行政院國家科學委員會專題研究計畫 成果報告

第二群過敏原誘發上皮細胞與自體反應 B 淋巴球凋亡之蛋白體研究與免疫體分析(第 2 年)
研究成果報告(完整版)

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Proteomic and immunomic investigation for house dust mite allergens-induced apoptosis in epithelial cell and autoreactive B lymphocyte

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中文摘要

塵蟎為最重要的室內過敏源之一，而已知的塵蟎過敏原中以第一群與第二群塵蟎過敏原為主要過敏原(major allergen)，研究顯示這兩群過敏原的盛行率皆超過 85%。第一群塵蟎過敏原已知為具有蛋白酶活性，可以破壞呼吸道上皮細胞進入體內並誘發相關發炎與過敏反應。第二群塵蟎過敏原並不具有蛋白酶活性，但臨床證據顯示仍有過敏病人的血清 IgE 僅會與第二群塵蟎過敏原有結合反應，而對第一群塵蟎過敏原沒有結合反應。因此，本研究著重在瞭解第二群塵蟎過敏原如何穿過呼吸道上皮細胞，再進入體內誘發相關致敏機制。

本研究利用酵母菌表現重組的歐洲室塵蟎第二群過敏原 Der p 2 (DP2)，再以呼吸道上皮細胞株 A549 建立細胞模式實驗，進行 DP2 對 A549 細胞所產生的影響。第一階段的研究結果顯示 DP2 會導致 A549 細胞的黏附力下降，促進細胞的脫附現象，且此一現象並非因細胞凋亡(apoptosis)或壞死(necrosis)所造成。接著，我們進一步觀察細胞黏附分子(adhesion molecules)的表現與調控，更深入地研究 DP2 導致脫附現象的機制。本階段的研究結果顯示，利用西方墨點法發現 DP2 會抑制 A549 細胞 E-cadherin 以及提高 claudin-2 蛋白質的表現。再以 RT-PCR 的分析發現 DP2 會提高 claudin-2 mRNA 的表現量，且利用轉錄抑制劑(actinomycin D)處理後，DP2 誘發的 claudin-2 蛋白質表現便下降，顯示 DP2 係透過促進 claudin-2 的基因表現來提高其蛋白質量。更進一步的研究發現此一基因表現調控與β-catenin 及 GSK-3β有關。

綜合上述研究結果，本研究首次發現無蛋白酶活性的 DP2 具有調控呼吸道上皮細胞黏附分子表現的能力，進而促進上皮細胞的脫附現象，且此一調控係透過 β-catenin 及 GSK-3β的轉位(translocation)與降解有關。據此，我們推測第二群塵蟎過敏原利用此一機制破壞上皮細胞的完整性，進而有機會進入體內誘發相關的致敏反應。

Abstract

Non-proteolytic group 2 allergen, Der p 2 (DP2) is known as a major allergen derived from house dust mite *Dermatophagoides pteronissinus*. Paracellular epithelial barrier, being composed of a number of tight junction molecules, plays pivotal roles in resistance of pathogen invading. However, the effects of DP2 on epithelial barrier are not well understood.

In the present study, we aimed to investigate the effects of DP2 on epithelial integrity with focus on expression of claudin-2, and the mechanism regulating the expression of the junction molecules. Effects of DP2 on cell adhesion/dissociation and cell cycle of lung alveolar cell A549 were determined by MTT assay and flow cytometry. Expression of claudin-2, subcellular distribution of β-catenin, and Der p 2-induced kinase cascades were demonstrated by immunoblot. Our results revealed that DP2 increased cell dissociation and decreased cell adhesion of A549 cell without influencing cell cycle. Additionally, it elevated protein level of claudin-2 and increased the expression and nuclear translocation of β-catenin. Moreover, DP2 was found to enhance the phosphorylation of glycogen synthase kinase-3β (GSK-3β) and Akt, and the DP2-induced claudin-2 expression was suppressed in presence of an Akt inhibitor, wortamannin.

In conclusion, our findings shows that exposure of A549 cells to non-proteolytic DP2 resulted in increases of cell dissociation. This phenomenon may be conclusively attributed to the increase of claudin-2 through Akt/GSK-3β/β-catenin pathway. It is suggested that presence of DP2 may loosen epithelial integrity by alteration of junction molecule expression.

Introduction

House dust mite (HDM) is considered a contributing factor to asthma. The mites commonly found in house dust belong to the genus *Dermatophagoides* of which there are two species, *D. pteronyssinus* and *D. farinae* (1). Mite-derived allergens eliciting IgE immune response share structural similarities resulting in a cross-reactivity between *Dermatophagoides* spp (2-4). Of mite-sensitive individuals, approximately 90% generates IgE antibody responses to well-identified HDM allergens that are categorized into 24-kd group 1 such as Der p 1 and Der f 1 and the 14-kd group 2 allergens like Der p 2 (DP2) and Der f 2 on basis of IgE affinity (5,6). Der p 1 exerts proteolytic activity that is proposed to be associated with allergenicity (7). In contrast, DP2 lacks proteolytic activity and its biological functions remains to be elucidated.

Although lacking the enzymatic activity, the immunogenic properties of HDM group 2 allergens have been widely investigated for the past few years. Recent studies have demonstrated that HDM group 2 allergens share not only structural homology but also functional similarity in aspect of innate immunity with MD-2, the lipopolysaccharide-binding component of the Toll-like receptor 4 signaling complex (8-10). DP2 recently has been considered a factor to aggravating respiratory airway disorder. It is found that interaction of DP2 with respiratory cells resulted in upregulated secretion of inflammatory cytokines and expression of intercellular adhesion molecule-1 (11).

Tight junction (TJ) between epithelial cells serves as a paracellular permeability barrier regulating passage of ions and small molecules (12). Of respiratory epithelia, it is in a position to resist allergen penetration, whereas it is vulnerable to molecules with proteolytic activity. A TJ is composed of a number of transmembrane proteins including occludin (13), junctional adhesion molecule (14), tricellulin (15), and the claudin family

(16). Claudins locate on the apicolateral portion of epithelial cells, and their differential expressions play important roles in regulation of paracellular permeability. On basis of biological function, claudins have been divided into barrier-forming and channel-forming claudins (17). Claudin-1 is crucial for the tightening of the epithelial barrier in native skin epithelium (18). In contrast, upregulation of claudin-2 markedly attenuates the tightness of the epithelial barrier (19) and induces the paracellular cation channels (20). These observations imply that difference in permeability of epithelia at various regions is attributed to the protein composition of tight-junction.

Appreciating the etiology of respiratory allergy such as asthma, interaction of allergen with frontline of physical barrier such as respiratory epithelia remains to be elucidated. The present study was aimed to investigate airway epithelial cells responding to DP2 with emphases on cell adhesion and dissociation. Appreciating the role of claudin-2 in epithelia formation, mechanisms underlying regulation of claudin-2 expression through β-catenin and phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathways is of interest. The alveolar epithelial cell line A549 was used to investigate the effects of DP2 on cell adhesion/dissociation, expression of claudin-2, translocation of β-catenin, phosphorylation of glycogen synthase kinase (GSK)-3β and activation of Akt.

Materials and methods

Cell culture

A549 cells, the human type II alveolar epithelial cell line, purchased from the American Type Culture Collection, was cultured in 10-cm sterile Petri dish in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS)(Invitrogen Life Technologies, Carlsbad, CA) and 100 U/mL penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2. The passage

and the harvest of A549 cells were performed by using non-proteolytic CDS reagent (Sigma, St. Louis, MO) to detach the cells from the culture plates according to the manufacturer's instructions. Prior to allergen treatments, A549 cells were starved and allowed to attach by incubating with serum-free DMEM for 16 hours (h).

Expression and purification of DP2

Recombinant DP2 was expressed in *Pichia pastoris* and purified as previously described (21). Briefly, *Pichia pastoris* that encoded DP2 DNA grew at 28^{o}C in buffered glycerol-complex medium for 48 h using an orbital shaker at 200 rpm, and then transferred to buffered methanol-complex medium for 72 h. Soluble DP2 in culture supernatant was precipitated by adding solid ammonium sulfate to 50% saturation. The precipitant was collected by centrifugation (20,000 g for 20 min) and then the pellet was resuspended with phosphate buffered saline (PBS). After dialyzed against PBS, the DP2 solution was passed through 0.22 μm filter and stored at -80^{o}C.

Determination of adherent cells and detached cells

The assessment of cell adhesion and detachment was performed as previously described (22). In brief, A549 cells were seeded in 24-well tissue-culture plates at 4x10^{4} cells/mL and cultured in DMEM supplemented with 10% FBS until 80% confluent. After starvation by serum-free DMEM for 16 h, the cells were incubated with a series of concentrations of DP2 for 24 h. After the incubation, the detached cells from the culture medium and the cells that remained attached to the culture plates were collected separately, and then the cell numbers were quantitated with MTT assay as described previously (23).

SDS-PAGE and immunoblot

A549 cells were seeded in 6-cm dish at $1.5x10^5$/mL. After 16 h incubation with serum-free DMEM, the cells were treated with a series concentration of DP2 for indicated times. After washing with PBS, the treated cells were collected and lysed by Triton lysis buffer [10 mM Tris-HCl, pH7.5; containing 1% v/v Triton X-100, 150 mM NaCl, 0.5 mM EDTA, 1 mM phenylmethanesulfonylfluoride (PMSF), 1 mM NaF, 1 mM $Na_4P_2O_7$, 10 μg/mL aprotinin and leupeptin](Sigma-Aldrich). After centrifuging to remove insoluble pellet, the supernatants were collected for SDS-PAGE analysis. Protein concentration of supernatant was quantitated by Bradford method using protein assay kit (Bio-Rad Laboratory, Hercules, CA). The crude proteins (30 μg/lane) were separated in 12.5% SDS-PAGE, and then transferred onto nitrocellulose membrane (Millipore, Bedford, MA). After blocking with 3% w/v skimmed milk, the membranes were incubated with antibodies against human claudin 2 (Abcam, Cambridge, UK), phosphorylated Akt (pAkt; Cell signaling, Beverly, MA), phosphorylated GSK-3β (pGSK-3β; Millipore), histone H1 (Millipore) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam), respectively. After washing with PBS containing 0.1% v/v Tween-20, the reacted membranes were incubated with anti-IgG antibodies conjugated with peroxidase (Abcam). The detection of antigen-antibody complex was performed by using ECL reagent (Millipore) and luminescence image system (LAS-4000; Fujifilm, Tokyo, Japan).

Subcellular fractionation

Adherent A549 cells were washed with PBS and incubated with lysis buffer (10 mM HEPES, pH7.6; containing 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.05% v/v Igepal CA-630 and 1 mM PMSF, 1 mM sodium orthovanadate,

50 mM sodium fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) for 10 min. Cell lysates were collected by a centrifugation at 2,500 g for 10 min at 4 °C. The supernatant containing the cytosol was further centrifuged at 20,000 g for 15 min at 4 °C, namely cytosolic fraction. The pellets containing nuclei were washed with PBS, resuspended in nuclear buffer (25 mM HEPES, pH7.6, 0.1% v/v Igepal CA-630, 1 M KCl, 0.1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 2 mM sodium fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin), and centrifuged at 10,000 g for 15 min at 4 °C. The resulting supernatants were collected, namely nuclear fraction.

Statistical analysis

Data were expressed as means ± SEMs of the three independent experiments. Statistical significance analysis was determined by using 1-way ANOVA followed by Dunnett for multiple comparisons with the control or the impaired 2-tailed Student *t* test. The differences were considered significant for *p* values less than 0.05.

Results

DP2 induces cell detachment and diminishes cell adhesion of alveolar A549 cells without interference of cell cycle

To investigate the effects of DP2 on cell adhesion/dissociation, the attached and the detached A549 cells were separately determined. As shown in Fig. 1A, 5 μg/mL DP2 treatments significantly decreased cell adhesion of A549 cell to 74.3 ± 1.6% of control, and higher than 20 μg/mL DP2 treatments significantly increased the cell detachment up to 163.6 ± 26.2% of control. To further examine whether the observation is attributed to cell death induced by DP2, A549 cells treated with DP2 was analyzed for cell cycle status. As shown in Fig. 1B, no significant cell cycle arrest was found in A549 cells in presence

of DP2 at concentrations of 20 and 30 μg/mL compared with the control. The results show that DP2-induced cell detachment is not associated with cell death in A549 cells.

DP2 upregulates protein expression of claudin-2 through transcription regulation

As cell cycle arrest in A549 in response to DP2 was not significant, it was hence postulated that manipulated expression of junction molecules might contribute to the DP2 induced cell dissociation, and claudin-2 was therefore investigated. Our results revealed that protein expression of claudin-2 in A549 cells was increased in response to DP2 treatments in a dose-dependent manner (Fig. 2A). Additionally, the increased protein expression of claudin-2 by DP2 was diminished by pretreatment of actinomycin D, a transcription inhibitor (Fig. 2B).

DP2 induced accumulation of β-catenin and enhanced the nuclear translocation of β-catenin

β-catenin has been reported for its regulatory role in mRNA expression of claudin-2 via the lymphoid enhancer factor-1/β-catenin complex (24). Therefore, the level and the localization of β-catenin in response to DP2 exposure were investigated for its involvement in regulating claudin-2. As shown in Fig. 3A, a treatment of A549 cells with DP2 at a concentration of 20 μg/mL significantly increased the protein expression of β-catenin in 1 hour. Cytoplasmic accumulation and consequent nuclear localization of β-catenin have been reported to be correlated to the transcription activation of target genes of Wnt/β–catenin pathway (25). Nuclear-cytoplasmic distribution of β-catenin in response to DP2 treatment in A549 cells was determined. To examine the nuclear localization of β-catenin, the cytoplasmic and nuclear extracts from DP2 treated cells were subjected to immunoblot. As shown in Fig. 3B, the levels of cytoplasmic and

nuclear β-catenin were both elevated in A549 cells exposed to DP2 at a concentration of 20 μg/mL for 30 min, and reached a peak in 60 min. The increase in nuclear β-catenin was associated with similar trend of elevation in cluadin-2.

DP2 increases serine-9 phosphorylation of GSK-3β and phosphorylation of Akt involving in upregulation of claudin-2 expression

Having observed accumulation and elevated nuclear localization of β-catenin, regulation of β-catenin with focus on an upstream regulator, GSK-3β was subsequently of interest to investigate. The activity of GSK-3β is differentially regulated by phosphorylation in a site-specific manner. Phosphorylation of tyrosine-216 increases the activity of GSK-3β, whereas the activity is inhibited by phosphorylation of serine-9 (26). Thus, the serine-9 phosphorylation of GSK-3β was determined. As shown in Fig. 4A, serine-9 phosphorylation of GSK-3β in A549 cells was increased upon DP2 treatment (20 μg/mL) in 10 min and appeared to reach the peak at 20 min post exposure.

Serine-9 of GSK-3β has been shown to be phosphorylated by Akt through PI3K/Akt signal cascades (27); therefore, activation of Akt in A549 cells upon DP2 treatment was investigated. Our result showed that DP2 treatment (20 μg/mL) increased phosphorylation level of Akt (pAkt) in 10 min with the peak at 20 min (Fig. 4B). Moreover, inhibition of PI3K by wortamannin significantly diminished the elevated claudin-2 expression by DP2 treatment (Fig. 4C).

Discussion

The epithelial lining of the airways is a developmentally specialized barrier that exerts as the frontline encountering inhaled allergens. As TJs normally restrict the access of particles to paracellular channels, degree of disassociation of TJs has been found to be

associated with the level of permeability of epithelial barrier, resulting in increased delivery of allergens to antigen presenting cells such as dendritic cells underneath (28). In the present study, although no proteolytic activity for DP2 is reported, our results show that DP2, a house mite allergen without proteolytic activity, increased cell dissociation and decreased cell adherence in A549 cells. We also found that the DP2-induced cell dissociation of A549 was not affected by protease inhibitors (data not shown). Accordingly, it is suggested that DP2 might induce paracellular leakage in epithelial monolayer without proteolytic reaction and subsequently lead to a transepithelial cross of macromolecules and encountering with antigen-presenting cells. Additionally, DP2 is known to activate respiratory epithelial cells with results of upregulated secretions of granulocyte-macrophage colony-stimulating factor, IL-6, IL-8, monocytechemotactic protein-1 and macrophage inflammatory protein-3α (11), which may potentiate inflammatory reactions (29), prolong survival of eosinophils (30) stimulate collagen synthesis (31) and recruit dendritic cells to mucosal and epithelial surfaces (32). Taken together, these may explain that a population of patients with HDM-induced asthma is only allergic to DP2 in clinics.

Claudin-1 and claudin-2 play important but biologically opposite roles in epithelial permeability. High expression of cluadin-1 strengthens epithelial barrier and cleavage of claudin-1 leads to a nonspecific increase in epithelial permeability (28), whereas, elevated expression of claudin-2 loosens epithelial integrity and causes leakage (20). It is reported that rat lung alveolar epithelial cells express claudin-3, claudin-4 and claudin-5, but show little claudin-1 and claudin-2 (33). Recently, Peter et al reported that claudin-2 overexpression was involved in permeability and remodeling in A549 cell model, suggesting that claudin-2 could contribute to degree of metastasis of lung cancer, and found that claudin-1 and claudin-4 were undetectable in their cell experiments (34).

Consistently, the both claudin-1 and claudin-4 are also undetectable in our A549 cell model (data not shown). Taken together, these findings indicate that claudin-2 plays a pivotal role in maintaining integrity of A549 cell monolayer.

β-catenin is a multifunctional protein that plays an important role in a variety of cell biological activities including cell development, cell adhesion, repair of injury, cell cycle regulation and tumor formation (25,35). It acts as a key mediator in Wnt signaling pathway by which a variety of genes are regulated in association with its nuclear localization and interaction with transcription factor T cell factor/lymphoid enhancer factor (25,35). We demonstrated that treatment of A549 cells with DP2 triggered cytosolic accumulation of β-catenin and promoted nuclear translocation of β-catenin. GSK-3β, a protein kinase, is known for its multifunctional activities of which the classic Wnt/β-catenin signaling pathway is negatively regulated and down-regulation of β-catenin levels through phosphorylating β-catenin and leading to its degradation (36). We report that treatment of DP2 resulted in significantly increased serine-9 phosphorylation of GSK-3β by which kinase activity of GSK-3β is attenuated and level and phosphorylation of β-cateninare altered and affected as consequences. These findings implicate that DP2-induced signaling may be involved in or crosstalk with parts of classical Wnt/β-catenin signaling pathway.

PI3K/Akt signaling cascade is one of the signaling pathways that leads to inhibition of GSK-3β by increasing serine-9 phosphorylation (27). Additionally, serine-9 of GSK-3β also can be phosphorylated by protein kinase C (37), p90Rsk, p70 ribosomal S6 kinase (38), and protein kinase A (39). Thus, multiple mechanisms are evolved and employed to surveillance and control the activity of GSK-3β by phosphorylation. Our results showed that treatment of DP2 increased phosphorylation of Akt, and inhibition of PI3K by wortamannin significantly diminished DP2-induced claudin-2 expression, suggesting that

PI3K/Akt activation is crucial for DP2-induced claudin-2 expression. However, further investigations are required to elucidate the involvement of protein kinase A, protein kinase c and p70 ribosomal S6 kinase in DP2-induced claudin-2 expression.

The present study shows that treatment of DP2 lead to increased protein level of claudin-2 in lung alveolar cell A549, putatively resulting from, in a synergistic fashion, activation of PI3K/Akt activation, inhibition of GSK-3β, and accumulation and enhanced nuclear translocation of β-catenin to increase mRNA transcription of claudin-2. These findings provide evidences that non-proteolytic DP2 allergen can alter the TJ molecule expression through induction of PI3K/Akt/GSK-3β/β-catenin signaling cascades and result in leakiness or looseness of epithelial barrier.

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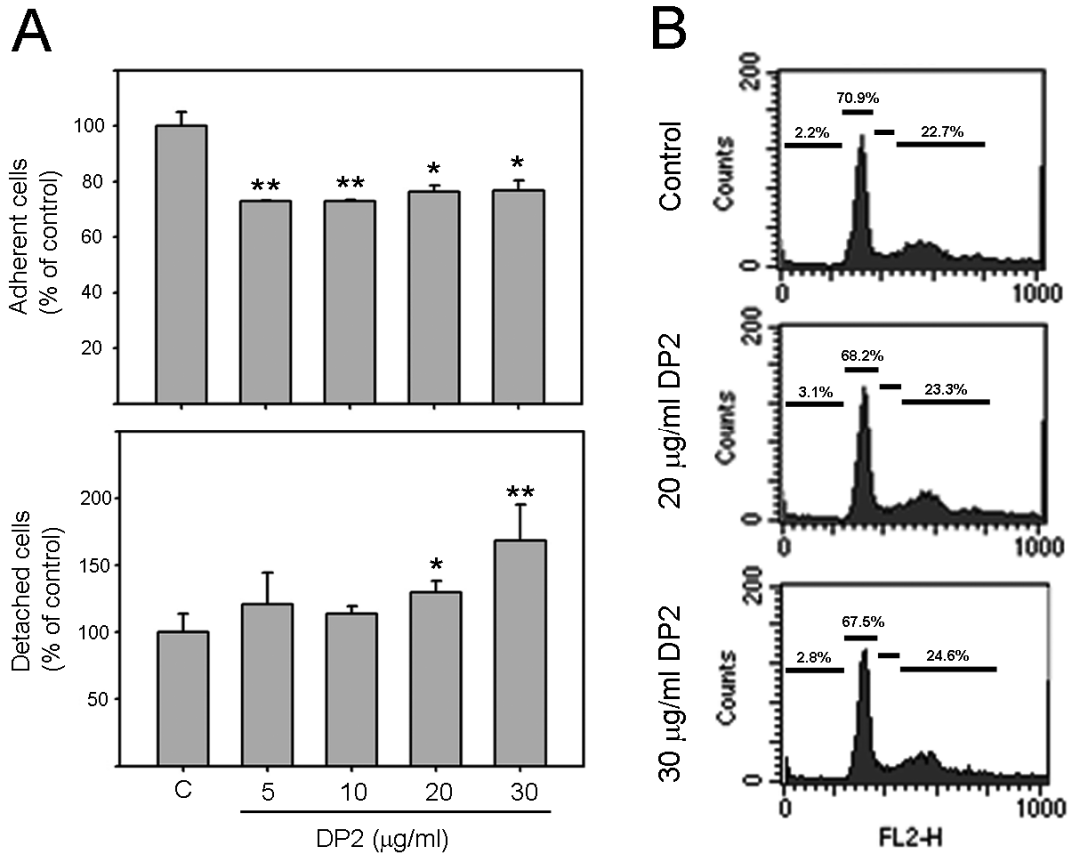

Figure 1. DP2 induced cell dissociation and decreased cell adherence in lung alveolar A549 cells. (A) A549 cells were treated with indicated concentration of DP2 for 24 h, and then the attached and the detached cells were quantitated by MTT assay. Data were shown as the means ± SD. Three independent experiments were performed for statistics. *, p <0.05 and **, p <0.01as compared with control. (B) A549 cells were treated with indicated concentration of DP2 24 h, and then the detached cells analyzed by flow cytometry. SubG1, G0/G1, S and G2/M phase were indicated, and the ratio of each phase showed no statistic significance as compared to which of control.

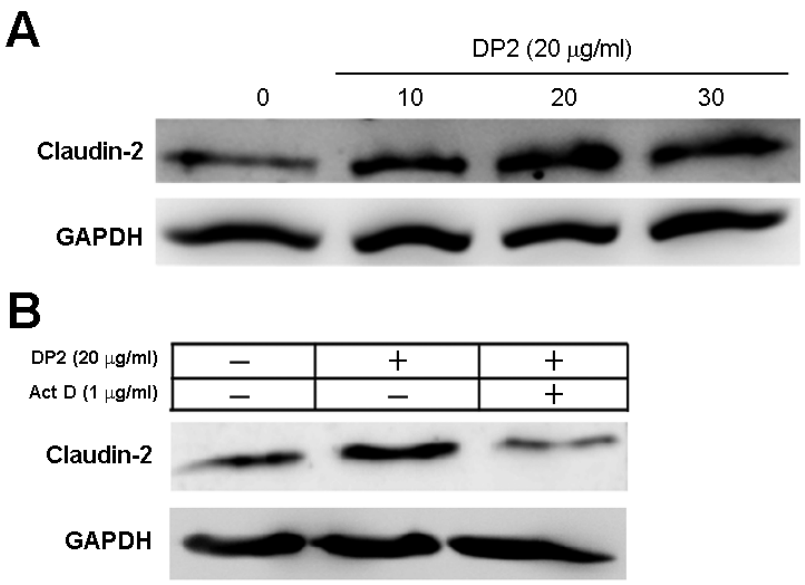

Figure 2. DP2 increased protein level of claudin-2 in lung alveolar A549 cells. (A) A549 cells were treated with indicated concentration of DP2 for 24 h, the levels of claudin-2 were determined by immunoblot. (B) A549 cells were pretreated with actinomycin D (Act D) for 3 h, and then incubated with 20 μg/mL DP2 for 24 h. The levels of claudin-2 were determined by immunoblot. GAPDH was used as control.

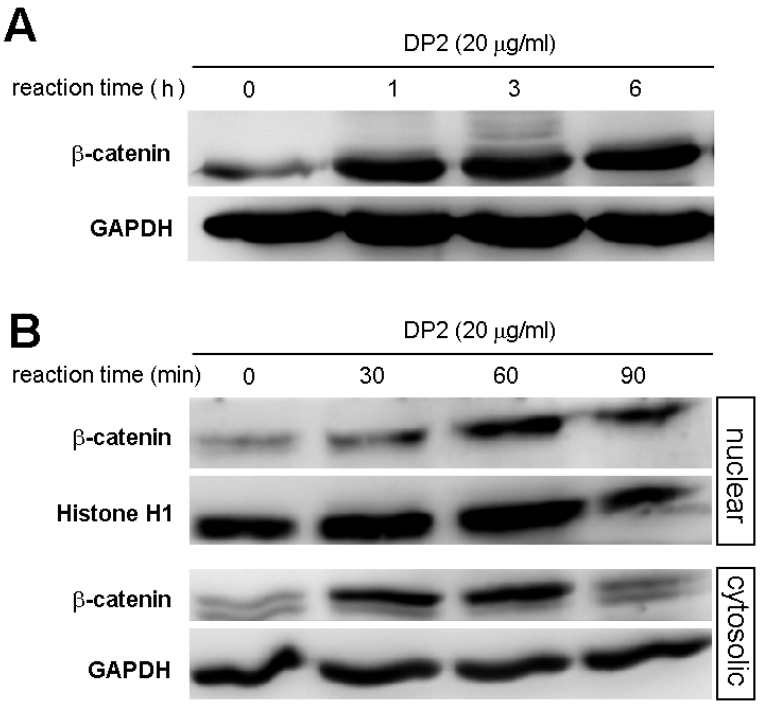

Figure 3. DP2 increased expression of β-catenin and induced nuclear translocation of β-catenin. (A) A549 cells were treated with 20 μg/mL DP2 for indicated time (h), and the levels of total β-catenin were determined by immunoblot. (B) A549 cells were treated with 20 μg/mL DP2 for indicated time (min), and the levels of cytosolic and nuclear β-catenin were determined by immunoblot. Histone H1 and GAPDH was used as control.

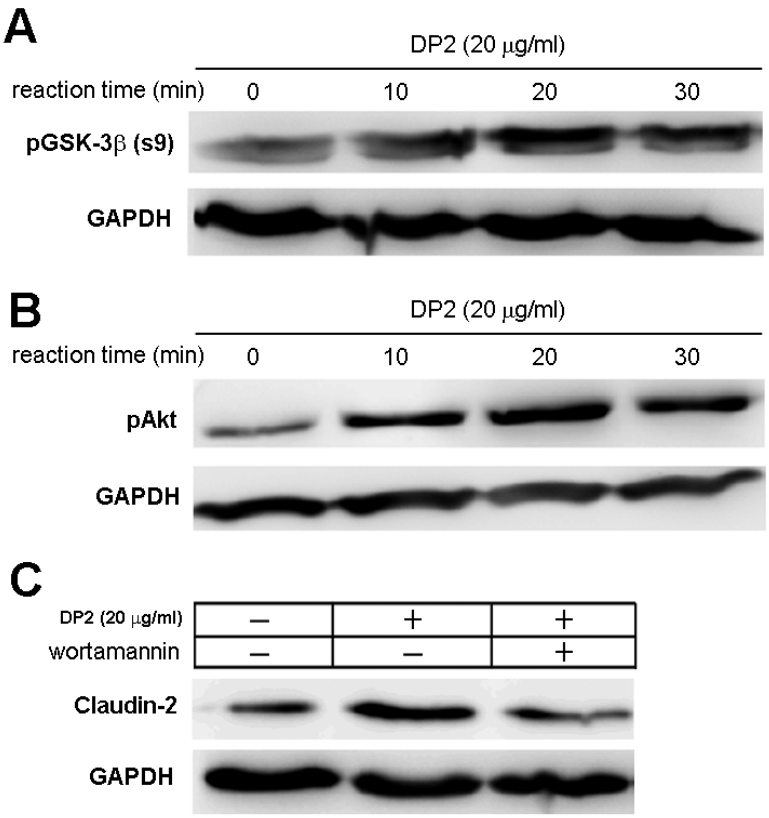

Figure 4. DP2 increased serine-9 phosphorylation of GSK-3β and induced Akt activation involving in claudin-2 expression. A549 cells were treated with 20 μg/mL DP2 for indicated time (min), and the serine-9 phosphorylation of GSK-3β (A) and phosphorylation of Akt (B) was determined by immunoblot. (C) A549 cells were pretreated with wortamannin for 1 h and then incubated with 20 μg/mL DP2 for 20 min. The levels of claudin-2 were determined by immunoblot. GAPDH was used as control.

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   - ☑ 達成目標
   - □ 未達成目標（請說明，以 100 字為限）
     - □ 實驗失敗
     - □ 因故實驗中斷
     - □ 其他原因

   說明：

2. 研究成果在學術期刊發表或申請專利等情形：

   論文：☑已發表 □未發表之文稿 □撰寫中 □無

   專利：□已獲得 □申請中 ☑無

   技轉：□已技轉 □洽談中 ☑無

   其他：（以 100 字為限）

本計畫已發表一篇 SCI 論文，以及一篇已投稿 SCI 並在修訂中的論文。已發表的 SCI 論文請詳見附錄一。

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

本研究結果首次揭露不具蛋白水解能力的第二群塵蟎過敏原具有改變呼吸道上皮細胞之表面結合分子(CAM)的能力，以及初步瞭解此一能力可能透過的訊息傳導路徑。此一發現有助於更清楚地瞭解非蛋白酶過敏原如何通過呼吸道上皮細胞進而致敏的機轉，擴展我們對於過敏原與呼吸上皮細胞之間相互影響之認知。未來更進一步的研究可以分析各種訊息傳導或是激酶抑制劑(kinase inhibitors)，對於阻斷這一類過敏原所誘發的過敏反應與發炎反應的效應，進而發展新型的抗敏或是減敏的療法與藥物。對於台灣地區一直不斷攀升的氣喘與過敏疾病盛行率，有相當的幫助。

附錄一：已發表的 SCI 論文（J Cell Biochem 2010 vol.110, pp660）

ARTICLE
Journal of Cellular Biochemistry 110:660–670 (2010)

Journal of Cellular Biochemistry

Proteomic Analysis for the Anti-Apoptotic Effects of Cystamine on Apoptosis-Prone Macrophage

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ABSTRACT

Increased macrophage vulnerability is associated with progression of systemic lupus erythematosus. Our previous studies have shown that cystamine, an inhibitor of transglutaminase 2 (TG2), alleviated the apoptosis of hepatocyte and brain cell in lupus-prone mice NZB/W-F1. In present study, we further investigated the effects of cystamine on apoptosis-prone macrophages (APMs) in the lupus mice. Using two-dimensional gel electrophoresis (2-DE) analysis, we found that cystamine induced a differential protein expression pattern of APM as comparing to the PBS control. The protein spots presenting differential level between cystamine and PBS treatment were then identified by peptide-mass fingerprinting (PMF). After bioinformatic analysis, these identified proteins were found involved in mitochondrial apoptotic pathway, oxidative stress, and mitogen-activated protein (MAP) kinase-mediated pathway. Further investigation revealed that cystamine significantly decreased the levels of apoptotic Bax and Apaf-1 and the activity of caspase-3, and increased the levels of anti-apoptotic Bcl-2 in APM. We also found that these apoptotic mediators were up-regulated in a correlation with the progression of lupus severity in NZB/W-F1, which were little affected in BALB/c mice. We also found that the reduced serum glutathione was restored by cystamine in NZB/W-F1. Interestingly, the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in APM and the phagocytic ability was diminished in presence of cystamine. In conclusion, our findings indicate that cystamine significantly inhibited mitochondrial pathway, induced antioxidant proteins, and diminished phosphorylation of extracellular ERK1/2, which may alleviate the apoptosis and the phagocytic ability of APM. J. Cell. Biochem. 110: 660–670, 2010.

KEY WORDS: APOPTOSIS; CYSTAMINE; LUPUS-PRONE MICE; APOPTOSIS-PRONE MACROPHAGES; CASPASE; ERK

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease, which is usually characterized by loss of tolerance to self-antigens and production of circulating autoantibodies to nuclear antigens, and consequent immune-mediated tissue injury of multiple organs [Munoz et al., 2005]. Apoptotic cells might provide self-antigens; therefore, persistence of apoptosis and impaired clearance of pro-apoptotic cells, causing the spillage of potential immunogenic macromolecules to the exterior, could enhance the production of auto-reactive T and B cells and then promote pathogenesis of SLE [Wu et al., 2001; Cohen, 2006]. Actually, increase of apoptosis in polymorphonuclear cells and macrophages leading to impaired clearance of pro-apoptotic cells was commonly observed in SLE patients [Ren et al., 2003]. Similarly, the apoptosis of lupus-prone macrophages (APM) in lupus-prone mice was markedly expended that it was proportional to the severity and development of lupus syndrome [Russell et al., 1985].

Shao-Hsuan Kao and Tsai-Ching Hsu contributed equally to this work.

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signaling. In vitro, MAPKAPK5 is reported as a novel substrate of the ERK and p38, which can be phosphorylated by ERK and p38 but not by JNK [Ni et al., 1998]. Similarly, we found that the level of MAPKAPK5 as well as the phosphorylation of ERK was decreased by cystamine treatment. However, the downstream signals mediated by MAPKAPK5 and their roles in the apoptosis of APM need to be further investigated.

Although the pathogenesis of SLE is not completely clear, many factors are reported to implicate in the development or progression of SLE, including altered cytokine levels [Waszczykowska et al., 1999], increased apoptosis [Kaplan, 2004] and elevated levels of oxidative stress. Oxidative stress has been demonstrated to associate with main pathological characteristics of SLE, for example, many of the autoantibodies produced in SLE patients exhibit a preference for the increased oxidized molecules [Cooke et al., 1997; Vaarala, 2000]. Additionally, overexpression of superoxide and reactive oxygen species (ROS) is also reported to induce oxidative stress and subsequently to cause various forms of apoptosis [Simon et al., 2000; Vincent et al., 2002; Kanno et al., 2004]. Our findings reveal that cystamine treatment enhances the protein expression of antioxidant proteins, including Mn-SOD, Cu/Zn-SOD, GSTP2, thioredoxin, and peroxidoxin-5. Cu/Zn-SOD has been reported to localize in mitochondria and to play important roles in removing superoxide in and around mitochondria as well as in protecting cells against mitochondria-derived oxidative damage and apoptosis [Okado-Matsumoto and Fridovich, 2001]. GST superfamily is also known to involve in the detoxification of ROS and the genetic polymorphism of GST may associate with susceptibility to SLE [Kang et al., 2005]. Free cysteamine in mouse tissues is mainly metabolized from pantothenic acid by vanin-1 [Martin et al., 2001]. It is reported that cystamine specifically restores hepatic GST-α3 level without upregulation of its mRNA expression or protein production in vanin-1 knockout mice, suggesting that cystamine may act on protein stability or folding [Di Leandro et al., 2008]. In lupus-prone mice, our findings reveal that exogenous cystamine upregulates the protein expression of GSTP2 in APM. The differences are supposed to be results of diverse cell types and animal models.

Macrophage activation induced by bacterial infection in vivo or by cytokine stimulation in vitro, is associated with enhanced superoxide production and cytolytic activity [Boraschi et al., 1982; Suzuki, 1991]. Previous study using vanin-1 knockout mice provides evidences that strong oxidative stress induced by chemicals or harmful irradiation increases cysteamine/cystamine production, subsequently inhibiting gamma-glutamylcysteine synthetase and diminishing GSH level in thymic tissue [Berruyer et al., 2004]. Although cystamine is reported to inactivate gamma-glutamylcysteine synthetase through S-cysteaminylation, it also has been demonstrated as a potent antioxidant and free radical scavenger [Revesz and Modig, 1965; Skrede and Christophersen, 1966; Stack et al., 2008]. Our previous study showed that cystamine significantly suppressed the expression of TNF-α and TGF-β and decreased the production of anti-cardiolipin autoantibody [Hsu et al., 2007]. The present findings show that cystamine both elevates the serum GSH in normal mice and lupus-prone mice, and the increase of serum GSH by cystamine in lupus-prone mice is higher than in normal mice. Taken together, the elevated serum GSH in lupus-prone mice by cystamine, at least partially, may be attributed to the antioxidant activity and the capability to inhibit inflammatory responses of cystamine. It is postulated that the intervention of macrophage activation in presence of cystamine contributes to attenuated phagocytic activity of both APM and normal macrophages.

In conclusion, the present study demonstrates that cystamine alters the protein expression profile of APM, inhibits the mitochondrial apoptotic pathway and enhances the antioxidant activity and survival signaling. These findings not only provide evidences that cystamine effectively alleviates the apoptosis of APM, but also indicates a possible anti-apoptotic mechanism induced by cystamine.

ACKNOWLEDGMENTS

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國科會補助出席國際會議報告

98 年 10 月 17 日

報告人姓名	高紹軒	服務機關名稱（請註明系所）及職稱	中山醫學大學 生化暨生物科技研究所 助理教授
會議期間及地點	自 2009 年 9 月 11 日至 9 月 13 日(新加坡)	國科會核定補助計畫編號	97 年 07 月 09 日 **NSC 97-2314-B-040-008-MY2**
會議名稱	（ 中文 ）第 4 屆亞洲自體免疫大會 （ 英文 ）The 4th Asian Congress in Autoimmune Disease (ACA)		
發表論文題目	（ 中文 ）第二群塵蹣過敏原 Der f 2 對同時患有紅斑性狼瘡與過敏性氣喘病人之 B 淋巴球的蛋白質體分析 （ 英文 ）Effects of Der f 2 on B lymphocyte from a patient with SLE and allergic asthma by using proteomic approach		

報告內容應包括下列各項：
一、參加會議經過
二、與會心得
三、建議
四、攜回資料名稱及內容
五、其它

務請配合

＊報告內容請以電腦繕打，並儲存成 word 檔，檔名請使用姓名，並請以電子郵件寄至 candy@mail.ntou.edu.tw，俾本組上網供各單位參考應用。

高紹軒參加 2009 年新加坡舉辦之國際學術會議報告

一、 參加會議經過

原本預計參加同年 (2009 年) 11 月於美國 Miami 舉辦的 ACAAI (American College of Allergy, Asthma and Immunology) 國際學術研討會，投稿摘要也已被接受 (如附件一)，但是基於經費因素、上課衝突以及擔心美簽的問題而作罷。因此轉而選擇參加新加坡舉辦的這個自體免疫學術研討會：亞洲自體免疫大會 ACA (Asian Congress on Autoimmunity)。ACA 為自體免疫研究者的重要國際研討會之一，雖然會議名稱加上「亞洲」，但是其主辦單位是同時承辦世界六大自體免疫國際研討會，並有 4 種重要自體免疫的國際期刊 (Autoimmunity Reviews, J Autoimmunity, Clinical Reviews in Allergy & Immunology, Annals of The NY Academy of Science) 所支持的國際學術研討會，而此次的 ACA2009 也有超過 47 個國家的學者參與。

ACA2009 於新加坡的 Suntec City 的國際會議廳舉辦，雖然只有三天但是議程相當緊湊，如附件二所示。由於議程從早上八點即開始第一場的演說，因此必須前一天先抵達新加坡。到達新加坡樟宜國際機場已是下午六點半，因此先到旅館去 check-in。晚上於確認明天步行至會場的路徑與時間後便在旅館動過在新加坡的第一晚。ACA2009 主席 Yehuda Shoenfeld 個人即有超過 250 篇有關自體免疫領域研究的發表，其中包括 131 篇的回顧文獻 (review)。本次與會的其他主要演講者也都是自體免疫領域中的重量級人物，因此對此次的會議可說是充滿著期待。

二、 與會心得

承蒙國科會於計畫中核列出席國際會議之預算，才能有機會參與這次的 ACA2009。本次與會人數約 500 人，有超過 350 人是預先註冊，其餘為新加坡的學者直接來現場註冊。雖稱不上是算是動輒千人以上的大型國際會議，但與會的學者皆為自體免疫領域之專家，會場內外的討論相當熱烈。其實這也是中型會議的最大好處：領域範圍較小但是較為專精，可以有較為專注且深入討論的機會。

這是我第一次到新加坡，因為本次的議程相當緊湊，早上八點開始第一場的演講，加上我是訂到比較遠的旅館 (步行約需 15-20 分鐘)，因為怕遲到所以我七點就離開旅館開始步行前往會場。在步行途中發現新加坡的市容的確相當的乾淨，行人或是車輛都嚴守交通規矩。(如果是在清晨的台北或是台中的街頭，比較會出現無視紅綠燈的車輛或是行人。) 步行約 15 分鐘即到了這次 ACA2009 的國際會議所在地 Suntec City。Suntec City 是新加坡的地標之一，主要由五棟建築所環繞而成，至少可以規劃同時有 10 場以上的中大型國際會議，且周邊約步行五分鐘範圍內還有兩家大型商務旅館。到達會場還不到七點半，但是所有的工作人員已經就位，且已有不少人已經完成報到並開始張貼 poster。顯示與會者都非常重視本次的研討會。進入主要演講廳，工作人員已經在測試投影機與試播 powerpoint，我也趁機先拍了一張會場的照片(**附件三**)。看來主辦單位的確具有相當豐富舉辦國際會議的經驗。

本次於 ACA2009 展示的是有關自體免疫與過敏之間的一些研究成果，因為參與本次會議中的學者有很大的一部份是兼具有醫師身份，所以他們對於我所提出的發現表示相當有興趣，因為臨床經驗上確實存在不少這樣同時呈現自體免疫與過敏病徵的病人。除此之外，隨著與這些醫師學者的交流中也發現，自體免疫疾病在近五年來有著加速增加且變得更為劇烈與複雜的趨勢，然而其原因不是基於太過複雜而難以有所定論，就是還有許多未知

的環節與變因。因此對於我所提出是否可能在過敏與自體免疫之間存在某種特定的關連，以及相關的初步結果，也是這些醫師學者覺得有趣且值得投入的一個研究方向。參加本次 ACA2009 的另外一個收獲是看到一些新的檢測儀器與檢驗試劑，以及這些儀器與試劑的發展過程。讓我體驗到國外的產學合作確實要比國內更加的緊密與快速。

三、 建議

自體免疫疾病在台灣地區有顯著上升的趨勢，而其複雜的致病機轉且日趨嚴重的病徵實為值得重視與同入研究的一個重要議題。本次 ACA2009 讓我在在自體免疫領域中增加許多學術交流與國際視野。在與各個國際知名學者交流的過程中，可從提問與答辯間解答許多的疑惑。而在私下討論時，也可了解不同學者間所關注的焦點，使我們對整體的研究趨勢有更多的了解與認知，對於提出新的研究方向與激發新的研究思維有相當的幫助。然而出席國際學術研討會無法避免經濟考量與語言因素。後者因近年來各大專院校重視國際化以及國內舉辦國際研討會的次數急速上升，使得不論是教師或是研究生在英語能力方面都有一定提升。然而對於研究生甚至是教師，一趟出國動輒 5-10 萬元的經濟考量難免會讓人卻步。以本次為例，如果選擇參加美國的 ACAAI2009 (7 天的議程)，註冊費、來回機票、住宿加上膳雜費初步估計至少要 9 萬元以上，與原核定的補助短缺將近 1/3 的費用。

因此，我們真的希望教育部或國科會往後能繼續支持補助國內教師與博士班研究生參與國際學術會議，不僅可以開拓其國際視野，同時也可以激發新的研究創意與方向。此外，也能多支持國內大專院校承辦一些中大型的國際會議，使無法獲得出國補助的學生及國內年輕老師，也能透過此一方式參與國際會議，增加與國外學者進行交流的機會，並提升台灣在國際上的知名度以及增加舉辦大型國際會議的實務經驗。

四、 攜回資料名稱及內容

本次會後攜回的主要資料，包含本次會議的詳細議程以及完整內容的會議 VCD。

五、其他

本次經費支出係由國科會專題計劃(**NSC 97-2314-B-040-008-MY2**)出席國際會議經費補助。

附件一 投稿 ACAAI 摘要接受與壁報編號

ACAAI | American College of Allergy, Asthma & Immunology

Follow the Leader Into the Future

August 26, 2009

Abstract ID # 649243

Shao-Hsuan Kao
No.110, Sec.1, Jianguo N.Rd.
Taichung 402
Taiwan

Dear Dr. Kao:

I am pleased to report that the abstract shown below* has been accepted for a **Poster Session** at ACAAI's 2009 Annual Meeting, November 5-10, in Miami Beach. You have been assigned **Poster # P117**. We are proud of the outstanding posters this year. We plan to make them the highlight of our meeting. Here's how:

- Authors are requested to: (a) mount their posters between 7 am – 5 pm, Friday, November 6, or 7 – 11 am, Saturday, Nov. 7 and (b) follow the enclosed guidelines in their presentation.
- **Posters will remain on display all day Saturday and until 1:00 pm, Sunday,** November 7-8, to enable as many physicians as possible to view them.

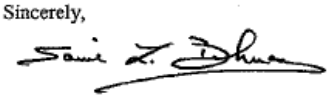
  Authors are requested to man their posters from 12:30 – 1:30 pm, Saturday and Noon – 1:00 pm, Sunday, in Exhibit Hall B of the Miami Beach Convention Center.
- Your abstract will be published in the Abstract Book and the *Annals of Allergy, Asthma & Immunology*.
- **Please return the enclosed abstract reply form confirming your plans to present your poster.**

As all poster presenters are required to register for the meeting, **please register online at www.acaai.org**, if you have not already done so. Pre-registration fees are:

- $275 for ACAAI members and $465 for non-members. **Any physician who joins ACAAI prior to the meeting – or whose application is pending – will be registered at the $275 rate.** (Registration fees increase $50 after September 14.)
- Waived for Fellows-in-Training (until September 14), provided registration is accompanied by a letter from their Allergy/Immunology Program Training Director.

If you have any questions, please call Dianne Kubis at (847) 427-1200 or email: diannekubis@acaai.org. I look forward to your poster presentation in Miami Beach.

Sincerely,



Sami L. Bahna, MD, DrPH
Program Chair and President-Elect

* Effects of allin lyase, a garlic allergen, on epithelial cell BEAS-2B revealing its plausible allergenic mechanism

85 West Algonquin Road, Suite 550 | Arlington Heights, IL 60005-4460
Tel: 847.427.1200 | Fax: 847.427.1294 | www.acaai.org

附件二 ACA2009 會議議程

THE 4th ASIAN CONGRESS ON AUTOIMMUNITY
Singapore, September 11 – 13, 2009

HOME
WELCOME LETTER
COMMITTEES
TOPICS
INTERACTIVE SCIENTIFIC PROGRAM
TIMETABLE
CERTIFICATE OF ATTENDANCE
REGISTRATION
GENERAL INFORMATION
INSTRUCTIONS FOR PRESENTERS
SPONSORSHIP & EXHIBITION
SPONSORS AND EXHIBITORS
ABOUT SINGAPORE
OPTIONAL TOURS
INVITATION LETTER
CONTACT US
PRELIMINARY PROGRAM
LINKS

TIMETABLE

	Friday September 11, 2009	Saturday September 12, 2009	Sunday September 13, 2009
08:00 – 10:00	Plenary Session: Diagnostics and Pathogenesis	Plenary Session: Autoimmunity and Associated Conditions	Plenary Session: Autoimmunity as a Glance
10:00 – 10:30	Coffee Break	Coffee Break, Exhibition and Poster Viewing	
10:30 – 12:30	Bio-Rad Satellite Symposium	Mitsubishi Tanabe Pharma Corporation / Schering-Plough Satellite Symposium	Workshop: Autoimmune Diagnostics *Supported by GA Generic Assays & Euroimmun*
12:30 – 14:00	Lunch Break	Lunch Break, Exhibition and Poster Viewing	
	13:00 – 14:00 Free Communication Sessions	13:00 – 14:00 Free Communication Sessions	13:15– 15:15 Free Communication Sessions
14:00 – 16:00	Plenary Session: The Mosaic of Autoimmunity	Plenary Session: Therapy	
16:00 – 16:30	Coffee Break	Coffee Break, Exhibition and Poster Viewing	
16:30 – 18:30	Plenary Session: Etiology	Free Communication Sessions	
18:30 – 19:30	Welcome Reception	Farewell Dinner (Additional Cost)	

附件三 ACA2009 會場照片

無研發成果推廣資料

97 年度專題研究計畫研究成果彙整表

計畫主持人：高紹軒	計畫編號：97-2314-B-040-008-MY2
計畫名稱：第二群過敏原誘發上皮細胞與自體反應 B 淋巴球凋亡之蛋白體研究與免疫體分析	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	2	2	100%		參加 2009 生醫年會並以壁報形式發表。
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	1	2	70%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	1	100%		出席 2009 於新加坡舉辦之亞洲自體免疫大會並以壁報形式發表。
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	4	4	100%	人次	蘇熙元、徐偉佑、陳宜靖、蔡佳昀
		博士生	2	2	100%		王威鈞、甘博介
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)

本研究計畫目的係以以蛋白體與免疫體進行自體免疫之相關研究，研究主題為自體免疫動物模式與分離自 SLE 病人的 B 淋巴球。動物模式研究部分對於胱氨之改善自體免疫機制有更深入的發現並已順利發表於 SCI 期刊；而 B 淋巴球的研究部分也進一步瞭解塵虫滿第二群過敏原 DP2 對 SLE 病人之 B 淋巴球之相關免疫體與蛋白體的影響。這些研究成果有助於發現改善自體免疫疾病的標的物及其分子作用機制，以及深入評估具改善效果之標的物是否具有發展成減緩或是治療自體免疫疾病之藥物的潛力。