in PBS, pH 7.0 (GE Healthcare, Lifesciences, UK). The chromatogram was constituted by only one chromatographic peak roughly symmetric, suggesting that rDer p 13 was pure (Figure 4.3.7). The SDS-PAGE analysis of fractions 25-31 confirmed the purity of rDer p 13 (Figure 4.3.8).

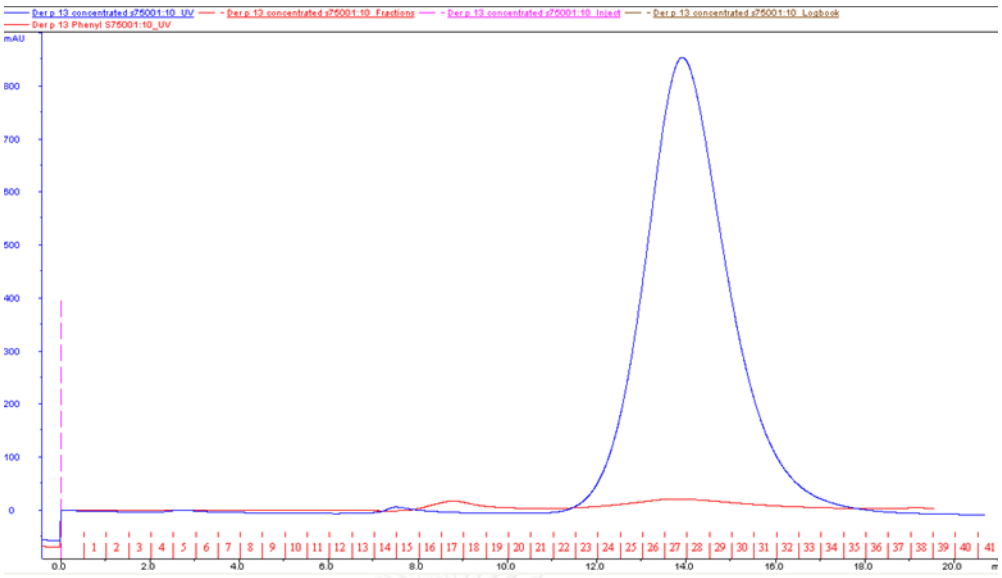


Figure 4.3. 7 Size exclusion chromatography of rDer p 13.

The IEX fractions positive for rDer p 13 were concentrated and applied onto a Superdex 75 HR column. Fractions 25-31 were analyzed by SDS PAGE.

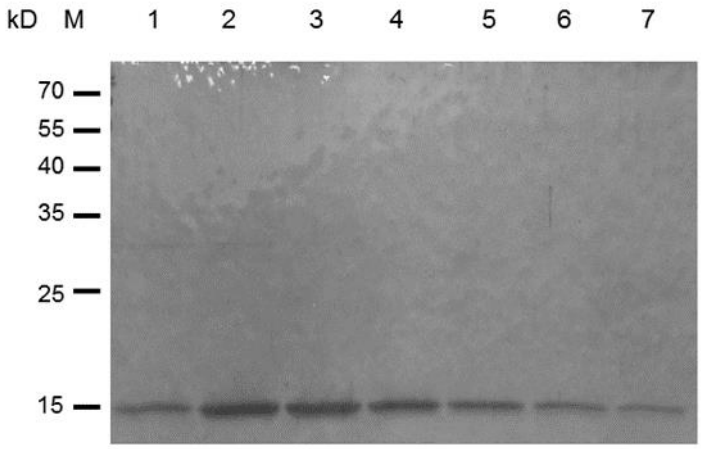


Figure 4.3. 8 Gel filtration profile of rDer p 13.

Lane 1-7: fraction 25-31.

The whole batch of purified rDer p 13 was measured by the BCA method using the BSA as protein standard. The total amount of rDer p 13 purified from 1L of culture supernatants was 4mg. The protein concentration was measured at 0.27 mg/mL (Figure 4.3.9).

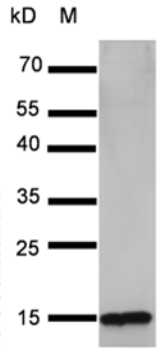


Figure 4.3. 9 Purified rDer p 13 following IEX and Size exclusion chromatographies.

The protein concentration = 0.27 µg/mL

4.4 rDer p 13 identification by mass spectrometry

Analysis of the intact protein by MS indicated that rDer p 13 displays amass of 15276.9 Da. This mass corresponded to the deduced from the mature Der p 13 sequence (amino acids 2-131) with four extra N terminal amino acids derived from the expression vector (Glu-Ala-Val-Ala) (Figure 4.4.1). This result was unexpected from our aim that was designed to prevent any addition of extra amino acid into the target protein, including the c-Myc and the His tag at the c-terminus. Although the result

wasn't as we expected, the explanation of the presence of 4 N-ter residues is due to inefficient cleavage of the leader sequence by the protease Ste 13 (184). Besides, this extra AA are commonly found in rec proteins produced in *P. pastoris*.

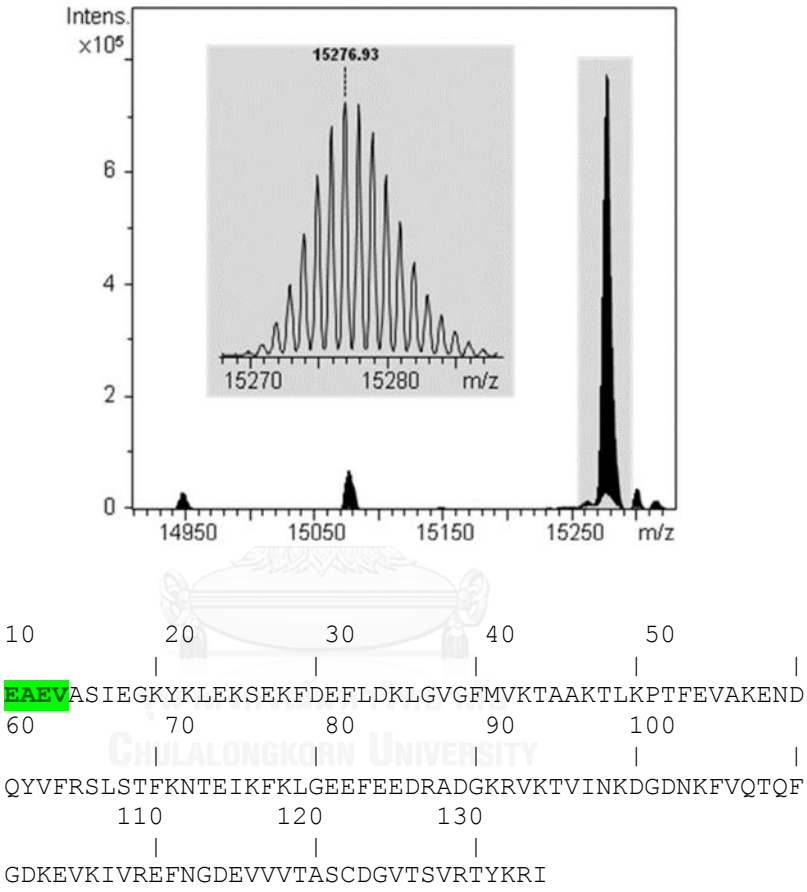


Figure 4.4. 1 MS analysis of the purified rDer p 13

Deconvoluted mass spectrum of purified and reduced rDer p 13 obtained using nanaLC-ESI-Qq-ToF MS (top panel). The rDer p AA sequence with 4 extra AA at N-terminal (down panel).

As the amino acid sequence of rDer p 13 is rich in Lysine (Lys, K) and Arginine (Arg, R) residues, tryptic rDer p 13-based peptides were generated and submitted to nanoLC-ESI-Qq-ToF MS analysis. MS analysis could be appropriately digested by trypsin to generate, the mass spectrometry (MS) analysis was then digested in solution with trypsin and subjected to nanoLC-ESI-Qq-ToF MS analysis.

More than 90% of the Der p 13 AA sequence was covered by the identified sequence from the analyzed peptides (Figure 4.4.2).

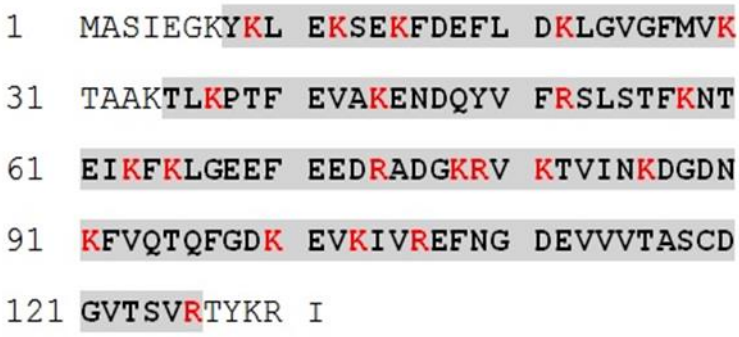


Figure 4.4. 2 Position of the tryptic peptides identified by MS.

The K and R in red represented the trypsin cleavage sites.

4.5 Far-UV circular dichroism analysis of purified rDer p 13

To predict the compositions of rDer p 13 in secondary structures, the amino acid sequence of rDer p 13 was subjected to the RaptorX bioinformatics software (www.raptorx.uchicago.edu/). Such prediction evaluated that rDer p 13 contained 38% of β-sheets, 16% of α-helix and 44% of coiled structure or unstructured domains.

Such secondary structure contents were clearly confirmed through the circular dichroism analysis of rDer p 13. The CD spectra profile matched the prediction results and stated that the compositions of purified rDer p 13 contain mainly 37.5% of β-sheet, 12.5% of α-helix, 19% of turn and 31% of unstructured (Figure 4.5.1, panel A). CD analysis under temperature changes from 25°C to 100°C revealed that rDer p 13 adopt a similar CD profile, suggesting that the allergen structure is very stable (Figure 4.5.1, panel B).

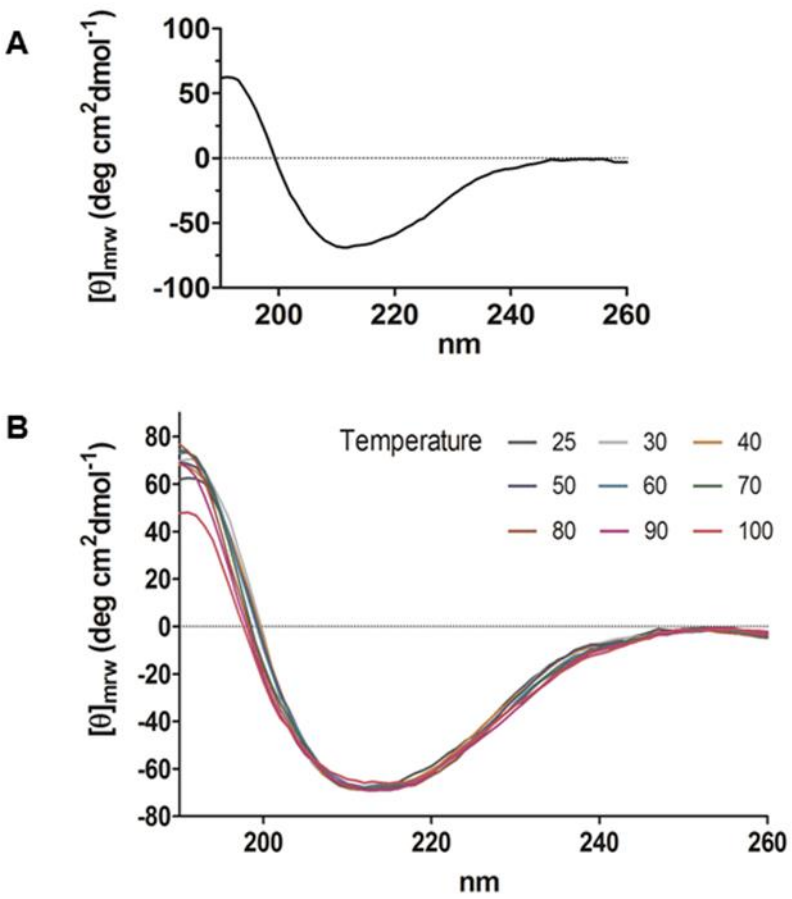


Figure 4.5. 1 UV-Circular dichroism analysis of purified rDer p 13.

CD spectra profile of rDer p 13 at 25ºC from 190-280nm (panel A), CD spectrum of rDer p 13 from 25ºC to 100ºC (panel B).

4.6 Production of polyclonal antibodies to rDer p 13

Anti-rDer p 13 polyclonal antibodies were produced in mice. Thanks to these antibodies, it would be possible to detect natural Der p 13 inin HDM extracts. Following three intraperitoneal immunizations with purified rDer p 13 formulated with alum, the presence of specific polyclonal antibodies in the serum of the immunized animals was

evidenced by a direct ELISA in which the plates were coated with rDer p 13. As negative controls, wells were also coated with purified rDer p 23, rDer p2, rDer p 5, rDer p 7, rDer p 21. The result confirmed the production of high titer of anti-rDer p 13 polyclonal antibodies (titer value around 100,000). These antibodies reacted very poorly with the other tested HDM allergens. As expected, no anti-rDer p 13 antibodies were detected into the preimmune serum (Figure 4.6.1).

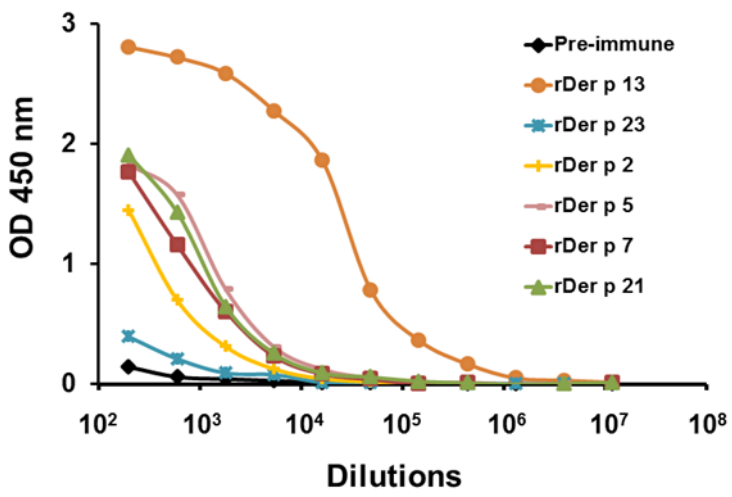


Figure 4.6. 1 Titer and specificity of anti-rDer p 13 polyclonal antibodies.

The specificity of anti-rDer p 13 antibodies was tested using rDer p 2, rDer p 5, rDer p 7, rDer p 21, rDer p 23 as coated antigens. The ELISA assay was performed with series dilutions of anti-rDer p 13 polyclonal antibodies.

4.7 Detection of nDer p 13 in HDM extracts

As our generated anti-rDer p 13 polyclonal antibodies were highly specific to rDer p 13, we decided to detect the natural form of Der p 13 in different HDM extracts by western blot; three different feces extracts (Stallergenes-r, France, Universite Libre de Bruxelles-ULB, Belgium and Siriraj Dust Mite Center, Mahidol University), and one commercial mite bodies extract (Greer, USA) were used for that purpose. The result showed that anti-rDer p 13 was able to detect the natural Der p 13 only in mite bodies extract but not in the fecal pellet extracts (Figure 4.7.1). For the future experiments, anti-rDer p 13 polyclonal antibodies were purified using immobilized rDer p 13 matrix (GE Healthcare Life sciences, UK).

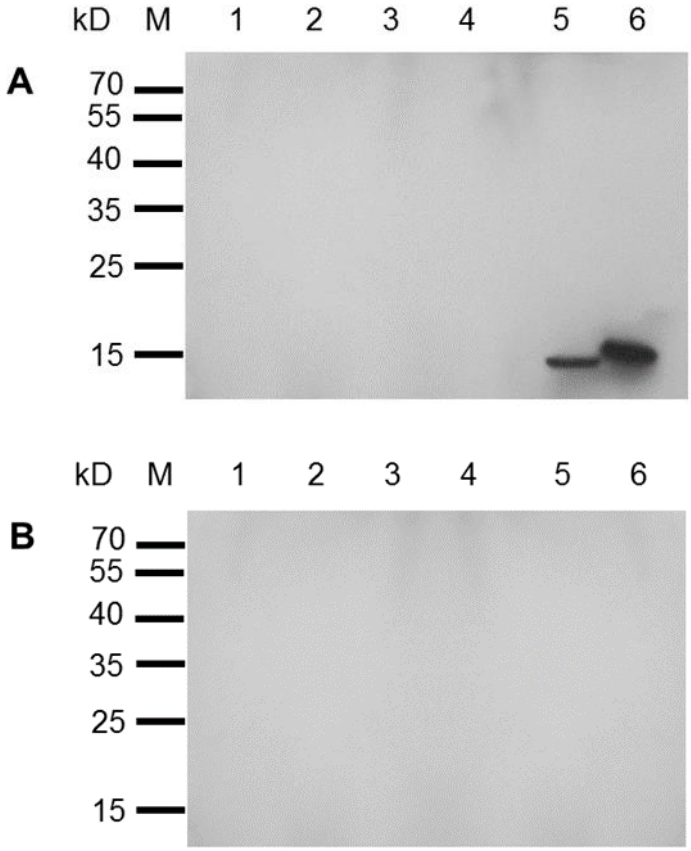


Figure 4.7. 1 Detection of natural Der p 13 in mite bodies and fecal extracts from D. pteronyssinus.

The anti-rDer p 13 antibodies (panel A) or pre-immune serum (panel B) were incubated after the proteins were transferred onto nitrocellulose membrane. (Legend; Lane 1: Fecal pellet from Stallergenes-Greer (1mg/mL), Lane 2: Fecal pellet extract from Stallergenes-Greer (2mg/mL), Lane 3: Fecal pellet extract from ULB,Belgium (2mg/mL), Lane 4: Fecal pellet extract from Siriraj Dust Mite Center (2mg/mL), Lane 5: HDM extract Greer, USA (100µg/ml), Lane 6: rDer p 13 (2µg/mL) (panel A)).

4.8 Human IgE reactivity to rDer p 13

Using a cohort of 224 sera from Thai HDM-allergic patients with positive skin prick test to *D.pteronyssinus* and displayed the class 3 ImmunoCAP value (>3.5 kU_A/L specific to *D.pteronyssinus*). The sera from patients were collected from four different hospitals in Bangkok; including King Chulalongkorn Memorial hospital, Phramongkutklao hospital, Ramathibodi hospital and Children hospital. To determine the threshold of positivity, sixty-seven Immunocap negative sera were used to calculate a cut-off value (mean OD negative sera ± 2SD).

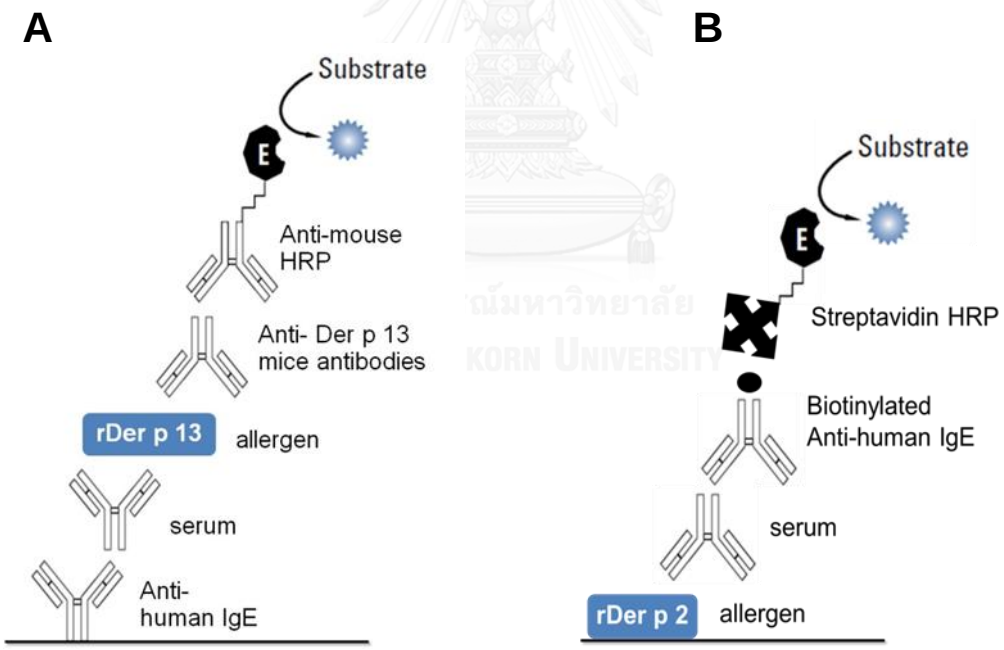


Figure 4.8. 1 ELISA assays for specific IgE detection.

IgE reactivity to rDer p 13 was performed using sandwich ELISA (panel A), whereas, IgE reactivity to rDer p 2 was performed with direct ELISA (panel B).

IgE detection to rDer p 13 was first performed in direct ELISA but we observed a very huge background due to unspecific IgE binding (Data not shown). Since the specific IgE detection to rDer p 13 was difficult by direct ELISA, we decided to shift to sandwich ELISA which might improve the specific IgE binding and reduce the high background. The anti-human IgE antibodies were coated onto the ELISA plates and followed by Thai HDM-allergic sera, rDer p 13 allergen, anti-rDer p 13 mice antibodies, and anti-mouse HRP-conjugated, respectively (Figure 4.8, panel A). The result showed much improvement in background reduction with the cut-off value of 0.166.

In order to compare the IgE reactivity results, rDer p 2 as the major HDM allergen was selected as a protein control for the IgE reactivity measurements. The IgE specific ELISA to rDer p 2 was performed using direct ELISA (Figure 4.8, panel B). The cut-off value of rDer p 2 was calculated to 0.157.

Our ELISA assays showed that 7%, from our cohort based on 224 patients developed specific IgE to rDer p 13 (15/224) whereas IgE reactivity to rDer p 2 reached 75% (169/224) in the same population. Therefore, Der p 13 is considered as a minor allergen.

4.9 Rat basophil degranulation by rDer p 13

The allergenic activity was measured using a genetically modified rat basophil leukemia cell line expressing human Fc**ε**RI (RBL-SX38), to estimate its capacity to degranulate such cells in contact with bound specific IgE. The allergenicity of rDer p 13 was also compared with the one displayed by rDer p 2 using six HDM positive sera with and two HDM negative sera. The degranulation rate was quantified through the realase of hexosaminidase activity in the cell culture supernatant. A typical dose-response following a bell-shaped curve was observed when both rDer p 13 and rDer p 2 at different concentrations were incubated with RBLSX-38 cells preloaded with appropriate specific IgEs. The maximum release of hexosaminidase (7-10%) was monitored in all three positive sera specific to rDer p 13at the allergen concentration 0.01 mg/mL (Figure 4.9.1, panel A). Similar results were evidenced with rDer p 2 but the degranulation rate reached 20-35% (Figure 4.9.1, panel B). For the positive control, similar levels of cell degranulation were observed when preloaded RBL cells were stimulated with polyclonal anti-human IgE (0.1 µg/mL), causing around 50% of the total hexosaminidase release ($p<0.01$). Although Der p 13 is a minor allergen, this protein displays a certain allergenicity, which is weaker when compared to Der p 2.

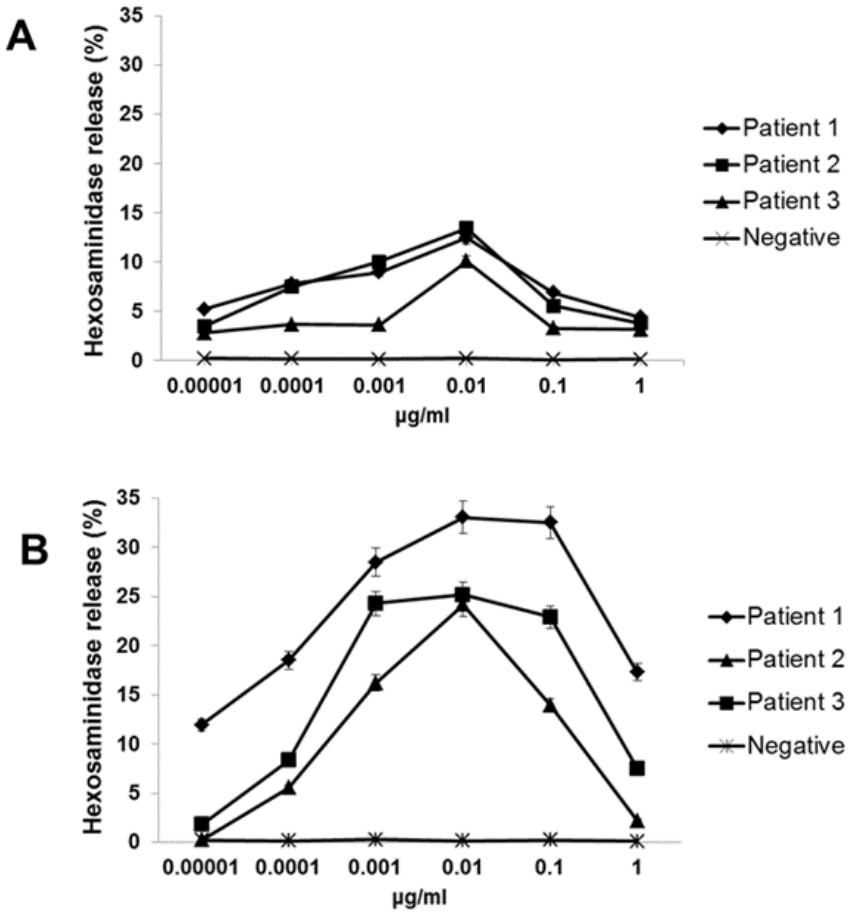


Figure 4.9. 1 RBL-SX38 cell degranulation by rDer p 13 and rDer p 2.

Cells were primed for 16 h with sera from three HDM allergic patients (specific immunoCAP value to *D.pteronyssinus*: patient 1 (15.90 kUA/L), patient 2 (9.21 kUA/L), patient 3 (23.70 kUA/L), and negative sera patient 4 (0.030 kUA/L)), containing rDer p 13 (panel A) or rDer p 2-specific IgE (specific immunoCAP value to *D.pteronyssinus*: patient 1 (>100 kUA/L), patient 2 (80.3 kUA/L), patient 3 (>100 kUA/L), and negative sera patient 4 (0.020 kUA/L), panel B), and subsequently stimulated with serial dilutions of purified rDer p 13 or rDer p 2 for 30 min. Degranulation was measured through β-

hexasominidases activity. Percentage of degranulation was presented as subtraction of spontaneous released over total lysis with Triton X-100.

4.10 Lipid binding assay

To demonstrate that rDer p 13 produced in *P. pastoris* is a fatty acid binding protein, lipid binding assays were performed using the naturally fluorescent fatty acid cis-parinaric acid (cPnA) as a ligand. The fluorescence emission of the natural, non-conjugated fluorescent fatty acid cPnA was significantly enhanced when mixed with rDer p 13, indicating the entry of the fatty acid into a non-polar site (Figure 4.10.1, panel A). No binding by the fluorophore-conjugated fatty acids bodipy-C16, DAUDA or DACA was observed. Addition of oleic acid to pre-formed Der p 13: cPnA complexes failed to show any significant competitive displacement, whereas control experiments with cPnA and a well-characterized fatty acid binding protein, β-lactoglobulin, showed efficient dose-dependent competitive displacement (Figure 4.10.1, panel B).

The titration curve of rDer p 13 to cPnA was plotted. The K_d value was calculated as 0.4 μM (Figure 4.10.2). This evidence confirmed a 1:1 binding mode of rDer p 13 to cPnA. rDer p 13 was unable to bind the other tested fluorescent lipid probes: Bis-ANS, Bodipy C16, 1- anilinonapthalene-8- sulfonate (ANS), ergosterol (DHE), and a set of DAUDA, DACA, Dansylglycine, (data not shown).

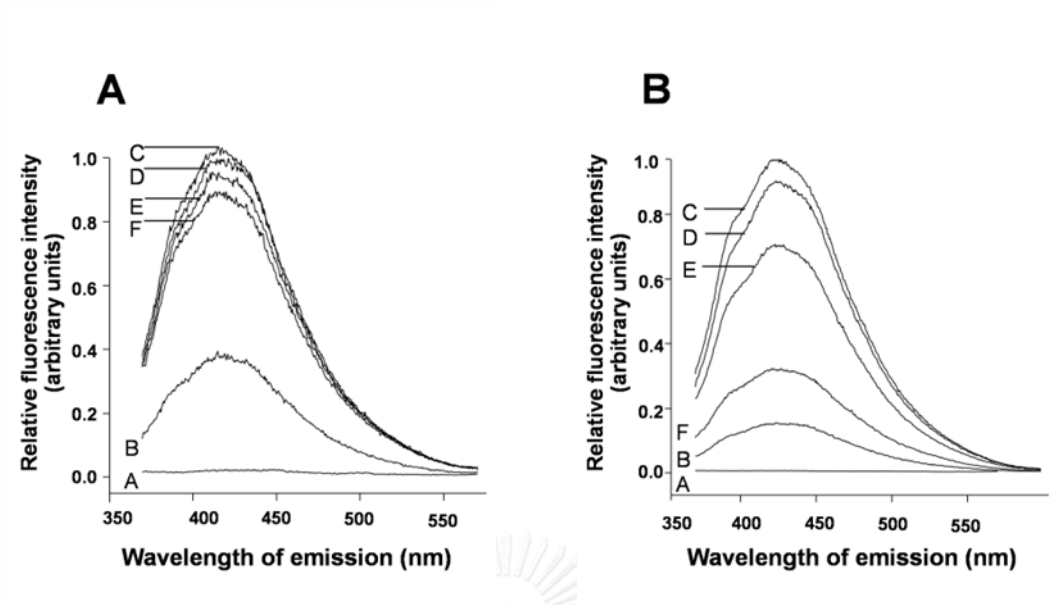


Figure 4.10. 1 Lipid binding assay of rDer p 13.

Fluorescence emission spectra of cis-parinaric acid (cPnA, $E_x$max = 319nm) bound to purified rDer p 13 (Panel A) and β-lactoglobulin (Panel B). The competitive binding of oleic acid used at different concentrations (7.9μM, 79μM, and 790 μM) is also shown (Curve A: PBS, curve B: cPnA alone, curve C: cPNA + protein, curve D: cPNA: protein complex + 7.9 μM oleic acid, curve E: cPNA: protein complex + 79 μM oleic acid, curve F: cPNA: protein complex + 790 μM).

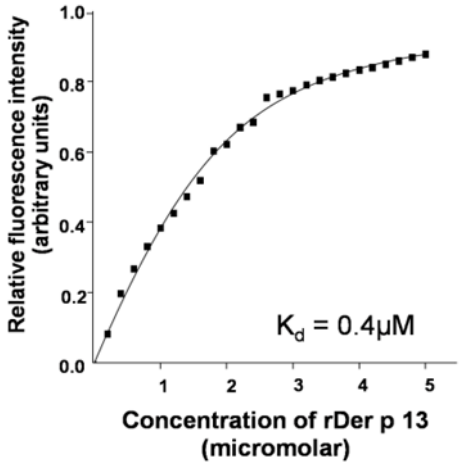


Figure 4.10. 2 Titration curve for the binding of cPnA to rDer p 13.

Increase in relative fluorescence intensity on progressive addition of rDer p 13 to cPnA in PBS. The solid line is a theoretical binding giving an apparent K_d of 0.4 μM, and is consistent with 1:1 binding.

4.11 In-silico prediction of the Der p 13 tertiary structure

The analysis of the empirical structure of Der f 13 has previously shown that it, in common with other family 13 members, shares close structural similarities to cytoplasmic FABPs. FABPs adopt an apo form in the absence of ligand, lipid binding then inducing detectable conformational changes. In human muscle FABP, the main difference between apo and holo forms is the orientation of residue Phe57, and other slight changes in the portal residues of the binding cavity involving Val25, Thr29, Lys58, Ala75 and Asp76. Interestingly, all these residues are conserved in Der p 13, with the

exception of position 29, where there is a Val. MODELLER 9.14 software (salilab.org) was used to predict the 3D structure of an apo form of Der p 13 based on the NMR structure of Der f 13 (PDB code 2A0A) (Figure 4.11.1, panel A) and of a holo form using as template the structure of myelin P2 protein in complex with ligand (PDB code 1YIV) (Figure 4.11.1, panel B).

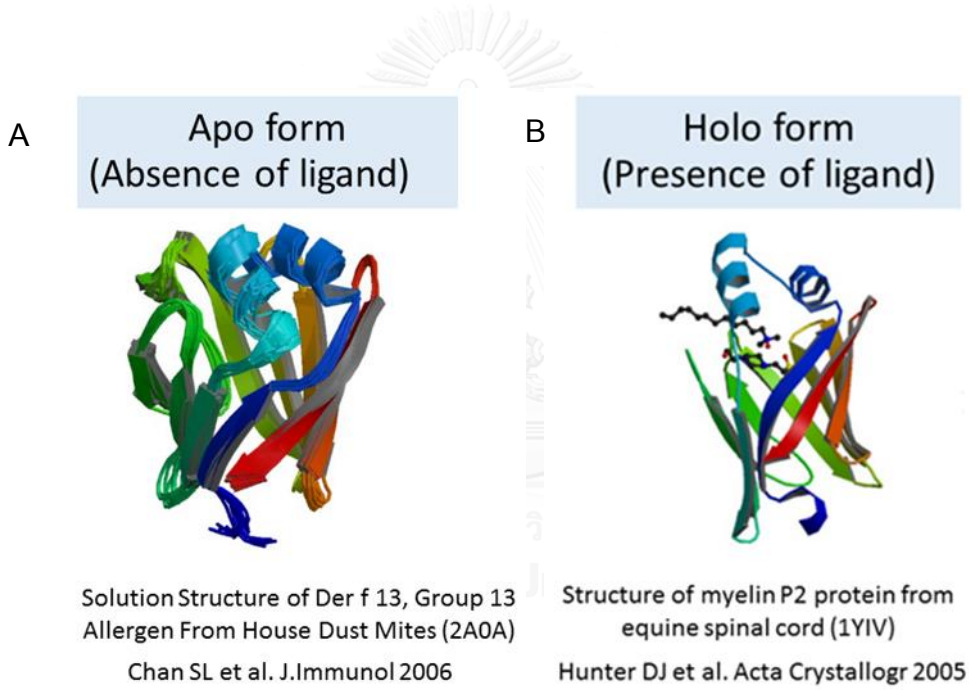


Figure 4.11. 1 Protein templates (two forms) for Der p 13 3D model prediction.

Der f 13 (PDB code 2A0A, apo form, panel A) and myelin P2 (PDB code 1YIV, holo form, panel B).

The amino acid sequence Der f 13 and myelin P2 showed the similarities to amino acid from Der p 13 as 95% and 38%, respectively, we then build the best 2 models of the of Der p 13 structures in MODELLER9.14; Der p 13 based on Der f 13 (Figure 4.11.2, panel A) and Der p 13 based on myelin P2 (Figure 4.11.2, panel B).

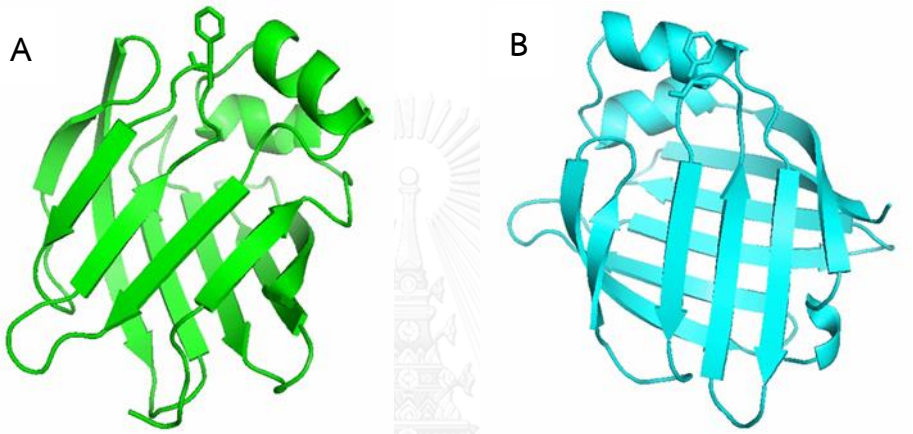


Figure 4.11. 2 Der p 13 3D models in apo and holo forms.

Der p 13 based on Der f 13 was represented in green (panel A) and Der p 13 based on myelin P2 was represented in light blue (panel B).

The interesting result was observed in the superimposition of the 2 models of Der p 13 at the orientation of Phe position 57 (Figure 4.11.3). This orientation is well-known in FABPs family which represent as a controllable gate that allows the free lipids to enter into the hydrophobic cavity.

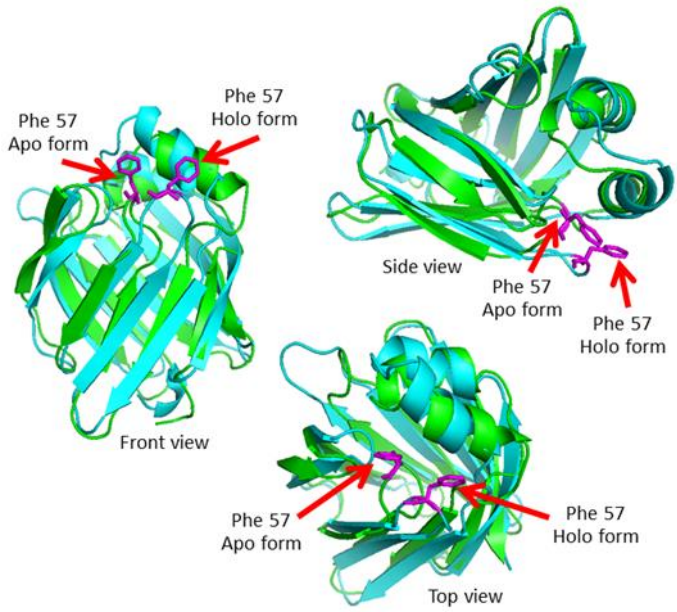


Figure 4.11. 3 Superimposition of the Der p 13 in apo and holo forms.

Der p 13 based on Der f 13 was represented in green, whereas, Der p 13 based on myelin P2 was represented in light blue. Red arrows indicated the positions of Phe 57 (purple) in both forms (front view, side view, and top view).

Comparison of central cavity in Der p 13 (apo form) showed an estimated volume of 590 Å3, in contrast, Der p 13 (holo form) showed bigger estimated volume of 995 Å3 by CASTp software (Figure 4.11.4).

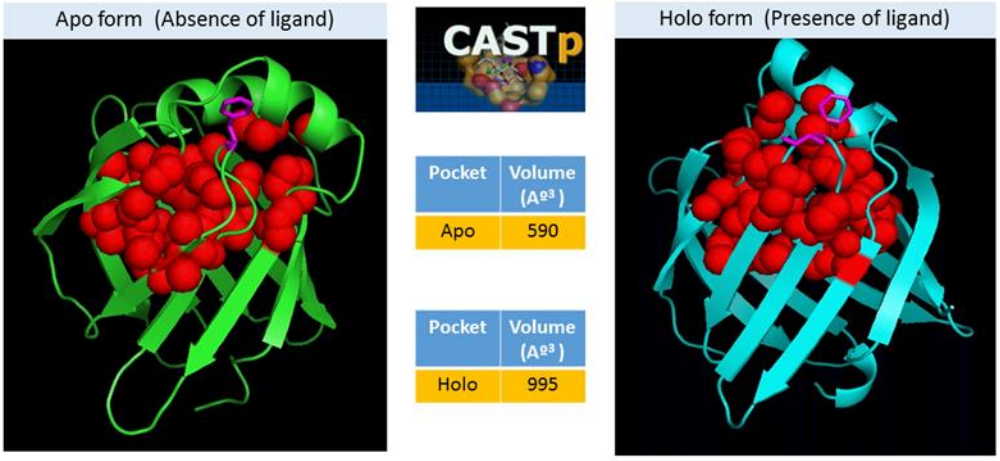


Figure 4.11. 4 Hydrophobic cavity evaluation by CASTp.

590 Å3 were estimated in apo form of Der p 13 (green) and 995 Å3 were estimated in holo form of Der p 13 (light blue). The red balls represented pocket's volume estimation. Position of Phe 57 was in purple.

As our results on lipid binding assays stated that rDer p 13 is a lipid binding protein that could interact with the natural fatty acid or cis-parinaric acid. The corina program was used to manually drawn cPnA (Figure 4.11.5). Once the preparation of

cPnA ligand was achieved, the protein-ligand docking test was performed the in order to mimic the lipid binding experimental condition.

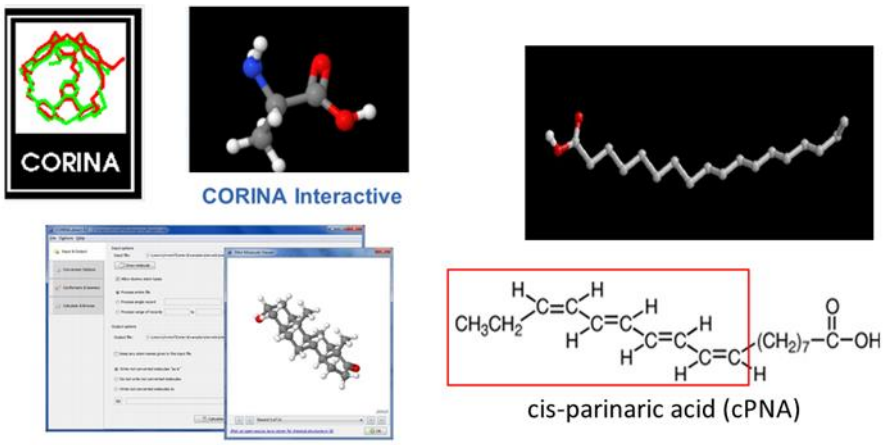


Figure 4.11. 5 cPnA ligand preparation.

Lipid was manually drawn in corina program to achieve the 3D format (red box indicated the rigid area of cPnA).

The protein-lipid docking simulation was then performed using Autodock 4.2. The docking result showed that cPnA could interact to the hydrophobic cavity of Der p 13 based on the structure of myelin P2 protein from equine spinal cord (PDB code 1YIV) with the open position of Phe 57 (Figure 4.11.6), whereas, the cPnA could stick outside the hydrophobic cavity of Der p 13 based on NMR structure of the solution structure of Der f 13, group 13 allergen from house dust mites (PDB code 2A0A) (figure

4.11.7). Our result suggested that Der p 13 structure is dynamic and orientate itself in the environment to bind with the free lipids/fatty acids.

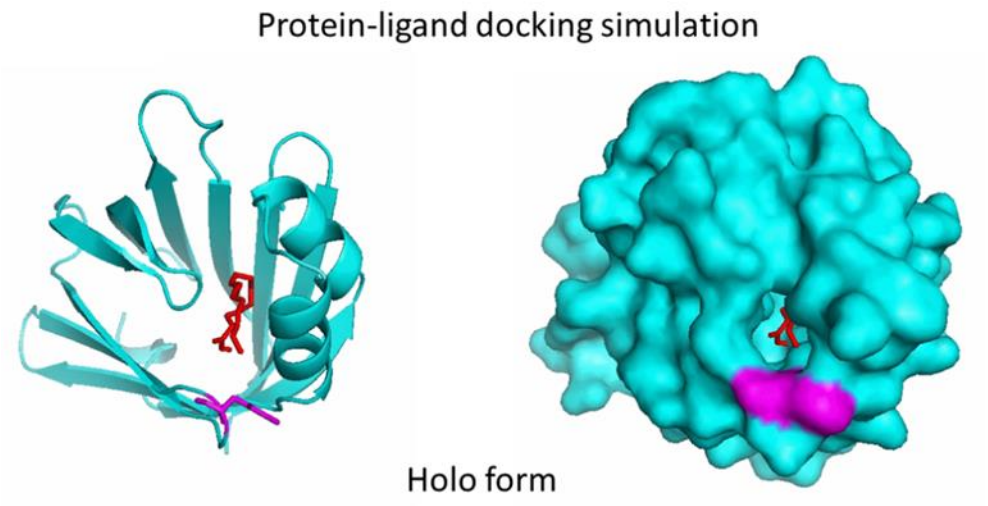


Figure 4.11. 6 Protein-ligand docking simulation in holo form.

Holo form of Der p 13 (light blue) with the presence of cPnA (red stick) into the hydrophobic cavity (left panel) and the surface area indicating the opened-gate of Der p 13 (position of Phe 57, purple, right panel).

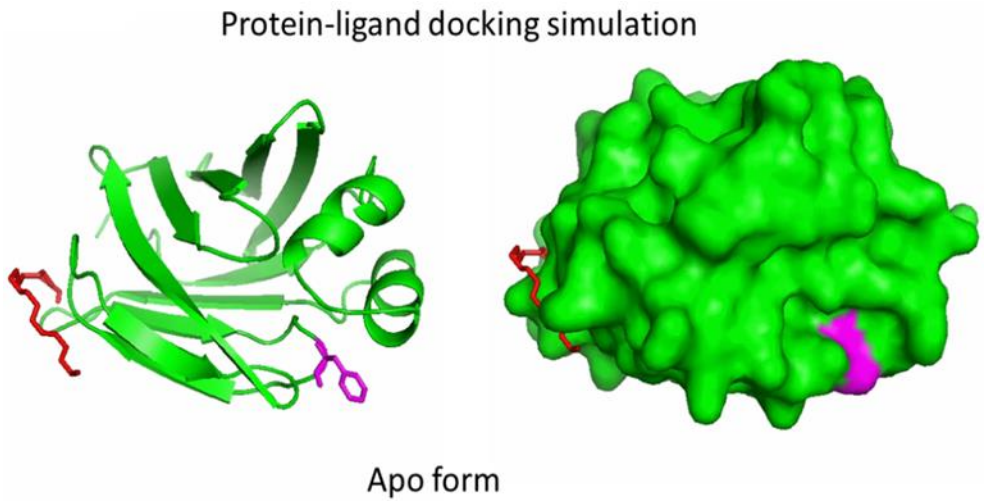


Figure 4.11. 7 Protein-ligand docking simulation in apo form.

Apo form of Der p 13 (green) with the presence of cPnA (red stick) on the outside of the hydrophobic cavity (left panel) and the surface area indicating the closed-gate of Der p 13 (position of Phe 57, purple, right panel).

4.12 Airway epithelial cell activation by rDer p 13

To investigate whether rDer p 13 can activate airway epithelial innate immune signaling pathways, we first incubated the bronchial epithelial BEAS-2B cells with different concentrations of rDer p 13. The cell activation was assayed through the production of IL-8 and GM-CSF cells. produced in *P. pastoris* can trigger IL-8 secretion from BEAS-2B cells in a concentration-dependent manner. The IL-8 and GM-CSF cytokines were detected in the presence of rDer p 13 in several concentrations (1-20 μg/mL) when compared with control medium (WT KM71). The result confirmed that

rDer p 13 successfully stimulated the cells to produce both IL-8 and GM-CSF cytokines in dose and time-dependent manner (Figure 4.12.1 and Figure 4.12.2).

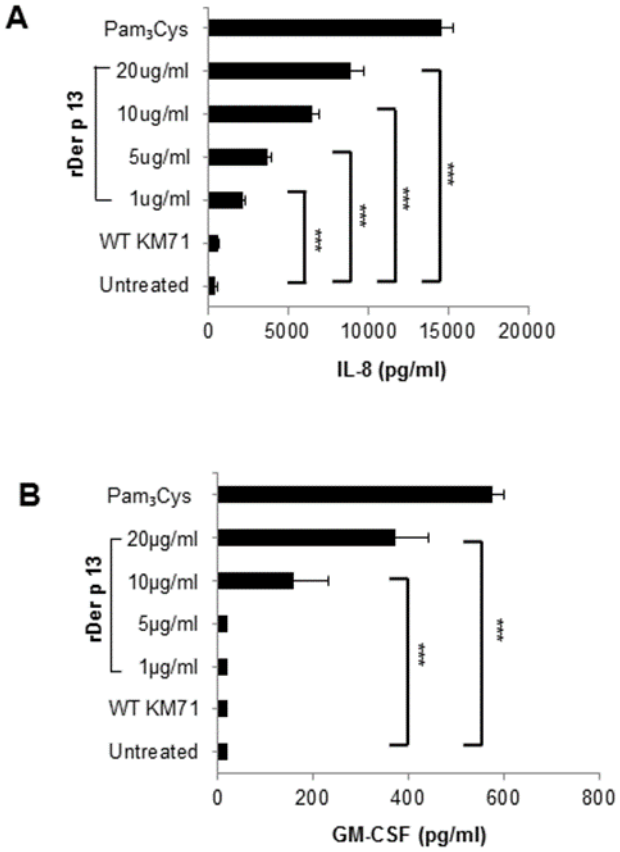


Figure 4.12. 1 IL-8 and GM-CSF cytokines detection in rDer p 13 cell activation.

BEAS-2B cells were stimulated with different concentration of rDer p 13 (1-20 µg/mL) under serum-free conditions. The production of IL-8 cytokine was shown in panel A, whereas, the production of GM-CSF was shown in panel B. Pam_3CSK_4 (100 ng/mL) was used as a positive control, as well as, wild type KM71 media (WT KM71) prepared by

using the rDer p 13 purification protocol. Data shows a mean average ± SE of 3 experiments. *** indicated P<0.001 versus untreated cells.

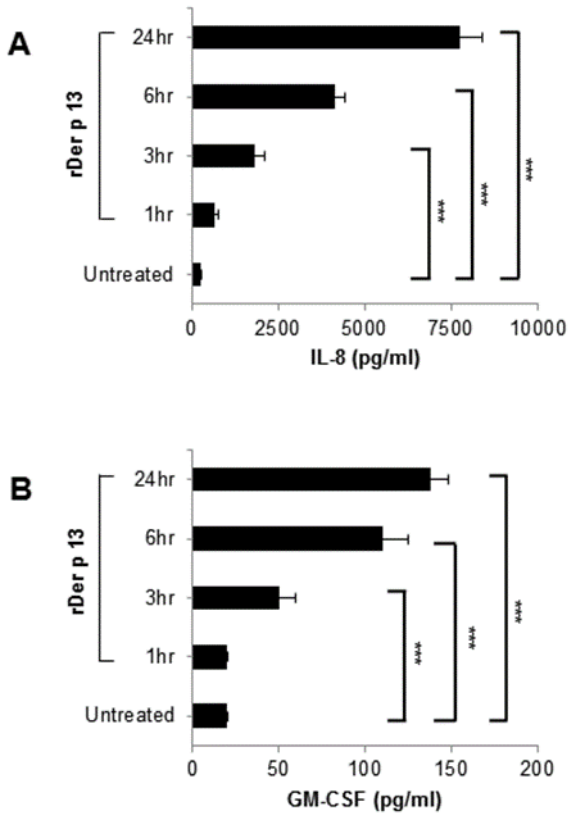


Figure 4.12. 2 IL-8 and GM-CSF cytokines detection in rDer p 13 cell activation.

BEAS-2B cells were stimulated with rDer p 13 (10 µg/mL) under serum-free conditions at 1-24 hr. The production of IL-8 cytokine was shown in panel A, whereas, the production of GM-CSF was shown in panel B. Data shows a mean average ± SE of 3 experiments. *** indicated P<0.001 versus untreated cells.

Hence, Der p 13 displays homologies with fatty acid/lipid binding proteins, this allergen could trigger cell activation through TLR2 signaling. To evaluate the TLR2 dependence of the rDer p 13-induced IL-8 production in BEAS-2B cells, the IL-8 secretion was compared in cells pre-incubated with a blocking antibody to TLR2. From the concentration-dependent result, we decided to pick the appropriate amount of 10 µg/mL rDer p 13 that could induce IL-8 and GM-CSF cytokines secretion for the further cell activation experiments.

Not only the positive control (Pam_3CSK_4), rDer p 23 and rProDer p 1, were selected as protein controls in this experiment. In our previous study, we demonstrated that rDer p 23 (the chitin binding protein) could stimulate the cells and induced IL-8 secretion; whereas, the rProDer p 1 was a pro form of Der p 1 which couldn't directly stimulate the cells unless this protein was enzymatically activated.

As expected, this pretreatment abolished the IL-8 production trigger by the TLR2 ligand (Pam_3CSK_4). Our results showed also clearly that the cytokine production by BEAS-2B stimulated with rDer p 13 was reduced by the anti-TLR2 blocking antibody (Figure 4.12.3). Neither, rDer p 23 cell activation showed any effect on the IL-8 secretion to the blockade of anti-TLR2, nor, rProDer p 1 cell activation showed no IL-8 cytokine production.

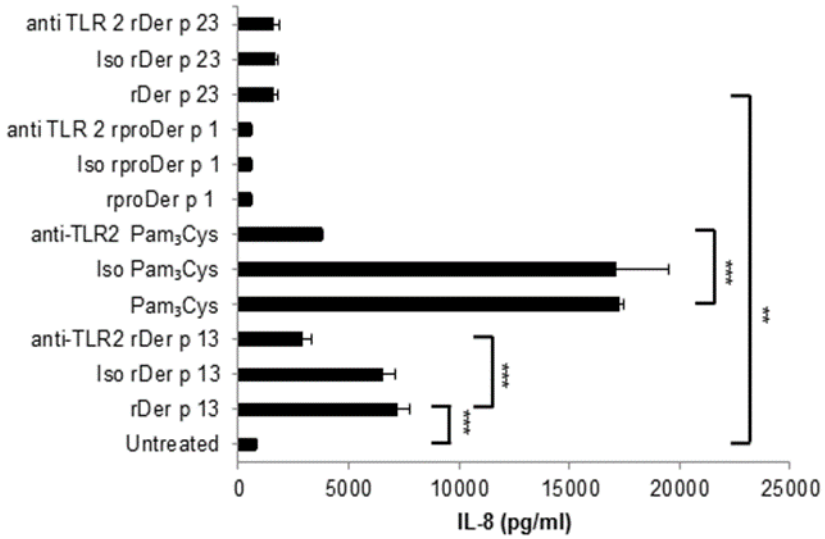


Figure 4.12. 3 Blockade of TLR2 in rDer p 13 cell activation.

The significantly difference was observed in the production of IL-8 cytokine from the cells pre-treated with a blocking antibody to TLR2 prior rDer p 13 stimulation (10μg/mL). Data shows a mean average ± SE of 3 experiments. *** indicated P<0.001, ** indicated P<0.05 versus untreated cells.

To down-regulate the expression of TLR2, the cells were then transfected with a plasmid expressing a Dominant Negative human MyD88 gene (pDeNy-hMyD88), together with the empty plasmid (pcDNA), as a negative control for 48 hr in culture condition. These transfected cells were then stimulated with rDer p 13 (10 μg/mL). Our result confirmed that both IL-8 and GM-CSF cytokines production were significantly reduced in Dominant Negative human MyD88 gene-transfected cells (Figure 4.12.4).

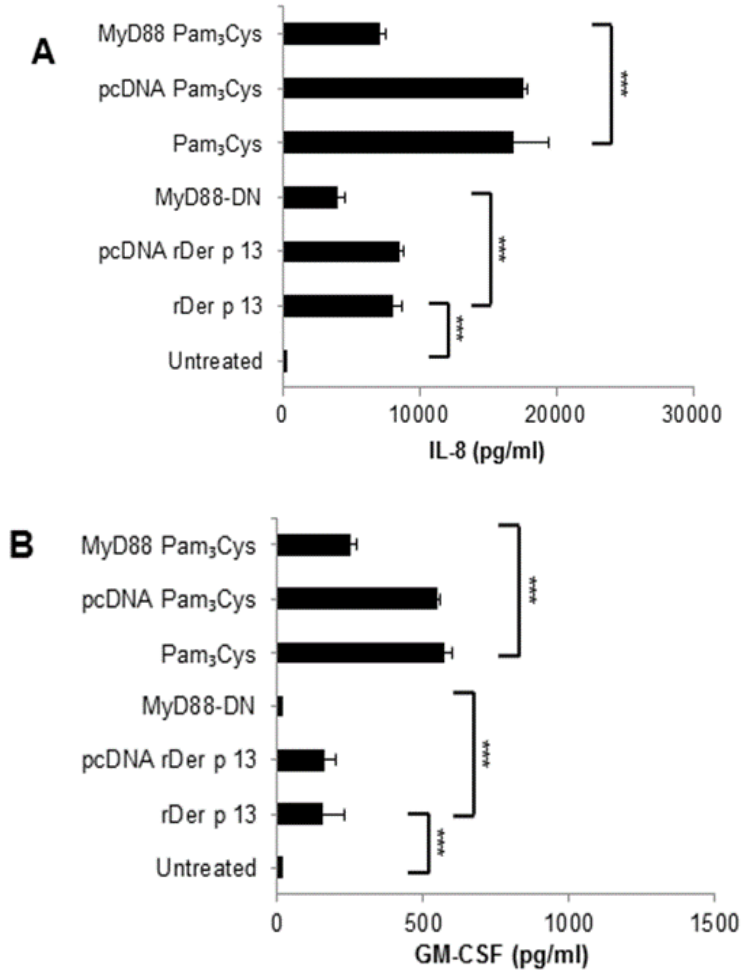


Figure 4.12. 4 rDer p 13 stimulation in Dominant Negative human MyD88 gene-transfected cells.

Down-regulate the TLR2 expression BEAS-2B cells were stimulated with rDer p 13 (10 µg/mL) under serum-free conditions. The production of IL-8 cytokine was shown in panel A, whereas, the production of GM-CSF was shown in panel B. Pam_3CSK_4 (100 ng/mL) was used as a positive control. MyD88-DN was stood for MyD88 gene-transfected cells prior rDer p 13 stimulation. Data shows a mean average ± SE of 3 experiments. *** indicated $P<0.001$ versus untreated cells.

The TLR2 expression was further investigated with several kind of the inhibitors including MEK1 and MEK2 Inhibitor (U0126), JNK inhibitor (SP600125), p38/RK (SB203580), Pan-Caspase inhibitor (z-vad-fmk), Inflammasome Inhibitor (Bay11-7082), and MG-132 (26S) proteasome inhibitor. This figure 4.12.5 illustrated the TLR2 signaling activation that were focused in our experiments.

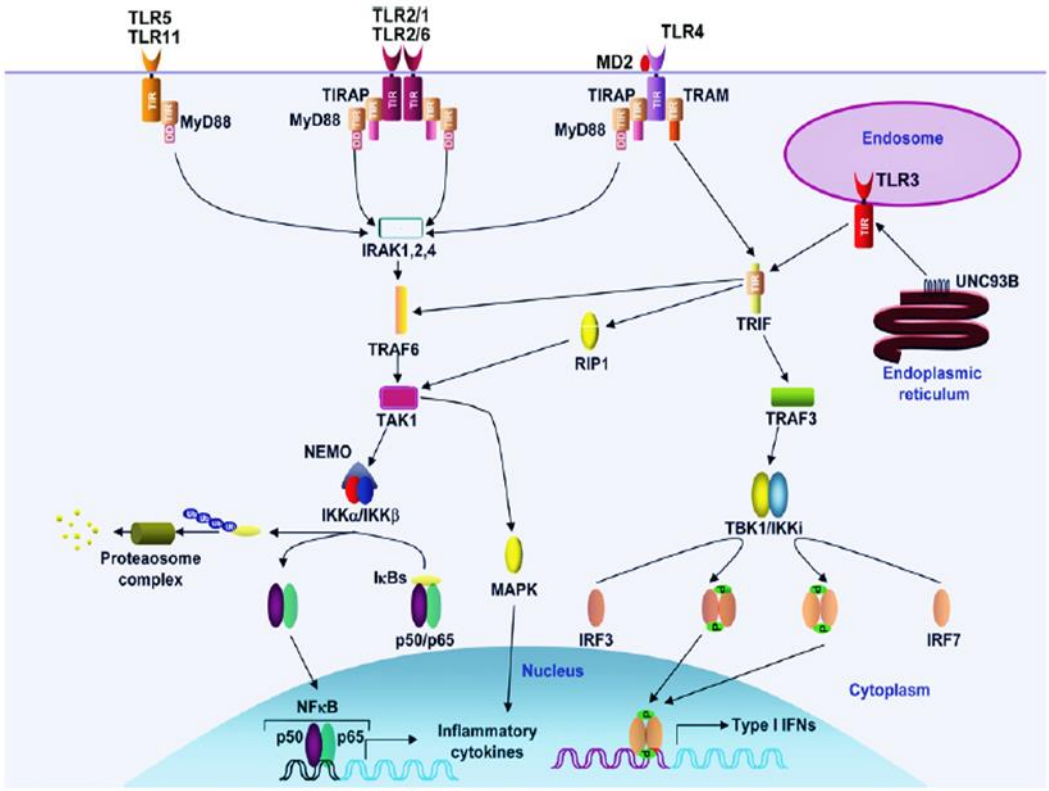


Figure 4.12. 5 TLRs signaling pathway.

TLR2 signaling pathway might be involved in Der p 13 activation [derived from (185)].

The cells were pre-treated for 1 hr with these inhibitors and followed by rDer p 13 stimulation (10 μg/mL). The result showed that both IL-8 and GM-CSF cytokines production were all inhibited under the serum-free conditions (Figure 4.12.6).

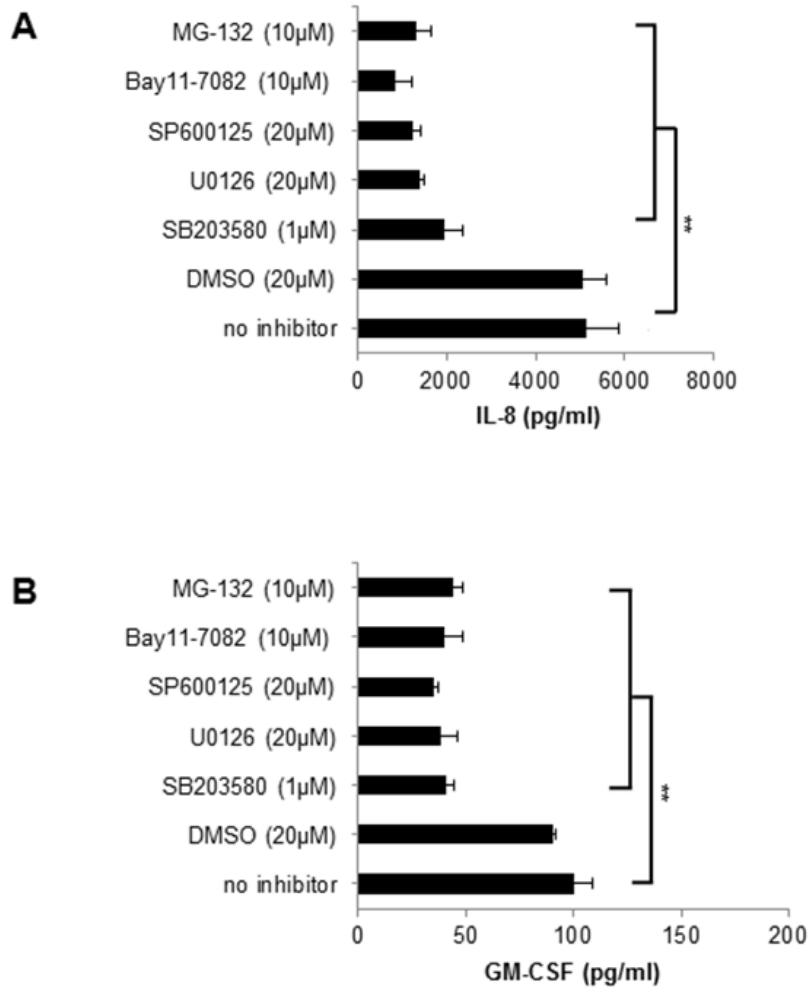


Figure 4.12. 6 IL-8 and GM-CSF cytokines detection in intracellular signaling.

BEAS-2B cells were pre-treated with inhibitors including U0126, SP600125, SB203580, Bay11-7082, and MG-132, as well as, DMSO (inhibitors solvent). The significantly reduced of IL-8 cytokine production (panel A) and GM-CSF cytokine production (panel B) were shown as $P<0.05$ versus untreated cells.

Still, the presence of lipid in rDer p 13 may trigger the TLR2 expression, rDer p 13 was digested with trypsin-EDTA before treating with serum-free cultured cells. The remaining trypsin-EDTA was removed by using immobilized benzaminidine matrix. As a control, trypsin-EDTA solution without the presence of rDer p 13 was used, as well as, the trypsin-EDTA alone. The undigested rDer p 13 and digested rDer p 13 was analyzed onto SDS-PAGE before starting the experiments (Figure 4.11.6, panel A). Our results indicated that the induced IL-8 cytokine production by both undigested rDer p 13 and digested rDer p 13 showed no significantly different. Therefore, the presence of lipid into rDer p 13 (digested rDer p 13 form) was clearly the activator of TLR2 and trigger the cytokine production (Figure 4.12.7, panel B).

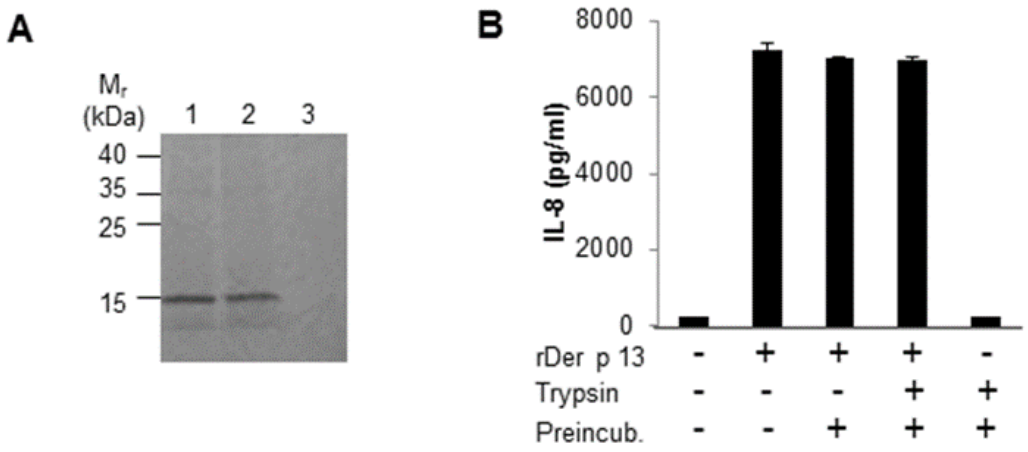


Figure 4.12. 7 Digested rDer p 13 (trypsin-EDTA) in cells activation.

15% SDS-PAGE analysis of digested rDer p 13, Lane 1: undigested rDer p 13 (control), Lane 2: pre-incubated undigested rDer p 13 at 37°C, Lane 3: digested rDer p 13 with 37°C pre-incubation (Panel A). IL-8 cytokine detection in different rDer p 13 forms; undigested rDer p 13, digested rDer p 13, trypsin-EDTA removal solution, and trypsin-EDTA alone (Panel B).

CHAPTER V

DISCUSSION

Our study mainly focused on the biochemical and immunological characterization of the HDM allergen Der p 13. Contrary to the well-studied Der p 1 and Der p 2, this allergen remained up to now poorly investigated. Its amino acid sequence revealed that this allergen similarity / identities with members of the cytosolic fatty acid binding protein (FABP) family which plays a critical role in the cellular uptake and transport of fatty acids. For example; the human heart FABP (protein accession number: P05413) or human brain FABP (protein accession number: 1JJX_A), showing 39% and 58% of amino acid identities and similarities, respectively. Within the group 13 mite allergens, Der p 13 showed 95%, 82%, 84%, 82%, and 83% homology with Der f 13, Blo t 13, Tyr p 13, Aca s 13 and Lep d 13, respectively (Figure 5.1). This high percentage of homology suggested that all the mite allergens from group 13 could share common IgE binding epitopes. It must be pointed out that, the lipid binding activity of group 13 mite allergens was only established for a recombinant form of Blo t 13 (40).

```
Der P 13          -MASIEGKYKLEKSEKFDEFLDKLGVGFMVKTAAKTLKPTFEVAKENDQYVFRSLSTFKN (59)
Der F 13          -MASIEGKYKLEKSEKFDEFLDKLGVGFMVKTAAKTLKPTFEVAIENDQYIFRSLSTFKN
Blo T 13          --MPIEGKYKLEKSDNFDKFLDELGVGFMVKTAAKTLKPTLEVDVQGDTYVFRSLSTFKN
Tyr P 13          --MTIEGKYKLEKSENFDAFLDKLGVGFMVKTAAKTLKPTLEVEVAGDTYTLRSLSTFKN
Aca S 13          --MSIDGKYKLEKSENFDVFLDKLGVGFMVKTAAKTLKPTLEVETQGDTYIFRSLSTFKN
Lep D 13          -MANIAGQYKLDKSENFDQFLDKLGVGFLVKTAAKTVKPTLEVAVDGDTYIFRSLSTFKN
FABPH_HUMAN       MVDAFLGTWKLVDSKNFDDYMKSLGVGFATRQVASMTKPTTIIEKNGDILTLKTHSTFKN
                      : * :** .*.:** ::..***** .: .*.  ***  :    *   ::: ******

Der P 13          TEIKFKLGEEFEEDRADGKRVKTVINKDGDNKFVQTQFGDKEVKIVREFNGDEVVVTASC (119)
Der F 13          TEAKFKLGEEFEEDRADGKRVKTVIQKEGDNKFVQTQFGDKEVKIIREFNGDEVVVTASC
Blo T 13          TEIKFKLGEEFEEDRADGKRVKTVVNKEGDNKFIQTQYGDKEVKIVRDFQGDDVVVTATV
Tyr P 13          TEIKFKLGEEFEEDRADGKKVKTVVNKEGDNKLVQTQFGDKEVKIIREFNGDDVVVTASV
Aca S 13          TEIKFKLGEEFEEDRADGKRVKTVVVKEGDNKFVQTQFGDKEVKIVREFAGDDVTVTATV
Lep D 13          TEIKFKLGEEFEEDRADGKRVKTVIVKDGDNKFVQTQYGDKEVKVVREFKGDEVEVTASV
FABPH_HUMAN       TEISFKLGVEFDETTADDRKVKSIVTLDGGKLVHLQKWDGQETTLVRELIDGKLILTLTH
                  ** .**** **:*  ** ::**:::  :* : .   ::  :*..::*::   .: :* :

Der p 13          DGVTSVRTYKRI- (131) (100%)
Der F 13          DGVTSVRTYKRI-       (95%)
Blo T 13          GDVTSVRTYKRI-       (82%)
Tyr P 13          DGVTSVRSYKRI-       (84%)
Aca S 13          ADVTSVRNYKRL-       (82%)
Lep D 13          DGVTSVRPYKRA-       (83%)
FABPH_HUMAN       GTAVCTRTYEKEA       (39%)
                    ....* *::
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Figure 5. 1 Multiple sequence alignment of Der p 13 with other HDM allergens from group 13 and human heart FABP.

Residues in bold represent the sequence of Der p 13 and the conserved Phe57 residue is highlighted in red.

Up to now, we can consider that the complete elucidation of the mechanism of the allergic response triggered by HDM allergens is incomplete and particularly at the initial step: the activation of innate immune signaling pathways. Many questions remain and need to be addressed:

All the HDM allergens are able to activate innate immunity? -)For any HDM allergen capable to trigger innate immunity, what is the molecular structure responsible for such activation? Is it the protein backbone? A post- translational modification? A ligand transported? Or the innate immune activation results from a microbial ligand which is present into the airways together with the HDM allergens?

A very important study demonstrated that Der p 2 can transport LPS (156), and it is well known that LPS is a key microbial compound to trigger HDM allergic response (76). The reason why Der p 2 was a potent activator of TLR 4 was that Der p 2 and MD2 (the co-receptor of TLR4) shared the same structure. Consequently, as a lipid cargo, Der p 2 could substitute MD-2 (111, 153, 156). Although the experimental evidences (in-vitro/in-vivo) to identify other HDM allergens involved in the transport of PAMPs are missing, the elucidation of the X-ray structure of recombinant forms of Der p 5 and Der p 7 suggested that these two proteins could also trigger innate immunity. Notably, Der p 5 is actually a homodimer and aa large hydrophobic cavity of 3000 A° 3 was identified at the interface of such dimer. Such pocket could represent a binding site to transport fatty acid/lipids (124). Whereas, it was shown that the crystal structures of Der p 7 and Der f 7 are similar to the one of LPS-binding protein (LBP), binding assays failed to demonstrate any interaction with LPS. In contrast, Der p 7 could bind the bacterial lipopeptide polymyxin B. Finally, a recent paper highlighted that a recombinant form of Der p 21 was able to stimulate airway epithelial cells through TLR2, a PRR activated by many lipid ligands (136).

Altogether, this information clearly suggested that any HDM fatty acid binding protein could play a key role for the initiation of the HDM allergic response through innate immune signaling. For all these reasons, our hypothesis was that Der p 13 could activate the allergic response at the level of TLR2-based innate immunity through the transport of a lipid ligand.

For the in-depth characterization of any protein, we need enough purified material. The isolation of appropriate amount of natural Der p 13 was impossible to be performed according to the difficulty to get large amount of house dust mite

organisms, even through in-vitro cultures and to extract appropriately natural Der p 13 for its purification. It was possible to initiate purification experiments from HDM cultures. To address this issue, we decided to produce a recombinant form of Der p 13. We selected the *P.pastoris* expression system for the allergen production as post-translational modifications take place in yeast, the expressed protein can be secreted into the culture medium and can commonly adopt a correct folding. Moreover, as we planned to evaluate the activation of innate signaling pathways by Der p 13, the selection of *E. coli* expression system for the protein production would induce problems in the interpretation of our results as the presence of residual bacterial LPS contaminating Der p 13 could also trigger innate immunity.

As the natural Der p 13 is sequestered into the cytosol, this protein could not display any leader sequence into its primary structure. A search for a leader sequence using the SignalP software (from Expasy) confirmed the absence for the signal sequence.

Therefore, we cloned the full length mature Der p 13 cDNA into a *P. pastoris* expression vector and directly downstream to the sequence encoding the yeast α-mating leader to drive the secretion of the expressed protein into the culture supernatant. Under these conditions, the purification of rDer p 13 must be highly facilitated as the protein content in such supernatant is drastically reduced compared with the one from the yeast cell. We successfully expressed and purified to homogeneity rDer p 13 and the purity was assessed by SDS-PAGE under reducing/non reducing conditions, MS, Gel filtration as well as dynamic light scattering (DLS, data not shown). Our results confirmed that:

Purified rDer p 13 was homogenous as the SDS-PAGE profile was similar under both reducing and non-reducing conditions, the protein displaying a molecular weight of around 15 kDa.

NanoLC-MS analysis of the intact protein confirms the expected mass at 14848.7 Da, corresponding to the mature sequence of the allergen plus an N-terminal extension of four amino acids. The four extra N-terminal Der p 13 residues corresponds to the C-terminal residues of the alpha mating factor signal peptide. Such extension is commonly present in recombinant proteins secreted by *P. pastoris* when the yeast dipeptidyl amino peptidase Ste13 processing to achieve the complete removal of the signal peptide is inefficient (182, 183).

Dynamic light scattering (DLS) experiment confirmed that the purified rDer p 13 contains no or traces amounts of polymers or aggregates.

Altogether, we could conclude that rDer p 13 is expressed as a monomeric protein.

The secondary structure analysis by CD evidenced that rDer p 13 display a large content of of β-sheets structures. This result was quite expected as FABPs adopt commonly atypical β barrel fold which create the inner hydrophobic pocket (184).

To further evidence that rDer p 13 is similar to natural Der p 13, we produced polyclonal antibodies to rDer p 13 and performed the western blot detection of the natural counterpart using different HDM allergen extracts: an extract from purified mite fecal pellets as well as a preparation derived from purified mite bodies. We successfully detected a specific 15kD band corresponding to nDer p 13 in the mite bodies extract. The absence of nDer p 13 into the mite fecal pellets was quite

expected as this protein is strictly sequestered into the producing mite cells. It must be point out that, other HDM allergens including Der p 8, Der p 11 are also uniquely detected into mite bodies (61, 128). The detection of natural Der p 13 by anti-rDer p 13 antibodies indicated that the natural and the recombinant forms of Der p 13 share at least some IgG binding epitopes.

Using this recombinant allergen, the IgE binding frequency of rDer p 2 reached 75% (169/224 patients), whereas, the IgE reactivity to rDer p 13 was only 7% (15/224 patients) in the present Thai HDM allergic population. In contrast, it reached around 20% in HDM allergic patients living in other various areas (61). Such low frequency of reactivity to Der p 13 was also observed with other members of group 13 mite allergens: IgE binding frequencies ranged from 6 to 20% for rBlo t 13, rTyr p 13, rAca s 13 and rLep d 13 (33-38). Our data clearly suggested that Der p 13 can be classified as a minor allergen triggering weak IgE responses. This could be explained by the fact that cytoplasmic FABP allergens are restricted to mite bodies and not present in the feces (61). Whereas fragmented mite body parts together with fecal pellets represent the main allergenic source, the deep penetration of particles with allergen cargo into the lung must be size-dependent (185). Consequently, the HDM allergens transported within mite fecal pellets (10 μm average diameter) should trigger airway inflammation more readily than mite body parts. To support this hypothesis, it has been recently demonstrated that HDM allergens detectable only in mite bodies display weak IgE reactivity in sensitized population with respiratory symptoms, but represent major allergens in patients suffering from atopic dermatitis (128). The HDM allergic in our cohort suffered only from allergic rhinitis or asthma, so it would be interesting to

determine whether HDM allergic with atopic dermatitis have similar or higher rates of IgE to Der p 13.

The low IgE reactivity to Der p 13 could also be likely related to the high protease-sensitivity of this allergen. Indeed, our preliminary results showed that nDer p 1 (cysteine protease) could cleave rDer p 13 whereas trypsin (serine protease) fully degraded the group 13 protein (data not shown). Der p 3 is a trypsin-like protease allergen which is present in mite fecal pellets, together with Der p 1 (27). Consequently, nDer p 13 released from mite bodies fragment could be degraded by nDer p 1/nDer p 3 from the fecal pellets during the sensitization.

Nevertheless, our basophil degranulation assays clearly confirmed that rDer p 13 displayed allergenic properties by its capacity to activate the basophil degranulation when in contact with specific IgE. But its allergenicity is weaker that the one from Der p 2, considered as a major allergen

Pertinent to the role of specific lipids in immune activation, we evidenced, using environment-sensitive fluorescent lipid probes, that rDer p 13 binds fatty acids and that the protein's binding to hydrophobic ligands is selective. Indeed, although Der p 13 and Blo t 13 share 80% sequence homologies and display the same range of K_d value for cPNA (0.4 10^{-6}M versus 1.31 10^{-6} M respectively), displacement of cPNA with oleic acid was quite ineffective for Der p 13. The tighter binding observed with cPNA could suggest that this highly conjugated fatty acid is more representative of the Der p 13 natural ligand. It must be pointed out that, using the same experimental conditions, a dose-dependent displacement of cPnA by oleic acid could be measured using b-lactoglobulin as control.

Whereas our lipid binding assays demonstrated that rDer p 13 could transport specifically cPNA such experiment could not evidence the presence of the ligand into the Der p 13 hydrophobic cavity.

Computer-based docking experiments performed with two different models of Der p 13 (one based on the apo form of Der f 13, (15) the other based on the holo form of myelin P2 protein (186), with the lipid ligand cPnA known to bind to the protein, predicted that the accessibility of the hydrophobic pocket could be controlled by the side chain orientation of Phe57. This residue is located immediately beside the portal of entry to the binding pocket of some FABPs (39). A similarly-positioned side chain is found in several FABPs (187), where it is speculated to regulate the entry of ligand to the proteins' binding pocket (188). The identification of the lipid ligand(s) naturally present in the Der p 13 hydrophobic pocket remains to be determined. Der p 13, like all FABPs other than those of nematodes (189), lacks a leader sequence, and is therefore probably confined to the cytosol of mite cells. The lipids it may present are therefore likely to be cytoplasmic lipids, though we cannot exclude the possibility that Der p 13 transports lipid ligands from endosymbiotic bacteria or microbes in house dust.

Using our 3D model of Der p 13 together with an alignment of Der p 13 and Blo t 13 AA sequences, we analyzed the residues from two allergens located in the hydrophobic cavity. The only notable difference at the level of the hydrophobic pocket is the presence of Phe40 residue in Der p 13 which is replaced by Leu in Blo t 13. The aromatic side chain of Phe40 in Der p 13 could provide **π-π** interactions with the double bonds of cPNA, which could stabilize this ligand into the pocket to reduce

its displacement by oleic acid. It would be interesting to validate our hypothesis through a lipid binding assay using a Phe 40 Leu Der p 13 mutant.

It is well accepted that HDM allergies may be initiated through activation of innate immunity (74), such that any mite component capable of stimulating innate immune signaling could be influential.

Based on the finding that Der p 13 binds fatty acids, we hypothesized that this allergen could activate TLR2 signaling in airway epithelium. This pathogen-associated molecular pattern receptor forms a heterodimer with either TLR1 or TLR6 and interacts with lipids/fatty acids or lipoprotein (190).

For such cell activation assays, we selected the human bronchial epithelial cell line BEAS-2B. Such cells can be easily cultured in-vitro and were shown to express TLR2 (191-193). It must be pointed out that such cellular system could be quite different from the primary bronchial epithelial cell culture isolated from patients, notably at the level of TLR2 expression (194), whereas, the use of primary cells can "mimick" more closely the in-vivo allergen interactions with the airway epithelium, these cells are quite difficult to isolate, the culture media are expensive, the cells cannot be propagated for many passages leading to reproducibility issues

Using the BEAS-2B cells, the activation of TLR4 in combination with MD-2 and CD14 (another receptor capable to recognize microbial lipidic ligands) by Der p 13 was not evaluated because the BEAS-2B airway epithelial cells were found to be hyporesponsive to the LPS needed for positive control (data not shown). Indeed, such cells were shown to poorly express MD-2 whereas intracellular localization of TLR4 is nevertheless apparent (195).

To determine whether rDer p 13 enhances innate responses through TLR2, proinflammatory cytokine production was measured from stimulated BEAS-2B. We focused on both IL8 and GM-CSF secretion because these cytokines are important chemoattractants and activators for immune cells such as neutrophils, basophils, eosinophils and dendritic cells (196). Our results showed that rDer p 13 stimulated the production of IL-8 and GM-CSF by airway epithelial cells in a time- and concentration-dependent manner. Through a combination of blocking antibodies, specific inhibitors, and depletion of MyD88, we found that Der p 13 triggers airway epithelial cell activation through TLR2-MyD88-NF-kB and MAPK-dependent mechanisms. Strikingly, this cell activation was shown to be independent of the persistence of intact Der p 13, thereby implicating the protein's lipid cargo.

We speculate that the Der p 13 protein backbone alone cannot activate TLR2. It must be pointed out that, with the exception of the proteins flagellins and RSV F (fusion protein from respiratory syncytial virus) which are agonists of TLR5 and TLR4 respectively, the common PAMPs activating TLR are non-protein molecules (5).

This result supported the hypothesis that Der p 13 facilitates the transfer of immunomodulatory fatty acid/lipid to TLR2 or to a TLR2 co-receptor such as CD14 or CD36 to trigger innate immune signaling.

To our knowledge, this is the first study to reveal the allergenic propensity of a group 13 mite allergen as well as its potential mechanism of action. Together with group 2 allergens and Der p 21 (136, 197), Der p 13 is the third TLR2 stimulator to be identified in HDM. Notably, the presence of hydrophobic cavities in the Der p 5 dimer and Der p 7 structures for the binding of lipid ligands suggests that HDM allergens transporting lipid cargo could act either synergistically or in a redundant fashion to

stimulate TLR2 signaling (124, 159). The immunomodulatory capacities of Der p 5 and Der p 7, however, await experimental confirmation. Although it was demonstrated initially that TLR2 ligands reduced Th2-biased allergic responses (198), recent studies indicated that TLR2 signaling could be critical for the development of HDM allergic rhinitis and asthma (80, 199-201) In that context, Der p 13 could represent an important factor in the initiation of the HDM allergic response because TLR2 engagement led to the activation of the epithelial NF-kB, which comprises an orchestrator of the HDM-induced airway inflammation, hyperresponsiveness, and fibrotic remodeling (202).

CHAPTER VI

CONCLUSION

In conclusion, Der p 13, through its ability to bind lipid and trigger TLR2-dependent innate immune signaling, must be considered as a potential contributor to the induction of the HDM allergic response. Although Der p 13 was strictly localized into mite bodies, we speculate that such TLR2 activation could occur *in-vivo* following deposition of mite bodies fragments onto the lung surface and allergen release by the unknown action of a dissolving media in the lung the epithelial lining fluid. It must be pointed out that Der p 2 was also mainly present in mite bodies and is one of the most important major allergen able to activate at least TLR2 and TLR4 (111, 197). Because the lipid environment in the mite, house dust or the *P. pastoris* yeast used to produce the allergen may differ considerably, it is important to identify the natural Der p 13 ligand(s) and to characterize its effects on immune cells. However, such characterization could be difficult to conduct according to the very tiny amount of natural Der p 13 which can be isolated from mite culture. Moreover, the lipid ligand(s) from natural Der p 13 isolated from mite culture could be also very different from the one(s) transported by Der p 13 from house dust. The lipid transfer mechanism involved in the Der p 13 enhancement of TLR2 signaling remains unclear, although FABPs of the family to which Der p 13 belongs are known to interact directly with membranes in the transfer lipid cargo.

Nevertheless, our results demonstrate that the HDM allergen hierarchy, based essentially on IgE reactivities, needs further refinement in order to take into account

the capacity of allergens to stimulate innate immunity. Consequently, minor HDM allergens such as Der p 13 require extensive characterization in order to elucidate their abilities to activate airway epithelial cells as well as keratinocytes Through production of other cytokines as IL-1β, TSLP, IL-33, IL-25.

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APPENDIX

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ORIGINAL ARTICLE EXPERIMENTAL ALLERGY AND IMMUNOLOGY

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The minor house dust mite allergen Der p 13 is a fatty acid-binding protein and an activator of a TLR2-mediated innate immune response

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Poster Discussion Session PDS 5. Clinical relevance of IgE to allergenic molecules

subjects sensitised only to the extract (n = 56). Furthermore, subjects sensitised to both cat extract and any component were more likely to develop asthma (P = 0.005) and rhinitis (P = 0.007) than subjects sensitised only to cat extract. Looking at individual components, Fel d 1 and Fel d 2 were associated with bronchial responsiveness (P = 0.001 and P = 0.02 respectively) and F_ENO_{50} (P = 0.002 and P < 0.001 respectively) but not Fel d 4 (P = 0.36 for bronchial responsiveness and P = 0.74 for F_ENO_{50}).

Conclusion: IgE sensitisation to cat allergen components appear to have a higher clinical value than extract-based measurement as it related better to airway inflammation and responsiveness and had a higher prognostic value for the development of asthma and rhinitis over a 12-yearperiod. Furthermore, sensitisation to certain cat allergen components might have a higher clinical value, but this should be further studied studied in asthma populations.

279
Sensitization to Fel d 1 in childhood predicts symptoms of cat allergy in adolescence - a BAMSE/MeDALL study

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Background: Sensitization to cat allergen molecules may contribute differently to development of cat allergy. The aim of the study was to investigate the association between sensitization patterns to cat allergen molecules during childhood and prediction of trajectories of symptoms to cat up to 16 years of age.

Method: Sera and questionnaire data from 779 randomly collected children from the BAMSE birth cohort at 4, 8 and 16 years were used. IgE to the cat allergen molecules Fel d 1, Fel d 2 and Fel d 4 were analyzed with an allergen chip based on ISAC technology (MeDALL chip). Allergy was defined as reported rhinitis, conjunctivitis or asthma at exposure to cat.

Results: Fel d 1 was the dominating cat allergen at all ages (8.7% at 4 years, 13.9% at 8 years and 20.3% at 16 years) and induced the highest median IgE levels, but with no significant increase over time (2.9–3.3 ISU-E at 4–16 years). Fel d 1 was the only cat allergen that induced significantly higher median IgE levels in children with symptoms to cat than in cat tolerant children at all ages (P-values < 0.001). Sensitization to Fel d 1 at 4–8 years of age was independently associated with symptoms to cat at 16 years (OR 13.7, 95% CI 8.3–22.7, adjusted for co-sensitization to other cat allergens). Sensitization to multiple cat allergens increased the likelihood of reporting cat symptoms longitudinally. However, positive predictive values for cat symptoms did not differ at any age between sensitization to Fel d 1 or cat extract.

Conclusion: IgE testing with just one cat allergen molecule, Fel d 1, is as good as testing IgE to cat allergen extract (ImmunoCAP) for prediction of cat allergy up to 16 years.

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Physico-chemical and lipid-binding characterization of the house dust mite allergen Der p 13 produced in *Pichia pastoris*

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Background: Amongst the allergens identified from the house dust mite (HDM) *D. pteronyssinus*, Der p 13 remains poorly characterized. According to its IgE reactivity, Der p 13 may be considered as a minor HDM allergen but it could nevertheless be important in the initiation of the HDM allergic response. This possibility is supported by Der p 13's sequence similarities with fatty acid binding proteins including the *B. tropicalis* lipid-binding allergen Blo t 13, indicating that Der p 13 could transport fatty acids/lipids able to activate innate immunity.

Objective: In the present study, Der p 13 was expressed in *P. pastoris* as a secreted protein. Physico-chemical characterization of rDer p 13 was performed to validate its structural integrity and its lipid-binding propensity was investigated for the first time.

Methods: The cDNA encoding full-length Der p 13 was cloned into the *P. pastoris* pPICZa A vector, downstream of the sequence encoding yeast mating factor leader peptide. Selected recombinant KM-71 *P.pastoris* clones were cultured in shake flask and rDer p 13 expression was induced by addition of methanol. After purification by cation exchange chromatography and ultrafiltration, the structure of rDer p 13 was characterized by circular dichroism (CD) and its molecular mass and sequence by mass spectrometry (MS). Polyclonal antibodies to rDer p 13 were produced to detect the natural allergen in HDM extracts. Finally, fluorescence-based lipid binding assays were performed to determine any hydrophobic ligand-binding activities exhibited by rDer p 13.

Results: rDer p 13 was expressed and secreted from KM71 *P. pastoris* strain as a 15 kDa soluble protein. MS analysis identified rDer p 13 with 90% sequence coverage. The physico-chemical characterization of purified rDer p 13 showed that the allergen preparation was homogenous, and displayed predominately β-sheet secondary structure content. Polyclonal antibodies to rDer p 13 were able to detect natural Der p 13 in HDM extracts. rDer p 13 was able to bind the hydrophobic surface probe 8-Anilinonaphthalene-1-sulfonic acid (ANS), and the natural fluorescent fatty acid *cis*-parinaric acid.

Conclusion: Our results not only suggest that rDer p 13 produced in *P. pastoris* is correctly folded but also support the hypothesis that Der p 13 is a member of the cytosolic lipid binding protein family. Such a recombinant product could be useful for future innate immune activation assays and HDM allergy diagnosis.

281
Prevalence of specific IgE to natural shrimp extracts and the allergens rPen a 1, nPen m 2, rPen m 3, rPen m 4 and rPen m 6 in shrimp allergic subjects

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Background: Shrimp is a common cause of severe food allergic reactions worldwide. Tropomyosin was initially considered to be the only relevant allergen in shrimp but several other components have since been identified. The aim of this study was to examine IgE antibody responses to natural extracts of different shrimp species (*Penaeus monodon, Pandalus borealis, Metapenaeus joyneri and Metapenaeus barbata*)

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Conclusion: The prevalence of RW and severity markers during the first year of life remained high between both surveys. The infants who suffer from RW have markedly high prevalence of severity markers as visits to ED and admissions for wheezing, indicating that an important group of these infants has a troublesome progression that certainly affect quality of life and put infants at risk of more severe complications.

A206

Successful Cyclophosphamide Desensitization in a Pediatric Patient with Systemic Lupus Erythematosus

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World Allergy Organization Journal 2016, **9(Suppl 1):**A206

Background: Cyclophosphamide (CYC) is an alkylating agent used to treat malignancies and autoimmune diseases. It is often given with mercaptoethane sulfonate (mesna) to prevent bladder toxicity. Hypersensitivity to CYC has been well described in adult patients with malignancy. These reactions are typically IgE-mediated to CYC or its metabolites after a previous exposure. Most of these patients have tolerated subsequent treatments by desensitization. We report the first described case of anaphylaxis after initial exposure of CYC in a pediatric patient with systemic lupus erythematosus (SLE) who successfully tolerated subsequent CYC by desensitization.
Method: Case description.
Results: An 11-year-old girl with SLE and Class III/V nephritis was admitted for hematuria, anasarca, and uncontrolled hypertension consistent with SLE nephritis flair. Her treatment was switched from mycophenolate mofetil to monthly CYC (945mg, 0.75 gm/m2) intravenously (IV) with mesna infusions given 30 minutes prior and 3, 6 and 9 hours after CYC infusion. Immediately after completing her first CYC infusion, she developed increased erythema at her IV site, angioedema of the lips and tongue, facial flushing, shortness of breath, chest tightness, and wheezing. She was treated for anaphylaxis with intramuscular epinephrine, IV diphenhydramine, methylprednisolone, and famotidine with An hour later, she became hypotensive (BP 91/31 from BP 147/69). Antihypertensives were held and she continued receiving IV diphenhydramine and methylprednisone. She received her post-treatment mesna infusions without any adverse event. She had no history of allergies or exposure to alkylating agents in the past. CYC was the preferred therapy as her renal function improved after her first treatment and Allergy was consulted to evaluate whether her anaphylaxis was secondary to mesna or CYC and for possible desensitization. Allergy evaluation revealed negative percutaneous testing to mesna (10mg/ml skin prick; 0.01mg/ml and 0.1mg/ml intradermal). CYC skin testing was deferred by the patient's guardian. She was admitted to the intensive care unit to receive CYC via a 12-step desensitization protocol. Her daily prednisone 40 mg dose was increased to methylprednisone 1 gm at 45 minutes prior to desensitization. She was also premedicated with diphenhydramine 25 mg and famotidine 20 mg at 20 minutes prior to desensitization. She tolerated desensitization without any adverse reactions. Since then, she has received CYC by desensitization every month at an outpatient infusion setting without any adverse event.
Conclusions: Hypersensitivity to CYC can be seen in pediatric patients with autoimmune disease. It can occur after initial exposure and can be as severe as anaphylaxis. Desensitization is one approach for continued treatment with CYC in pediatric patients with SLE which has been successful.

A207

The Fatty Acid Binding Protein Der p 13 Is a Minor House Dust Mite Allergen Able to Activate Innate Immunity

Pattraporn Satitsuksanoa[1], Narissara Suratannon[2], Jongkonnee Wongpiyabovorn[2], Pantipa Chatchatee[2], Kiat Ruxrungtham[2], Alain Jacquet[2]
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World Allergy Organization Journal 2016, **9(Suppl 1):**A207

Background: Compared with other group 13 house dust mite (HDM) allergens, Der p 13 remains very poorly characterized. We recently produced a recombinant form of Der p 13 in *P.pastoris* and demonstrated that this allergen binds lipids/fatty acids. This study investigated IgE sensitization to rDer p 13 in a large thai HDM allergic cohort as well as the allergen-induced airway epithelial cell activation.
Methods: The IgE reactivity to rDer p 13 was analysed by ELISA using 644 sera with a positive skin prick test (SPT) to *D.pteronyssinus* and collected from four different hospitals in Bangkok. The allergenic activity of rDer p 13 was tested using IgE-loaded rat basophil leukaemia cells (RBL) expressing human FcεRI. The direct airway epithelial cell activation by rDer p 13 was also evaluated.
Results: Only 6% of 644 HDM-allergic patients showed IgE-reactivity to rDer p 13 whereas the IgE binding frequency to rDer p 2 reached more than 60%. In RBL assays, rDer p 13 triggered basophil degranulation but the effector activity was lower than that measured for rDer p 2. Treatment of BEAS-2B respiratory epithelial cells with rDer p 13 triggered the production of IL-8 in a *concentration-dependent* manner.
Conclusions: Although rDer p 13 displays allergenic activity, its weak IgE reactivity clearly confirmed that Der p 13 is a minor allergen. We hypothesized that the poor IgE binding frequency of Der p 13 is in line with the apparent very limited amount of this allergen in mite fecal pellets aqueous extracts. Nevertheless, Der p 13, through its fatty acid binding capacity, could play a role in the activation of innate signaling pathways to initiate the allergic response.

A208

Epidemiology of Stevens-Johnson Syndrome and Toxic Epidemal Necrolysis: An Administrative Database Study

Min Suk Yang[1], Jin Yong Lee[1], Ja Yeun Kim[2], Han-Ki Park[3], Ju-Young Kim[4], Woo-Jung Song[4], Hye-Ryun Kang[4], Heung Woo Park[4], Yoon-Seok Chang[5], Sang-Heon Cho[4], Kyung-up Min[4], Chang-Han Park[6], Suk-Il Chang[6], Sook-Hee Song[7]
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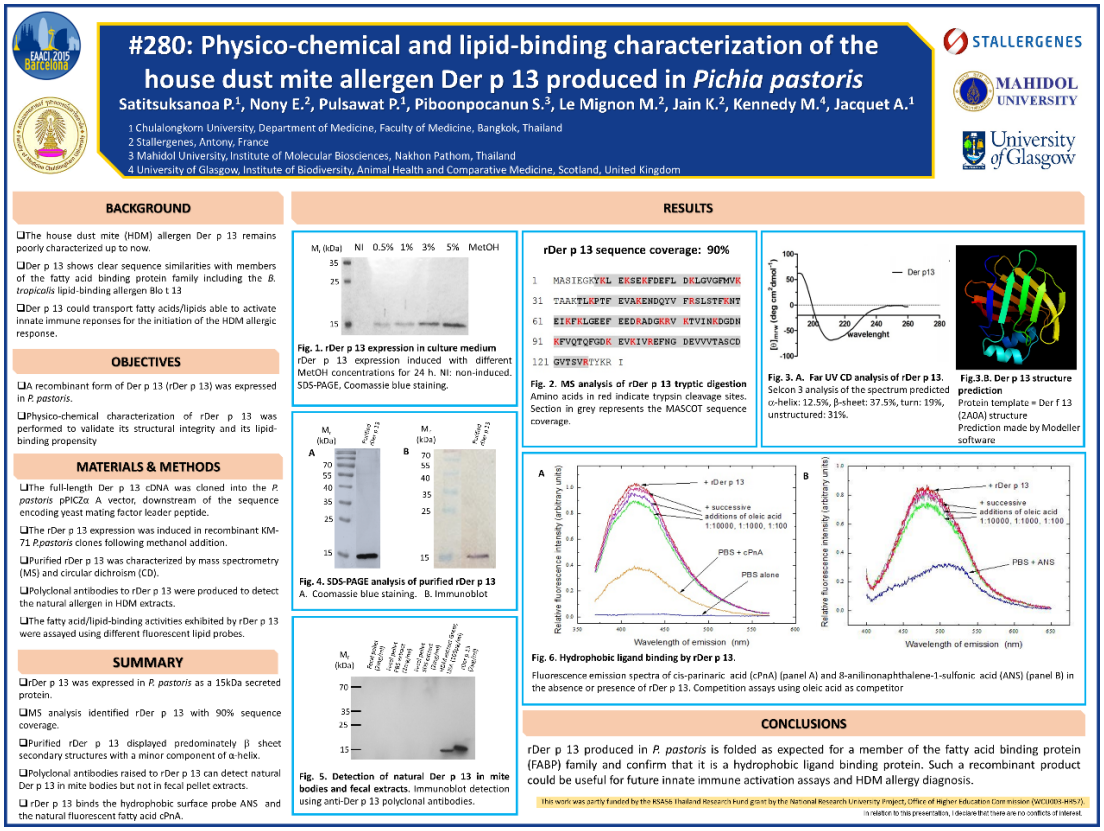
World Allergy Organization Journal 2016, **9(Suppl 1):**A208

Background: Nationwide incidence of Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) is hard to estimate. We report nationwide incidence of SJS and TEN using an administrative database.
Method: We used the database of the Health Insurance Review and Assessment Service (HIRA) in Korea. We employed the HIRA database from 2009 to 2013 to estimate the annual incidence, in-hospital mortality, related complications due to SJS and TEN. In this study, using the International Classification of Diseases-10th Revision (ICD-10), target study population was defined as patients with SJS or TEN, who had the primary diagnostic codes of L511 (SJS) or L512 (TEN), respectively.
Result: During four-years study period, estimates of annual incidence of SJS and TEN were 4.9-5.5 and 0.9-1.4 per million people. Mortality rate were 5.7% for SJS and 15.1% for TEN. Mean age was about 50 years old and female predominance was not so apparent in our data. Ocular and urethral sequelae were the most significant sequelae clinically that more than 40% of patients with both diseases suffered from ocular sequelae and about 6% of SJS and 9% of TEN patients were affected by the urethral sequelae. Mortality rate increased as the patients' age got older.
Conclusion: The incidence of SJS and TEN was not so much changed since 1990's. However, the mortality rate was decreased and this would be due to the evolution of supportive management.

Oral presentation in Poster discussion session 5 with Electronic-poster in EAACI Congress 2015, Barcelona, Spain



#280: Physico-chemical and lipid-binding characterization of the house dust mite allergen Der p 13 produced in *Pichia pastoris*

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BACKGROUND

- The house dust mite (HDM) allergen Der p 13 remains poorly characterized up to now.
- Der p 13 shows clear sequence similarities with members of the fatty acid binding protein family including the *B. tropicalis* lipid-binding allergen Blo t 13
- Der p 13 could transport fatty acids/lipids able to activate innate immune reponses for the initiation of the HDM allergic response.

OBJECTIVES

- A recombinant form of Der p 13 (rDer p 13) was expressed in *P. pastoris*.
- Physico-chemical characterization of rDer p 13 was performed to validate its structural integrity and its lipid-binding propensity

MATERIALS & METHODS

- The full-length Der p 13 cDNA was cloned into the *P. pastoris* pPICZα A vector, downstream of the sequence encoding yeast mating factor leader peptide.
- The rDer p 13 expression was induced in recombinant KM-71 *P.pastoris* clones following methanol addition.
- Purified rDer p 13 was characterized by mass spectrometry (MS) and circular dichroism (CD).
- Polyclonal antibodies to rDer p 13 were produced to detect the natural allergen in HDM extracts.
- The fatty acid/lipid-binding activities exhibited by rDer p 13 were assayed using different fluorescent lipid probes.

SUMMARY

- rDer p 13 was expressed in *P. pastoris* as a 15kDa secreted protein.
- MS analysis identified rDer p 13 with 90% sequence coverage.
- Purified rDer p 13 displayed predominately β sheet secondary structures with a minor component of α-helix.
- Polyclonal antibodies raised to rDer p 13 can detect natural Der p 13 in mite bodies but not in fecal pellet extracts.
- rDer p 13 binds the hydrophobic surface probe ANS and the natural fluorescent fatty acid cPnA.

RESULTS

Fig. 1. rDer p 13 expression in culture medium rDer p 13 expression induced with different MetOH concentrations for 24 h. NI: non-induced. SDS-PAGE, Coomassie blue staining.

Fig. 4. SDS-PAGE analysis of purified rDer p 13 A. Coomassie blue staining. B. Immunoblot

Fig. 5. Detection of natural Der p 13 in mite bodies and fecal extracts. Immunoblot detection using anti-Der p 13 polyclonal antibodies.

rDer p 13 sequence coverage: 90%

```
1   MASIEGKYKL EKSEKFDEFL DKLGVGFMVK
31  TAAKTLKPTF EVAKENDQYV FRSLSTFKNT
61  EIKFKLGEEF EEDRADGKRV KTVINKDGDH
91  KFVQTQFGDK EVKIVREFNG DEVVVTASCD
121 GVTSVRTYKR I
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Fig. 2. MS analysis of rDer p 13 tryptic digestion Amino acids in red indicate trypsin cleavage sites. Section in grey represents the MASCOT sequence coverage.

Fig. 3. A. Far UV CD analysis of rDer p 13. Selcon 3 analysis of the spectrum predicted α-helix: 12.5%, β-sheet: 37.5%, turn: 19%, unstructured: 31%.

Fig.3.B. Der p 13 structure prediction Protein template = Der f 13 (2A0A) structure Prediction made by Modeller software

Fig. 6. Hydrophobic ligand binding by rDer p 13.

Fluorescence emission spectra of cis-parinaric acid (cPnA) (panel A) and 8-anilinonaphthalene-1-sulfonic acid (ANS) (panel B) in the absence or presence of rDer p 13. Competition assays using oleic acid as competitor

CONCLUSIONS

rDer p 13 produced in *P. pastoris* is folded as expected for a member of the fatty acid binding protein (FABP) family and confirm that it is a hydrophobic ligand binding protein. Such a recombinant product could be useful for future innate immune activation assays and HDM allergy diagnosis.

This work was partly funded by the RSA56 Thailand Research Fund grant by the National Research University Project, Office of Higher Education Commission (WCU0001-AM57).

In relation to this presentation, I declare that there are no conflicts of interest.

Poster presentation in WAO Congress 2015, Seoul, South Korea

2138 The Fatty Acid Binding Protein Der p 13 Is a Minor House Dust Mite Allergen Able to Activate Innate Immunity

Pattraporn Satitsuksanoa[1], Narissara Suratannon[2], Jongkonnee Wongpiyabovorn[3], Pantipa Chatchatee[2], Kiat Ruxrungtham[1], and Alain Jacquet[1], and the Mite Allergy Research Cohort (MARC) study team

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(2) Department of Pediatrics, Chulalongkorn University, Bangkok, Thailand,

(3) Department of Microbiology, Chulalongkorn University, Bangkok, Thailand

Background:
Compared with other group 13 house dust mite (HDM) allergens, Der p 13 remains very poorly characterized. We recently produced a recombinant form of Der p 13 in *P.pastoris* and demonstrated that this allergen binds lipids/fatty acids. This study investigated IgE sensitization to rDer p 13 in a large thai HDM allergic cohort as well as the allergen induced airway epithelial cell activation.

Methods:
The IgE reactivity to rDer p 13 was analysed by ELISA using 644 sera with a positive skin prick test (SPT) to *D.pteronyssinus* and collected from four different hospitals in Bangkok. The allergenic activity of rDer p 13 was tested using IgE loaded rat basophil leukaemia cells (RBL) expressing human FcεRI. The direct airway epithelial cell activation by rDer p 13 was also evaluated.

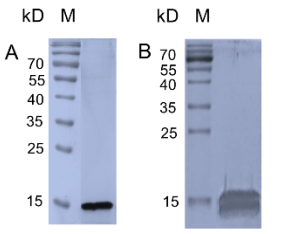
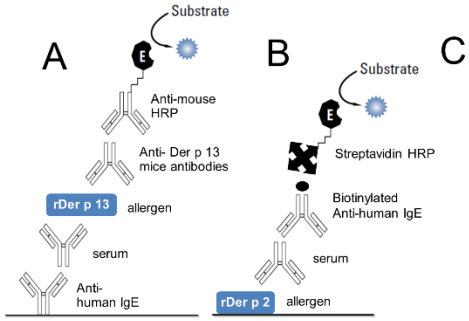


Fig.1. SDS-PAGE of purified rDer p 13 (A) and rDer p 2 (B)



Serum	
HDM allergic patient (SPT≥ 3 mm)	
N	644
ImmunoCAP Dpt	0.45 to >100 kU/L
HDM Non allergic (SPT negative)	
N	67
ImmunoCAP Dpt	<0.35 kU/L
IgE reactivity	
Positivity Threshold (Mean OD negative sera ±3 SD)	rDer p 13 = 0.166
	rDer p 2 = 0.157
rDer p 13	38/644 = 6%
rDer p 2	399/644 = 62%

Fig.2. IgE reactivity to rDer p 13 and rDer p 2
A) ELISA assays for the specific IgE detection. B) Characterization of the sera. C) IgE binding frequency. A serum was considered as positive when the OD value was higher than a cut off value, established as the mean OD values of negative control serum plus 3 standard deviations.

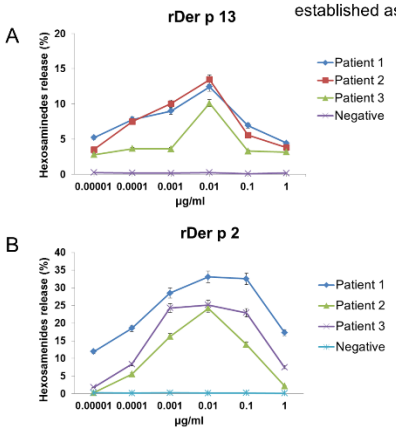


Fig.4. RBL-SX 38 degranulation assay
Allergenic activity of rDer p 13 (A) and rDer p 2 (B) using sera from three representative patients.

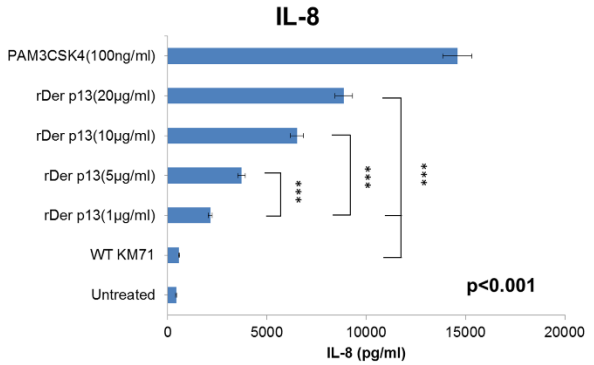


Fig.5. rDer p 13-induced IL-8 secretion in BEAS-2B cells
WT KM71: medium from cultured wild type KM71 strain and purified according to the purification protocol of rDer p 13. All data are expressed as the mean ± S.E. from at least two independent experiments performed in triplicate.

Conclusion
rDer p 13 is confirmed to be a minor allergen in Thailand, according to the low IgE binding frequency and its low allergenic activity. We hypothesized that the poor level of sensitization to Der p 13 is in line with the apparent limited amount of this allergen in mite fecal pellets. Nevertheless, Der p 13, through its fatty acid binding capacity, could play a role in the activation of innate signaling pathways to initiate the allergic response at the level of the airway epithelium.

Acknowledgements:
This work was partly funded by the RSA56 Thailand Research Fund grant by the National Research University Project, Office of Higher Education Commission (WCU003-HR57) and the 90th Anniversary of Chulalongkorn University, Rachadapisek Sompote Fund. Mite Allergy Research Cohort (MARC) study team: King Chulalongkorn Memorial Hospital (Pattarawat Thantiworasit, Pinya Pulsawat), Children hospital (Mukda Vangveravong,Tassalalpa Daengsuwan), Ramathibodi hospital (Ticha Rerkpattanapipat, Wannada Laisuan, Malinee Tongdee, Nizchapha Dchapaphapeaktak), Phramongkutklao College of Medicine (Atik Sangasapaviliya, Tadech Boonpiyathad)

In relation to this presentation, I declare that there are no conflicts of interest.

Poster presentation in CU MED Conference 2014, Bangkok, Thailand

CU MED2014 CONFERENCE 16 - 20 JUNE 2014
'100 Year - Experiences towards Excellence'
'๑๐๐ ปีแห่งประสบการณ์ สู่ศตวรรษแห่งความเป็นเลิศ'

Expression and purification of recombinant house dust mite allergen Der p 13

Satitsuksanoa P and Jacquet A
Department of Medicine, Faculty of Medicine,
Chulalongkorn University

Background: Der p 13 is a poorly characterized allergen from the house dust mite (HDM) *Dermatophagoides pteronyssinus*. As this allergen displayed sequence homologies with fatty acid binding protein/lipocalin, Der p 13 could activate innate immune pathways to initiate the allergic response.
Objectives: The goal of the present study was to produce a recombinant form of this allergen using the yeast *Pichia pastoris* expression system.
Materials & Methods: The Der p 13 cDNA was amplified by PCR and cloned into the pPICZa A expression vector. The *P. pastoris* KM71 strain was transformed with the recombinant vector and rDer p 13 expression was induced by addition of methanol in the culture medium. The supernatant containing secreted rDer p 13 was used to purify the allergen by cation exchange chromatography. Polyclonal antibodies were subsequently produced following mice immunizations with purified rDer p 13 to detect natural Der p 13 in HDM extracts.

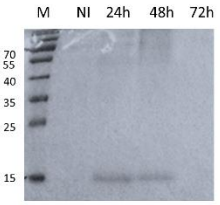


Figure 1. rDer p 13 expression from transformed *P.pastoris* KM71 strain. rDer p 13 expression was monitored in the culture supernatant following 0.5% methanol induction for 24h, 48h, 72h and NI (non induced). The highest level of expression was observed at 24 h.

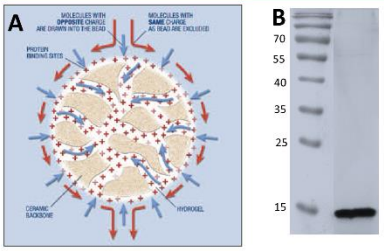


Figure 3. rDer p 13 purification cation exchange chromatography
A. Section of a ion exchange porous bead for efficient purification of biomolecules.
B. SDS-PAGE analysis of purifed rDer p 13

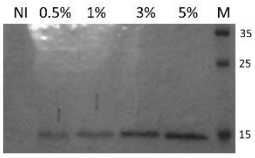


Figure 2. Optimization of rDer p 13 expression
rDer p 13 expression was induced with different methanol concentrations (0.5%, 1%, 3% and 5%) for 24 h. The highest level of rDerp 13 production was obtained with a 5% methanol induction.

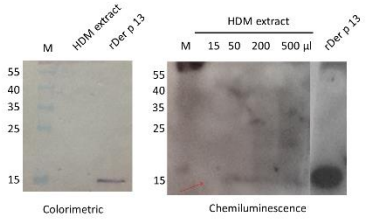


Figure 4. Detection of natural Der p 13 by Western blot in HDM extract
Colorimetric or chemiluminescent detection of natural Der p 13 by using polyclonal antibodies against rDerp 13

Results: The highest production of secreted rDer p 13 by *P.pastoris* was triggered with 5% methanol during 24h (figure 1 & 2). rDer p 13 was shown to adsorb aspecifically to any agarose-based matrices. The use of ceramic-based cation exchange resin not only improved the purification yield but also the removal of pigments from the yeast culture medium. Purified rDer p 13 migrated onto SDS-PAGE as a single 15kD band (figure 3B). Mice polyclonal antibodies to rDer p 13 was able to detect the corresponding natural allergen in HDM extracts, suggesting that rDer p 13 adopts a correct folding (figure 4).
Conclusions: rDer p 13 produced in *P. pastoris* is appropriate to further characterize the lipid binding properties of this allergen and to estimate its IgE reactivity in HDM allergic population.

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Patterns of IgE sensitization in house dust mite-allergic patients: implications for allergen immunotherapy

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The House Dust Mite Major Allergen Der p 23 Displays O-Glycan-Independent IgE Reactivities but No Chitin-Binding Activity

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VITA

Miss Pattraporn Satitsuksanoa was born on the 4th of July 1988 in Bangkok, Thailand. She is currently doing her doctoral degree in Medical Science (Cell biology and human molecular genetics) at the Faculty of Medicine, Chulalongkorn University. She completed her bachelor's degree in Genetics at the Faculty of Science, Kasetsart University and her master's degree in Medical science (Human reproduction and population planning) at the Faculty of Medicine Ramathibodi Hospital, Mahidol University. Once she started her master's degree, she was selected to be the first-runner up of University representative at the Faculty of medicine Ramathibodi hospital, Mahidol University. She also worked as the mistress of the ceremony in the Reproductive Health Conference and FIGO Joint Meeting (International medical conference), Bangkok, Thailand, 2011. Soon after, she received an Achievement award as the first student who graduated within 2 years from the Faculty of medicine Ramathibodi hospital, Mahidol University. When she started her doctoral degree in Medical science, she won 10,000 baht research grant for the full workshop training of the International workshop on Protein Expression and Purification Strategies 2012, at Chulalongkorn University and then became the teacher assistant of this International workshop in the following years of 2013 and 2014. She was the winner of the UK-SE Asia Collaborative Development Awards that received £2,000 grant to support the initial stages of new collaborations between the UK and South-East Asian researchers from the British council. The science experiment training was supervised by Prof. Malcolm Kennedy, PhD at the University of Glasgow, Scotland, 2013. In the year 2015, she won both of the Junior member travel grants from the European Academic of Allergy and Clinical Immunology (EAACI), for the oral presentation in Barcelona, Spain and also from the World Allergy Organization (WAO), for the poster presentation in Seoul, Korea. Since she visited the international science laboratory and international scientific meetings, she realized that science is the most powerful tool for all mankind. Not only that, she was inspired by her foreigner professors that everyone has the capability of thinking scientifically and solving scientific problems. So, she put a lot of effort to achieve her doctoral degree. Her inspiration quote is "A dream doesn't become reality through magic; it takes sweat, determination, and hard work. However, her life was not only about academic achievement, she also spending her free time practicing her English and working for society. She has become a Thai-English interpreter for tourists at Suan Lumpinee police station since 2011.