

# Effects of Mix-1 on Anti-CD40 Antibody and Recombinant IL4- Induced Cytokine Production and Immunoglobulin E in Highly Purified Mouse B Cells

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In the oriental medicine, a mixture of herbs has been commonly used as important components to control allergic and inflammatory diseases. In the present study, we prepared a mixture of *Dictamni Radicis Cortex*(Baiksunpee), *Houttuyniae Herba*(Uhsungcho), and *Aurantii Immaturus Fructus*(Jisil) to examine its anti-allergic effects in activated mouse splenic cells and found that Mix-1 is involved in regulating levels of B cell activating factors (CD23 and CD11a), IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ , and IgE as well as HRF expression. It was observed that Mix-1 did not have cytotoxic effects on mLFC. Mix-1 showed inhibition of CD23 and CD11 alpha expression in mouse B cells, and also decreased the production of IL-6, TNF- $\alpha$ , and IgE. Both RT-PCR and ELISA analyses indicated that IL-6 and TNF alpha production were regulated at the gene expression level. In contrast, IL-10 mRNA and protein levels were increased in activated B cells by Mix-1 treatment. We also found that Mix-1 inhibited B cell proliferation and inhibited histamine releasing factor(HRF) expression, suggesting its inhibitory effect on histamine secretion. These data indicated that Mix-1 has an anti-allergic effect in activated macrophages and further suggest the possible application of Mix-1 as a therapeutic agent for the treatment of allergy-related diseases.

Key words : Anti-allergic, RT-PCR, ELISA, B cells, cytokine, CD23+, CD11a+, IgE, HRF

## Introduction

Statistical data showed an increased incidence of allergy and anaphylaxis particularly in the developed countries. In Korea, recent data indicate an incidence of 10% in adults and 20% in children. The causes of allergy are diverse including genetic alterations due to increased exposure to environmental pollutants and chemicals, and westernized life style which might result in abnormal regulation of immune system. Improved environmental sanitation leads to reduced opportunity to pathogenic infections, resulting in less chance of immune system to confront with pathogens, debilitate body's ability to distinguishing between pathogen and antigens, which lead to in increases in allergic diseases<sup>1)</sup>. This hypothesis is supported by the observation that the habitants in an area with higher risks in parasite infection show lower allergic diseases.

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· Received : 2004/09/10 · Revised :2004/10/20 · Accepted : 2004/11/17

There are also reports that lower chances of infection in infants or children are inversely related to higher allergy incidence rate. Allergic disease can be caused by genetic and environmental factors, both of which are associated with one's physical condition in a complicated manner. Therapy for allergy is not simple and thus requires a long-term treatment. Currently used therapies include environmental control to avoid causative antigens and aggravating environmental factors, chemotherapy and immuno- therapy<sup>2)</sup>.

In the context of environmental control, it is important to minimize the contact with potential allergens. Since the allergen sensitivity are generally different among individuals, case-specific treatment is particularly important. Chemotherapy has drawn a particular attention for allergy treatment since numerous drugs recently developed are known to be effective for allergy treatment<sup>3)</sup>. For instance, drugs such as glucocorticoid, anti- histamine, tarolimus, and sympathetic nerve stimulants inhibit the specific steps in the allergic reaction or alleviate allergic symptoms. Finally, immunotherapy is applied under the custody of the specialists when environmental control and chemotherapy do not work.

In immunotherapy, once the causative antigen is carefully examined and decided, doses of injection gradually increased to induce the resistance to hypersensitivity. Antigen injection can reduce the function of antigen-specific Th2 cells and activate IFN- $\gamma$ -predicting cells, which leads to decrease IgE-mediated immune responses<sup>4</sup>). Despite diverse approaches mentioned above, most of the traditional therapies are not highly successful and thus, modified allergen, peptide immunotherapy, oral immunotherapy, DNA vaccination, fusion proteins with augmented antigenicity, and adjuvant such as ISS(immunostimulatory sequence) have recently been considered<sup>5</sup>). These new approaches are expected to have a long-term anti-allergic effect without enduring treatments or reinforcing immunization. Moreover, recent efforts on identifying genes related to allergic relation would be critical to disclose molecular mechanisms underlying allergy and for the new drug development<sup>6,7</sup>). Major molecular factors which have been characterized include cytokines, chemokines, cytokine receptors, MHC complexes, transcription factors (see Table 1). While these molecules are involved in IgE production and inflammation reactions, their specific roles remains to be elucidated with diverse molecular genetic approaches<sup>8</sup>).

In oriental medicine, a mixture of several herbal drugs are known to be effective for the treatment of diverse disease including allergy. For example, the efficacy of herbal plants such as Baiksunpee (BSP), Uhsungcho (USC), and Jisil (JS) has been well documented in relation to allergic diseases. The Mix-1 used in the present study represents a mixture of three different herbs, Baiksunpee (BSP), Uhsungcho (USC), and Jisil (JS). BSP (Dictamni Radicis Cortex) is obtained from the root skin of *Dictamnus dasycarpus* Turcz. The major components identified are dictamnine, dictamnolacton, trigonellin, sitosterol, obacunonic acid, choline, fraxinellone. Dictamni radix has been used for treating diverse skin diseases including psoriasis, anti-inflammatory effects.

JS (*Aurantii Immaturus Fructus*) is prepared from the dried young fruits of the trees *Citrus aurantium* L. and *Poncirus trifoliata* Rafin. The major chemical components are flavonoid such as poncirin, hesperidine, neohesperidin, rhoifolin, naringin, aurantiamarin, aurantin, 5-hydroxyaurantin, and 5-O-desmethyl nobiletin, synephrine, and N-methylamine. Also, they contain vitamin C and acetate. Pharmacological studies indicated that JS is effective for the suppression of anti-anaphylaxis(e.g., skin allergy), inhibition of histamine release from the mast cells by inhibiting intracellular calcium increase and including cAMP production in cells. Physiologically, JS has been used for increasing gastric activity, uterus muscle contraction, and regulation of blood vessels. USC

(*Houttuyniae Herba*) is obtained from the upper part of the part of *Houttuynia cordata* Thunb. Important components are anti- microbial components such as decanoyl acetaldehyde, methyl-n- nonyl-ketone, alpha-pinene, linalool, camphene, d-limonen, bornyl acetate, laurine aldehyde and cordine. Several studies have reported that USC can suppress hypertension, increases immunity by acting on B-and T cells, and has antibacterial and anti-viral activities, and anticancer diuretic and anti-rheumatic activities. In the present study, a mixture of above three herbs were used to investigated their effects on regulation of major parameters related to allergy in cultured macrophage cells.

Table 1. Major molecular mediators in allergy

Chromosome	Candidate gene	Function
1p31.2	IL-12 receptor $\beta$ 2 chain	Signal transducer of IL-12
2q33	CD28	Co-stimulator in T-cell activation
	CTLA-4	Co-stimulator in T-cell activation
5q31	IL-4	Differentiation of Th2 cells/ induction of IgE production
	IL-5	Eosinophil growth and activation/ promotion of IgE production
	IL-13	Induction of IgE production
10p14	GATA3	Transcription factor in Th2 differentiation
12q13-14	STAT6	Transcription factor in IL-4 signaling
12q2	IFN- $\gamma$	Inhibition of Th2 activity/ inhibition of IgE isotype classswitch
16p12	IL-4 receptor $\alpha$ chain	Signal transducer of IL-4

## Materials and Methods

### 1. Materials

#### 1) Animals

BALB/c mice (4 week old) obtained from Korea Research Institute of Chemical Technology, were used in the present study. The animals were fed with food pellets (Samyang Co, Inc., no antibiotic additives) and water. Animals were adjusted at least 2 weeks in a room maintained at 22±2°C before the experiment.

#### 2) Herbal drugs

Mix-1(mixture of three different herbs, Dictamni Radicis Cortex, *Aurantii Immaturus Fructus*, and *Houttuyniae Herba*) used in this study was purchased from local manufacturer and further purified before use.

Table 2. Prescription of Mix-1

Drug name	Scientific name	Amount(g)
Baiksunpee (白鮮皮)	Dictamni Radicis Cortex	6
Jisil (枳實)	Aurantii Immaturus Fructus	6
Uhsungcho (魚腥草)	Houttuyniae Herba	6
		18

### 3) Reagents and instruments

Diethyl pyrocarbonate(DEPC), chloroform, RPMI-1640 culture medium, isopropanol, ethidium bromide (EtBr), dulbecco's phosphate buffered saline(D-PBS), and magnesium chloride ( $MgCl_2$ ) were obtained from Sigma (USA), Taq polymerase and deoxynucleotide triphosphate (dNTP) from TaKaRa Co. (Japan), moloney murine leukemia virus reverse transcriptase (MMLV RT) and RNase inhibitor from Promega Co. (Madison, USA), RNA- zolB from Tel-Test (USA), fetal bovine serum (FBS) from Hyclone (Logan, USA), agarose from FMC (USA), phycoerythrin -conjugated anti-rat Ig, fluorescein isothiocyanate (FITC)- conjugated anti-CD23, anti-IgE, anti-CD40, anti-B220-FITC were from Pharmingen (Torreyana, U.S.A).  $^3H$ -thymidine and Sephadex G-10 were purchased from Amersham Pharmacia (Buckinghamshire, UK). Other general chemicals were purchased with the highest quality available.

The instruments used in this study are as follows; Bright microscope and inverted microscope (Nikon, Japan), flow cytometry (Becton Dickinson, U.S.A), spectrophotometer (Shimadzu, Japan),  $CO_2$  incubator (Napco, Germany), imager II photo-system (Bioneer, Korea), Centrikon (Sigma, USA), bio-freezer (Sanyo, Japan), Primus 96 thermocycler system (MWG, Germany), ice-maker (Vision Science, Korea) and homogenizer (OMNI, USA).

## 2. Methods

### 1) Mix-1 preparation

Fifty grams of dried Mix-1 were dissolved in 1,000 ml distilled water, extracted for 3 hr. After filtration using the rotary evaporator (Büchi B-480, Switzerland), the purified powder was obtained by using the freeze dryer (EYELA FDU-540, Japan), and kept at  $-84^\circ C$  until use. The powder obtained was dissolved in distilled water and filtered with syringe filter ( $0.22 \mu m$ , Falcon) before use.

### 2) Cell culture

#### (1) Isolation and culture of mouse lung fibroblast cells

Mouse lung tissues were dissected, washed three times with cold PBS and cut into small pieces. The tissues in 15 ml conical tube were centrifuged at 1400 rpm for 5 min. After adding DMEM containing collagenase A ( $5 mg/ml$ , BM, USA), DNase type I ( $0.15 mg/ml$ , Sigma, USA), and antibiotics (penicillin  $104 U/ml$ , streptomycin  $10 mg/ml$ , amphotericin B  $25 \mu g/ml$ ), the dissociated tissue was incubated at  $37^\circ C$  for 2 hr, and further incubated for more 30 min in the presence of 0.5% trypsin-0.2% EDTA. The tissue was washed twice with cold PBS, centrifuged at 1500 rpm to remove the supernatant, resuspended in DMEM-10% FBS, and cultured for 7 days. Then, the cells were detached from the plate by 0.5% trypsin-0.2% EDTA

treatment, and plated on 96 well plate with cell concentration of  $10^5$  cells/ml in DMEM-5% FBS culture medium.

#### (2) Mouse B cell isolation and primary culture

Spleen tissues were dissected out from BALB/c cells, and used to prepare dissociated primary cells. Cells were harvested by centrifugation at 2000 rpm for 5 min, and cell pellets were suspended in 2 ml of saline solution at  $37^\circ C$  for 5 min. Then, 10 ml of D-PBS was added and centrifuged at 2000 rpm for 5 min. After treatment with J1J, GK153, and M1/70 culture medium (1 ml per  $10^8$  cells), cells were kept on ice for 30 min, washed twice with D-PBS, followed by treatment with 0.5 ml of rabbit complement (Serotec, U.K). The sample was incubated at  $37^\circ C$  for 1 hr, washed with complete medium, and B cells were purified by sephadex G10 gel filtration column chromatography (Amersham Pharmacia, USA) (Fig. 1). The amount of B cells were measured by flow cytometry using a -B220-FITC.

Table 3. Monoclonal Antibody used for Immunofluorescence Staining

Immune cell types	Markers	Monoclonal antibody
T cells	Thy1.2	J1J10
helper T cells	Thy1.2	GK1.5.3
Macrophages	CD4	M1/70

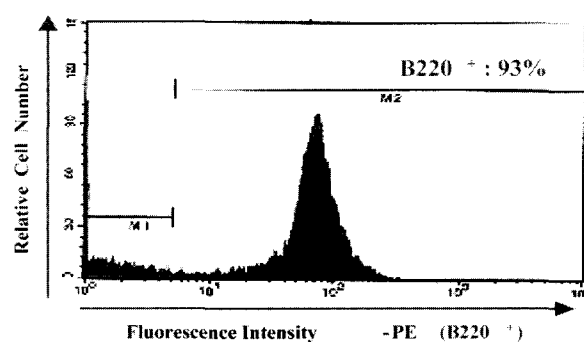


Fig. 1. Purity of B-cells isolated in Balb/c spleen cells. The splenic cells prepared from Balb/c mice were treated with RBC lysis solution,  $\alpha$ -thy1.2 (J1J10, GK1.5.3, M1/70), and complement. Then, B-cells were purified by sephadex G10 column.

#### 3) Measurement of cytotoxicity

The cytotoxicity was measured by modified MCB method. After incubation in 5%  $CO_2$  incubator at  $37^\circ C$  for 1 hr, mLFC or mouse macrophage cells were treated with  $200 \mu g/ml$ ,  $100 \mu g/ml$ ,  $50 \mu g/ml$ ,  $10 \mu g/ml$ ,  $1 \mu g/ml$  of Mix-1 for 48 hr. The culture medium was then removed and cells were washed with PBS twice. Cells in each well were treated with  $50 \mu l$  of 50% TCA (trichloroacetic acid) for 1 hr at  $4^\circ C$ , washed with distilled water five times, and air dried. Cells were stained with MCB solution (0.4%/1% acetic acid;  $100 \mu l/well$ ) for 30 min at room temperature, and washed with 0.1% acetic acid for 4-5 times. Cells were air dried, solubilized in 10 mM Tris base ( $100 \mu l$

/well). Cells in the plate were dispersed in the culture medium by using a plate shaker (Lab-Line, USA) for 5 min and used for the measurement of optical density at 540 nm using ELISA LEADER (molecular devices, USA).

4) Measurement of IgE, IL-4, and IFN- $\gamma$

Isolated B cells were seeded on 96 well plates ( $2 \times 10^5$  cells/well) and treated with 100  $\mu\text{g}/\text{ml}$  or 10  $\mu\text{g}/\text{ml}$  of Mix-1 extract. One hour later, cells were treated with anti-CD40 monoclonal antibody (500 ng/ml) and recombinant mouse IL-4 (rmIL-4, Pharmingen, USA), and incubated for 10 days and 48 hr respectively. Recombinant mouse interleukin-10 (rmIL-10, 50 ng/ml, Endogen) which inhibits the production of IgE, IL-4 or IFN- $\gamma$  was treated to cells as a positive control. Cells were then harvested by centrifugation at 2000 rpm for 5 min and used for ELISA. ELISA for IFN- $\gamma$ , IL-4 and IL-10 was performed for 48 hr after culture (Endogen, USA), and ELISA for IgE (Pharmingen, USA) was done 10 days later. Microwells were coated with antibody-diluted coating buffer, and then, 100  $\mu\text{l}$  of cell culture supernatant was treated to each well for 1 hr, rinsed twice with washing buffer, treated with 100  $\mu\text{l}$  of avidin-HRP conjugated antibody for 1 hr and washed again. Then, 100  $\mu\text{l}$  of TMB substrate was treated for 30 min, and 50  $\mu\text{l}$  of stop buffer added. Chromogenic reaction was monitored using spectro- photometer at 450 nm wavelength.

5) Measurement of B cell proliferation

B cells were dispensed into 96 well plates ( $2 \times 10^5$  cells/well) and treated with 100  $\mu\text{g}/\text{ml}$ , 10  $\mu\text{g}/\text{ml}$ , or 1  $\mu\text{g}/\text{ml}$  of Mix-1 for one hour later. Then, cells were treated with anti-CD40 monoclonal antibody (500 ng/ml) and recombinant mouse interleukin-4 (rmIL-4, 500 U/ml, Pharmingen) for 72 hr. The cell group treated with recombinant mouse interleukin-10 (rmIL-10, 50 ng/ml, Endogen) was used as a positive control. After 40 hr culture, cells were treated with 50  $\mu\text{Ci}/\text{ml}$  of [methyl- $^3\text{H}$ ] thymidine (Amersham, USA) and further incubated for 48 hr. In order to determine the radioactivity incorporated into the cells, cells were collected onto the glass fiber filter (Whatman, USA) using the cell harvester (Amersham, USA), and the incorporated radioactivity was measured by liquid scintillation counter (LSC, LKB, USA).

6) Histamine-Release Factor binding assay

Mouse splenocytes were dispensed in 24 well plate ( $1 \times 10^6$  cells/well) and cultured for 24 hr in the presence of anti-CD40 monoclonal antibody (500 ng/ml), rmIL-4 (500U/ml), and 100  $\mu\text{g}/\text{ml}$  or 10  $\mu\text{g}/\text{ml}$  of Mix-1. After culture, cells were washed twice with PBS containing 3% fetal bovine serum and 0.1%  $\text{NaN}_3$  and used for immunofluorescence staining at 4 $^\circ\text{C}$ . Cells were reacted with biotin-conjugated rHRF for 30 min and washed with PBS followed by streptavidin-conjugated

PE(SA/PE) reaction on ice. After washing with PBS three times or more, cells were reacted with FITC-labeled anti-CD3 antibody, and finally cells bound to HRF were analyzed with flow cytometer (Becton Dickinson, USA).

7) Statistical analysis

Data obtained from various treatments were represented as mean  $\pm$  standard error of mean (SEM), and Student's t-test was employed to determine statistical differences between groups.

## Results

### 1. Measurement of the cytotoxicity of Mix-1 to mouse lung fibroblast cells(mLFC).

Mouse lung tissues was dissected out and used to prepare dissociated cell culture. The cytotoxicity of Mix-1 to mLFC was determined by SRB method. Levels of cytotoxicity of Mix-1 on mLFC were 85.4 $\pm$ 5.1 with 200  $\mu\text{g}/\text{ml}$  Mix-1, and 91.3 $\pm$ 4.6, 95.7 $\pm$ 5.6, 95.4 $\pm$ 7.4 and 97.6 $\pm$ 6.5(%) for 100  $\mu\text{g}/\text{ml}$ , 50  $\mu\text{g}/\text{ml}$ , 10  $\mu\text{g}/\text{ml}$  and 1  $\mu\text{g}/\text{ml}$  of Mix-1 concentrations respectively (Table 4, Fig. 2). These data suggest that Mix-1 in a concentration range of 1 - 200  $\mu\text{g}/\text{ml}$  does not have cytotoxic effects on cultured LFC cells.

Table 4. Cytotoxic Effect of Mix-1 Extract on Mouse Lung Fibroblast Cells(mLFCs)

Drug	Dose( $\mu\text{g}/\text{ml}$ )	% of Control Data mLFC
Control	0	100 $\pm$ 5.7
	1	99.6 $\pm$ 5.8
	10	96.3 $\pm$ 4.2
Mix-1 extract	50	93.2 $\pm$ 6.4
	100	87.8 $\pm$ 5.4
	200	82.1 $\pm$ 4.7*

Mouse lung fibroblast cells (mLFCs) were pretreated with various concentration of Mix-1 extract. The results are expressed the mean  $\pm$  S.E (N=6). Statistically significant value compared with control group data by T test (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001).

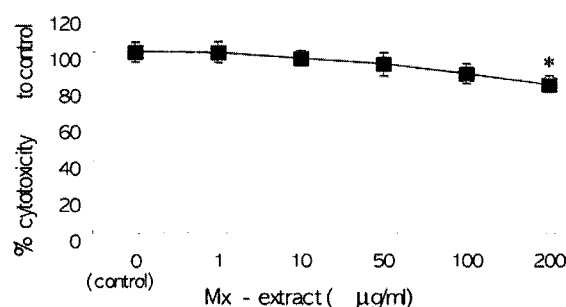


Fig. 2. Inhibitory effect of Mix-1 extract on CD23 $^+$ /B220 $^+$  expression by Mix-1 extract plus anti-CD40 mAb plus rIL-4-stimulated murine splenic B cells. Mouse B cells from healthy Balb/c mice were either stimulated with anti-CD40 mAb and recombinant interleukin-4 (500 U/ml) or treated with Mix-1 extract (100 mg/ml and 10 mg/ml) or rIL-4 (500 U/ml) plus anti-CD40mAb plus Mix-1 extract. B cells (A) were cocultured with anti-CD40 mAb plus rIL-4 (Control, B), and with anti-CD40 mAb plus rIL-4 plus Mix-1 extract (100 mg/ml (C), and 10 mg/ml (D) for 68 hrs. After 68 h, the cells were harvested, and assayed for CD23 $^+$ /B220 $^+$  expression by flow cytometry, and the other methods for assay were performed as described in Materials and Methods.

2. Expression of CD23 in mouse B cells

B cells isolated from mouse spleen were stimulated with anti-CD40 antibody and rIL-4 and cultured for 68 hr in the presence or the absence of Mix-1 extract. Then, levels of B cell surface molecule CD23 (FCεRI) were measured. In cells with no treatment, CD23+/B220+-positive B cell number was 11.9% of the total cells, and positive number of cells when treated with anti-CD40 antibody and rIL-4 was 20.4%. When anti-CD40 and rIL-4 stimulated cells were treated with Mix-1, cell numbers were reduced to 12.0% with 100 µg/ml Mix-1 treatment and 18.4% with 10 µg/ml Mix-1 (Fig. 2). These data suggest that Mix-1 may have an inhibitory effect on CD23 expression in differentiating B cells.

3. Expression analysis of CD11a in mouse B cells.

B cells isolated from mouse spleen were stimulated with anti-CD40 antibody and rIL-4 and cultured for 68 hr in the presence or the absence of Mix-1 extract. Then, levels of B cell surface molecule CD23 (FCεRI) were measured. In cells with no treatment, CD11a+/CD19-positive B cell number was 1.8% of the total cells, and % CD11a positive cells when treated with both anti-CD40 antibody and rIL-4 was 12.5%. When the cells group stimulated with both anti-CD40 antibody and rIL-4 were treated with Mix-1, cell numbers were reduced to 7.1% with 100 µg/ml Mix-1 treatment and 10.5% with 10 µg/ml Mix-1 (Fig. 3). These data suggest that Mix-1 may have an inhibitory effect on activation of differentiating B cells.

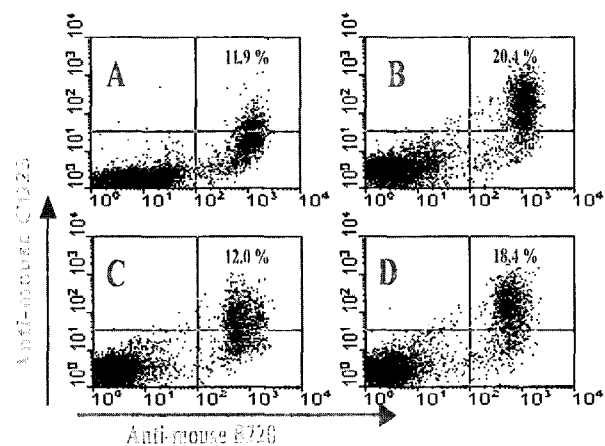


Fig. 3. Inhibitory effect of Mix-1 extract on CD11a+/CD19+ expression by Mix-1 extract plus anti-CD40 mAb plus rIL-4-stimulated murine splenic B cells.

4. mRNA expression analysis of mouse B cell cytokines

B cells isolated from mouse spleen were treated with various combinations of drugs as shown in Table 4 and cultured for 6 hr. Expression levels of several cytokines were determined by RT-PCR using isolated RNA.

Table 5. Inhibitory Effects of Mix-1 Extract on Cytokines Transcript Expression in Murine Splenic B Cells

α-CD40	rIL-4	Mix-1 (mg/ml)	Cytokines m-RNA expression (Ht)			
			IL-1b	IL-6	IL-10	TNF-a
RPMI1640-media		0	45	35	25	152
+	+	0	96	68	57	195
+	+	100	47	59	27	140
+	+	10	62	55	55	153
+	+	1	84	65	50	158

Mouse B cells from healthy Balb/c mice were either stimulated with anti-CD40 mAb (500 ng/ml) and recombinant interleukin-4 (500 U/ml) or treated with Mix-1 extract (100, 10, 1 mg/ml) or rIL-4 plus anti-CD40mAb plus Mix-1 extract or rIL-10 (50 ng/ml). B cells were cultured or cocultured with anti-CD40 mAb and Mix-1 extract for 6 hrs. The gene expression of productive IL-1b, IL-6, IL-10 and TNF-a transcripts was evaluated by reverse transcriptase-PCR. Amplified PCR products were electrophoresed on 1.2% agarose gel, and the analysis (Ht) was used to 1D-density program (USA). The other methods for assay were performed as described in Materials and Methods.

1) IL-1β mRNA expression

IL-1β mRNA expression level in B cells with no treatment (medium control) as represented by Ht value was 45 in one dimensional densitographic representation Ht, mRNA levels in cells treated with both anti-CD40 antibody and rIL-4 was 96. Ht values in cells co-stimulated with anti-CD40 antibody and rIL-4 were decreased to 47, 62, and 84 by 100, 10, and 1 µg/ml of Mix-1 treatments respectively. These data suggest that Mix-1 have an inhibitory effect on B cell differentiation (Fig. 4).

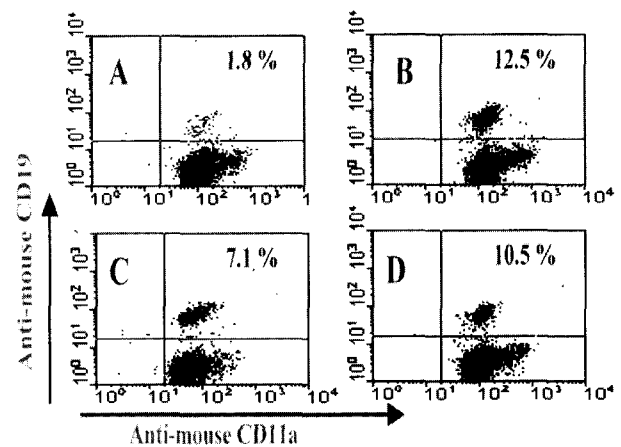


Fig. 4. Inhibitory effects of Mix-1 extract on IL-1β transcript expression in murine splenic B cells. Mouse B cells from healthy Balb/c mice were either stimulated with anti-CD40 mAb (500 ng/ml) and recombinant interleukin-4 (500 U/ml, line 2) or treated with Mix-1 extract (100, 10, 1 mg/ml, line 3, 4, 5) and not treated group (only B cells, line 1). B cells were cultured or cocultured with anti-CD40 mAb and Mix-1 extract for 6 hrs. The gene expression of productive IL-1b transcripts was evaluated by reverse transcriptase-PCR. Amplified PCR products were electrophoresed on 1.2% agarose gel.

2) IL-6 mRNA expression

IL-6 mRNA expression level in B cells with no treatment (medium control) in Ht value was 45, and mRNA levels in cells treated with both anti-CD40 antibody and rIL-4 was 68. Then, mRNA expression in cells co-stimulated with anti-CD40 antibody and rIL-4 Ht values were decreased to 59, 55, and 65 by 100, 10, and 1 µg/ml of Mix-1 treatments respectively. These

data suggest that Mix-1 have an inhibitory effect on B cell differentiation (Fig. 5).

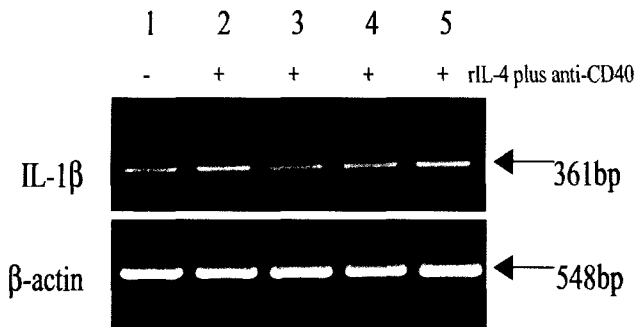


Fig. 5. Inhibitory effects of Mix-1 extract on IL-6 transcript expression in murine splenic B cells.

3) IL-10 mRNA expression

IL-6 mRNA expression level in B cells with no treatment (medium control) in Ht value was 25, and mRNA levels in cells treated with both anti-CD40 antibody and rIL-4 was 57. Then, mRNA expression in cells co-stimulated with anti-CD40 antibody and rIL-4 Ht values were decreased to 27, 55, and 50 by 100, 10, and 1 µg/ml of Mix-1 treatments respectively. These data suggest that Mix-1 have an stimulating effect on IL-10 expression which inhibits B cell differentiation (Fig. 6).

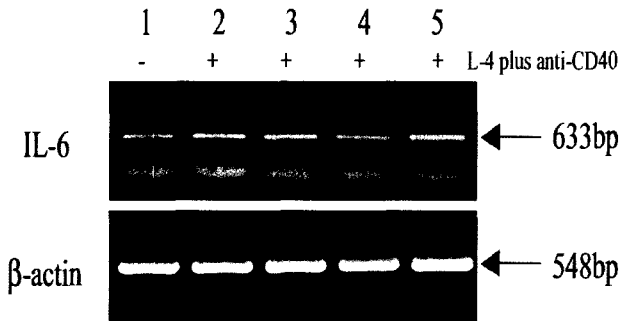


Fig. 6. Inhibitory effects of Mix-1 extract on IL-10 transcript expression in murine splenic B cells.

4) TNF-α mRNA expression

Level of TNF-α mRNA expression in terms of Ht value was 152 for non-treated control B cells and expression levels after stimulation with both anti- CD40 mAb and rIL-4 were increased to 195. Then, the presence of 100, 10, and 1 µg/ml of TNF-α decreased Ht values to 140, 153, and 158, indicating that Mix-1 has an inhibitory effect on B cell differentiation(Fig. 7).

5. Effect of Mix-1 on IL-6 production

B cells after culture were harvested by centrifugation at 2000 rpm for 5 min and used for ELISA to determine IL-6 protein levels. Levels of IL-6 production were determined by

measuring optical density at 450 nm for individual samples: rIL-4 treated group, α-CD40-treated group, α-CD40 plus rIL-4-treated group, α-CD40, rIL-4 and rIL-10-treated group, and α-CD40 plus rIL-4 with 1-100 µg/ml of Mix-1 stimulation. As shown in Fig. 8, Mix-1 treatment showed a significantly inhibitory action on IL-6 production.

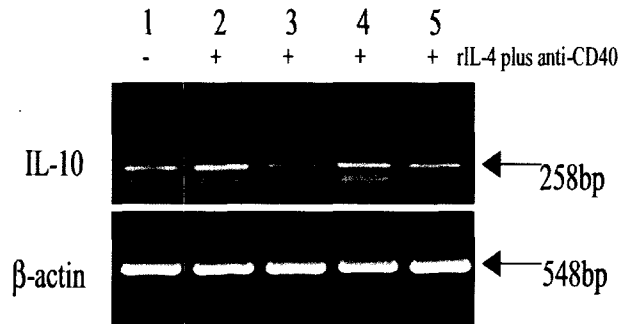


Fig. 7. Inhibitory effects of Mix-1 extract on TNF-α transcript expression in murine splenic B cells.

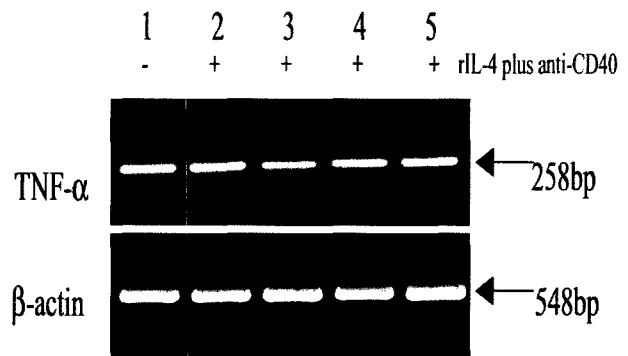


Fig. 8. Effect of Mix-1 extract on IL-6 production in murine splenic B cells. Mouse B cells from healthy Balb/c mice were either stimulated with anti-CD40 mAb (500 ng/ml) and recombinant interleukin-4 (500 U/ml) or treated with Mix-1 extract (100, 10, 1 mg/ml )or rIL-4 plus anti-CD40mAb plus Mix-1 extract or rIL-10 (50 ng/ml). Statistically significant value compared with control ( with rIL-4 (500 U/ml) plus anti-CD40mAb) data by T test (\*\*p<0.05, \*\*\*p<0.001)

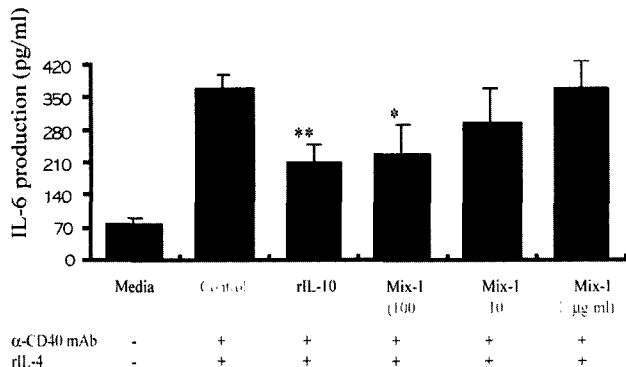
6. Effect of Mix-1 on IL-10 production

B cells after culture were harvested by centrifugation at 2000 rpm for 5 min and used for IL-10 ELISA. Levels of IL-10 production were determined by measuring chromogenic reaction at 450 nm for each group: groups with no treatment, rIL-4 treated group, α-CD40-treated group, α-CD40 plus rIL-4-treated group, α-CD40, rIL-4 and rIL-10-treated group, and α-CD40 plus rIL-4 with 1-100 µg/ml of Mix-1 stimulation. As shown in Fig. 9, Mix-1 treatment showed a significantly stimulatory activity on IL-10 production.

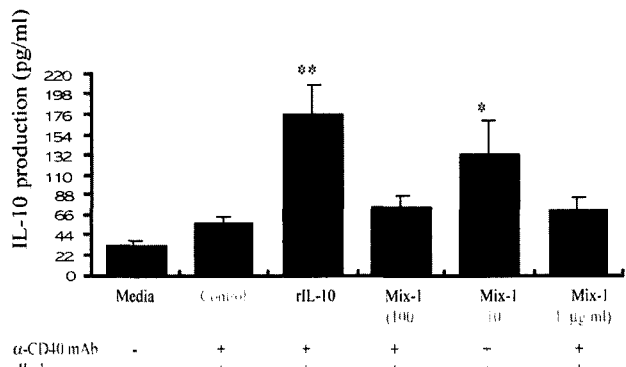
7. Effect of Mix-1 on TNF-α production

B cells after culture were harvested by centrifugation at 2000 rpm for 5 min and used for TNF-α ELISA. Levels of TNF-α production were determined by spectrophotometric

measurement of chromogenic reaction at 450 nm for following groups: groups with no treatment, rIL-4 treated group,  $\alpha$ -CD40-treated group,  $\alpha$ -CD40 plus rIL-4-treated group,  $\alpha$ -CD40, rIL-4 and rIL-10-treated group, and  $\alpha$ -CD40 plus rIL-4 with 1-100  $\mu\text{g}/\text{ml}$  of Mix-1 stimulation. As shown in Fig 10, Mix-1 treatment showed a significantly inhibitory effect on TNF- $\alpha$  production.



**Fig. 9.** Effect of Mix-1 extract on IL-10 production in murine splenic B cells. Statistically significant value compared with control (with rIL-4 (500 U/ml) plus anti-CD40mAb) data by T test (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001).



**Fig. 10.** Effect of Mix-1 extract on TNF- $\alpha$  production in murine splenic B cells.

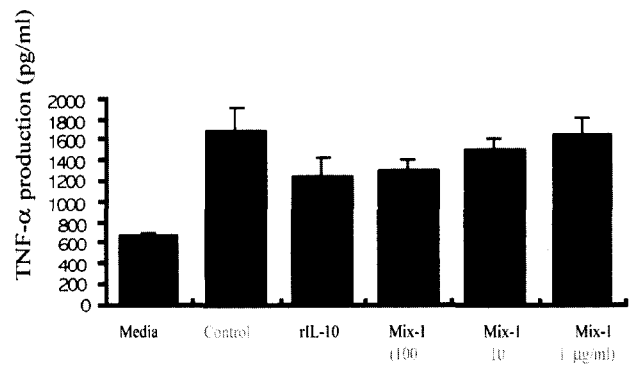
#### 8. Effect of Mix-1 on IgE production

The amount of IgE production was measured 10 days after culture. Levels of IgE production were determined by spectro- photometer for following individual groups: group with no treatment, rIL-4 treated group,  $\alpha$ -CD40-treated group,  $\alpha$ -CD40 plus rIL-4-treated group,  $\alpha$ -CD40, rIL-4 and rIL-10-treated group, and  $\alpha$ -CD40 plus rIL-4 with 1-100  $\mu\text{g}/\text{ml}$  of Mix-1 stimulation. As shown in Fig. 11, Mix-1 treatment showed significant inhibitory action on IgE production.

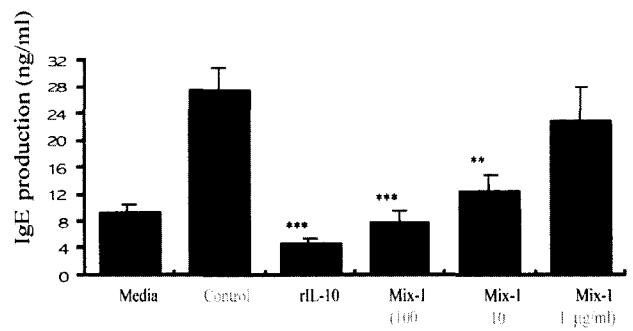
#### 9. Effect of Mix-1 on B cell proliferation

To measure the levels of [methyl- $^3\text{H}$ ] thymidine incorporated into the cells, collected cells were spotted onto the glass fiber filter. After drying the filters, the radioactivity was

measured by using liquid scintillation counter (LSC) for the individual groups; group with no treatment, rIL-4 treated group,  $\alpha$ -CD40-treated group,  $\alpha$ -CD40 plus rIL-4-treated group,  $\alpha$ -CD40, rIL-4 and rIL-10-treated group, and  $\alpha$ -CD40 plus rIL-4 with 100, 10 or 1  $\mu\text{g}/\text{ml}$  of Mix-1 stimulation. As shown in Fig. 12, Mix-1 treatment showed a significantly inhibitory effect on B cell proliferation.



**Fig. 11.** Effect of Mix-1 extract on IgE production in murine splenic B cells. Mouse B cells from healthy Balb/c mice were either stimulated with anti-CD40 mAb (500 ng/ml) and recombinant interleukin-4 (500 U/ml) or treated with Mix-1 extract (100, 10, 1 mg/ml) or rIL-4 plus anti-CD40mAb plus Mix-1 extract or rIL-10 (50 ng/ml). Statistically significant value compared with control (with rIL-4 (500 U/ml) plus anti-CD40mAb) data by T test (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001).

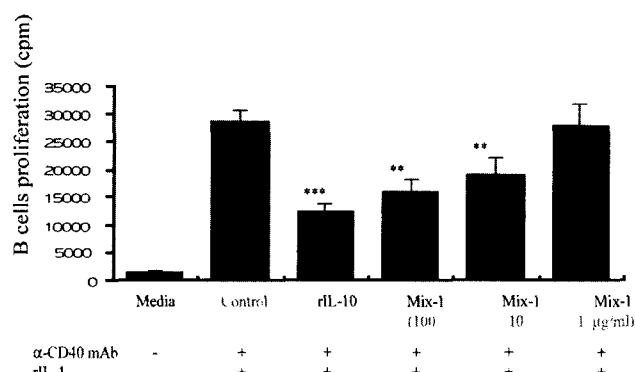


**Fig. 12.** Effect of Mix-1 extract on B cells proliferation in murine splenic B cells. Mouse B cells from healthy Balb/c mice were either stimulated with anti-CD40 mAb (500 ng/ml) and recombinant interleukin-4 (500 U/ml) or treated with Mix-1 extract (100, 10, 1 mg/ml) or rIL-4 plus anti-CD40mAb plus Mix-1 extract or rIL-10 (50 ng/ml). Statistically significant value compared with control (with rIL-4 (500 U/ml) plus anti-CD40mAb) data by T test (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001).

#### 10. Effects of Mix-1 on histamine releasing factor (HRF) expression

Mouse B cells were plated on 24 well plates ( $1 \times 10^6$  cells/well). After culture in the various combinations of anti-CD40 mAb(500 ng/ml), rIL-4(500 U/ml), rHRF (500 ng/ml), and Mix-1 (100 or 10  $\mu\text{g}/\text{ml}$ ), HRF expression was determined by immuno- fluorescence staining. The cells were incubated with biotin- conjugated rHRF on ice, and HRF

binding was assayed by flow cytometry. As shown in Fig 13, Mix-1 showed an inhibitory effect on HRF expression in mouse splenocytes.



**Fig. 13. Inhibitory effect of Mix-1 extract on histamine-releasing factor (HRF) expression in murine splenic B cells.** B cells from healthy Balb/c mice were either stimulated with anti-CD40 mAb and recombinant interleukin-4 (500 U/ml) or treated with Mix-1 extract (100 mg/ml, 10 mg/ml, and 1 mg/ml) or rIL-4 (500 U/ml), anti-CD40mAb plus Mix-1 extract. B cells (A) were cocultured with anti-CD40 mAb plus rIL-4 plus rHRF (Control, B), and with anti-CD40 mAb plus rIL-4 plus rHRF plus Mix-1 extract (100 mg/ml (C), and 10 mg/ml (D) for 48 hrs. After 48 h, the cells were harvested, and assayed for HRF expression by flow cytometry, and Statistically significant value compared with control (with rIL-4 (500 U/ml) plus anti-CD40mAb plus rHRF) data by T test (\*p<0.05,\*\*p<0.01,\*\*\*p<0.001).

## Discussion

In the present study, we investigated anti-allergic effects of Mix-1, a mixture of three different kinds of herbal drugs. Using cultured mouse B cells, levels of major cytokines and IgE levels which are known to be critical for regulating type I hypersensitivity (allergy) were determined in the presence or absence of Mix-1 extract. Our data indicate that Mix-1 treatment inhibited the levels of B cell surface molecules CD23 and CD11a, implying the inhibition of B cell activation by Mix-1. Also, determination of B cell proliferation by measuring the incorporation of radiolabeled thymidine into DNA showed decreases in B cell proliferation. Measurement of IL-1beta, IL-6, TNF-alpha, and histamine-releasing factor(HRF) at mRNA and /or protein levels in B cells showed decreased production in by Mix-1 treatment whereas levels of IL-10 mRNA and protein were increased by Mix-1 treatment. Finally, Mix-1 was not toxic to cultured mouse lung fibroblast cells. Together, the present data suggest that Mix-1 was effective in inhibiting the key molecules (or related molecular events) during allergy responses and further suggest possible application to type I hypersensitivity disease such as asthma which is primarily lung cell disease.

Sequence of events leading to allergic responses is initiated by the binding of extracellular allergens. Allergen can be involved directly in activating B cells by interacting with

surface IgE antibodies<sup>9-11</sup>). Alternatively, allergen activates CD4<sup>+</sup> T cells of TH2 type<sup>12</sup>). Activated TH2 cells secrete IL-4 which again activates IgE-B cells or IL-3, IL-5 and GM-CSF which are involved in eosinophil recruitment. Once B cell is activated, then IgE antibodies are released and involved in activating mast cells by binding to surface IgE receptor<sup>13,14</sup>). Consequently, activated mast cells transmit signals for degranulation, phospholipase A2 (PLA2), and cytokine gene activation<sup>15</sup>). Granule contents such as histamine, proteases and chemotactic factors including eosinophilic chemotactic factor (ECF) and neutrophilic chemotactic factor (NCF) are released. Activation of gene expression increases secretion levels of cytokines including TNF, IL-1, IL-4, IL-5 and IL-6<sup>16,17</sup>). Then, the release of phospholipids via the phospholipase A2 pathway increases levels of platelet-activating factor (PAF) and arachidonic acid which again induces the synthesis of leukotrienes B4, C4, and D4 and prostaglandin<sup>18</sup>). Also, a role of interferon-γ and IL-13 in the type I sensitivity has been shown to be important for regulating allergic reactions in skins, airway epithelial cells and lung, and others<sup>19-24</sup>). Several recent studies have reported that interferon-γ is involved in inhibiting the development of allergic response<sup>25-27</sup>) and its level is reversely regulated in tissues of atopy<sup>28,29</sup>). All these released mediators are involved in initial or late-phase allergic reactions. Thus, therapeutic approaches have been directed to control cytokines and IgE related with type I hypersensitivity<sup>30,31</sup>).

Individuals who are infected in childhood produce more Th1 cells known to inhibit allergic disease but less Th2 cell causing allergic diseases<sup>32</sup>). Cytokines produced from Th1 cells stimulate Th1 cell proliferation but inhibit Th2 cell growth whereas cytokines from Th2 cells induces Th2, but not Th1, cell proliferation. Thus, an imbalance between Th1 and Th2 cells is important for controlling immune responses and allergy reaction. Allergic disease can thus be seen as one of the immune diseases by balance shift from Th1 to Th2 cells<sup>33,34</sup>). Indeed, Th2 cells play a key role in the allergic reaction by producing cytokines such as IL-4, IL-10, and others which mediate IgE production, eosinophil activation, and inflammation. In an asthma as an example, Th2 cells produce β-chemokines such as eotaxin, RANTES, MIP-1α, and MCP-3 eosinophil, and help eosinophils enter into the lamina propria of the bronchioles and secrete IL-5, IL-3, and GM-CSF. These events are involved in proliferation, maturation, and increased life-span of eosinophils. Also, eosinophils infiltrated into the bronchial tissues secretes myelin basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin and induces bronchial damage<sup>35,36</sup>). IL-4 induces ε isotype switching in humoral reactions, which increases IgE production



and promotes the differentiation of Th0 cell to Th2 cells, and thus involved in inducing characteristic inflammatory responses in eosinophils, epithelial cell detachment, mucus secretion and bronchoconstriction. All these chronic asthma-related functions of eosinophils are known to be regulated by Th2 cell function<sup>36</sup>). Another important function of Th2 cell is that they inhibit IFN- $\gamma$  production in asthma patients. It is observed that IFN- $\gamma$  inhibits IgE production and Th2 cell differentiation in vitro. IFN- $\gamma$  also increases expression levels of CD69, HLA-DR, and ICM-1 from eosinophils, activates eosinophil, and exacerbates the bronchiole inflammation by ICAM-1. While more studies are required for elucidating actions of IFN- $\gamma$  in the onset and development progression of asthma, several lines of evidences indicate that Th2 cells are one of the critical factors regulating asthma<sup>37</sup>), and further implicate that several therapeutic applications for asthma would be possible by regulating the Th2 cell functions.

Recent studies also suggest that differentiation of Th1 and Th2 cells are controlled at the transcriptional levels. T cell receptor, co-stimulating factors, and cytokine environments are known to be important for the differentiation of Th1 and Th2 cells. First, a binding affinity of TCR to MHC class II is critical for the differentiation pattern of Th1 and Th2 cells; the higher the binding affinity, the more cells are differentiated into Th2 cells<sup>38</sup>). Also CD4<sup>+</sup> coreceptor after interacting with MHC class II transmits the signal via the activation of tyrosine protein kinase Ick and contributes the production of Th2 cells. This has been demonstrated by the fact that CD4<sup>+</sup> deficient Th2 cells are not able to differentiate into Th2 cells. Secondly, co-stimulating receptor CD28 interacts with B7.1 or B7.2 on the antigen presenting cells to activate and proliferate Th cells and also act as an essential Th2 cell differentiation factor. CD4<sup>+</sup> Th2 cells produce IFN- $\gamma$  (Th1 cell cytokine) via CD28 activation to induce the production of IL-4 and 5 which are Th2 cell cytokines<sup>39</sup>).

Our data showed inhibition of IL-1 beta, IL-4 and 6, and IgE levels in activated B cells. Mast cell-produced cytokines (TNF, IL-1, IL-4, 5, and 6) and chemokines play an important role in allergic reaction through their ability to recruit and activate a variety of inflammatory cells. TNF is an extremely potent mediator in leukocyte adhesion emigration, activation. IL-4 is also a mast cell growth factor and is required to derive IgE synthesis by B cells. It is known that IL-4 can stimulate B cell differentiation via the interaction with IgM and IgD receptors on the cell surface. IgE stimulates IgE-specific CD23 production and excretion, and induces the proliferation of B lymphocyte. After acting on resting B lymphocytes, IL-4 increases IgE producing B cells, and upregulates IgE

production by interacting with IL-13<sup>40</sup>). IFN- $\gamma$  has not only antiviral activity but also induces class II MHC and inhibits CD23 expression. IFN- $\gamma$  is involved in immunoglobulin production by inhibiting IL-4-dependent IgE production, and blocking IL-4-dependent CD23 expression which is antagonistic to IL-4 effects<sup>39</sup>). Thus, Mix-1 treatment which resulted in decreases in IL-1 beta, IL-4 and 6, and IgE production may have positively regulatory effects on allergy. Our data in which Mix-1 inhibited B cell proliferation and HRF levels, supports this interpretation.

The present data showed that levels of IL-10 production were increased by Mix-1 treatment in activated B cells. This regulation were assumed at the gene expression level since IL-10 mRNA, when analyzed by RT-PCR, was decreased. It has been reported that IL-10 inhibited proliferation of IgE producing B cells, and the inhibitory effect of IL-10 on IgE B cell was dependent upon the presence of monocytes<sup>41-43</sup>). The role of IL-10 as an anti-inflammatory cytokine in allergy has recently been reviewed<sup>44</sup>). IL-10 downregulates IL-4 and IL-5 expression by type 2 helper T cell<sup>45</sup>). IL-10 also inhibits accessory cell function<sup>42</sup>) and in the presence of IL-10, not only do T lymphocytes proliferate and produce cytokines in response to allergen, but those cells also rendered irreversibly nonresponsive (tolerant)<sup>44,46,47</sup>). Support for a modulating role for IL-10 in human allergic diseases is further derived from observations that IL-10 inhibits eosinophil survival<sup>48,49</sup>) and IgE synthesis<sup>50,51</sup>). Administration of IL-10 to sensitized mice abrogates allergen-induced airway inflammation<sup>52</sup>), and IL-10 knockout mice developed a lethal form of allergic bronchopulmonary aspergillosis with markedly elevated concentrations of interferon gamma (IFN- $\gamma$ ), IL-4, and IL-5<sup>53-55</sup>). Thus, increased expression of IL-10 by Mix-1 might be important for preventing allergy development.

Histamine-releasing factor or HRF is a collective term used for a heterogeneous group of factors with different modes of action<sup>40</sup>). The human recombinant histamine-releasing factor (HrHRF) acts as a complete stimulant for histamine release and IL-4 secretion from a subpopulation of highly allergic donor basophils. Additionally, IgE(+) basophils release histamine to other secretagogues, IL-3, and deuterium oxide. In the present study, Mix-1 decreased levels of HRF in activated B cells, suggesting decreased stimulation to mast cells or Th2 cells resulting in decreased production of histamine release and IL-4 in vivo.

## Conclusion

Accumulating evidences indicate that it is critical to

diagnose the allergic disease, pin-point, and eliminate the cause at the earliest stage as possible. One of the major targets for early diagnosis in allergic diseases such as asthma, nasobronchial allergy, atopy, urticaria (hives) is to determine IgE levels. The present data showed that Mix-1 showed clear inhibitory effects on the production of IgE though the levels of inhibition were smaller compared to positive control group. In summary, our data show that Mix-1 has significant inhibitory effects on allergy without cytotoxicity, and further implies its potential application for clinical medicine.

## References

1. Bradding P, Feather IH, Wilson S, Bardin PG, Heusser CH, Holgate ST et al. Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. *J Immunol* 151:3853-65, 1993.
2. Kips JC, O'Conner BJ, Langley SJ. Results of a phase I trial with SCH55700, A humanized anti-IL-5 antibody, in severe persistent asthma. *Am J Respir Crit Care Med* 161:A505 2000.
3. Foster P, Hogan S, Ramsay A, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 183:195-201, 1996.
4. Asakura K, Saito H, Watanabe M, Ogasawara H, Matsui T, Kataura A. Effects of anti-IL-5 monoclonal antibody on the murine model of nasal allergy. *Int Arch Allergy Immunol* 116:49-52, 1998.
5. Danzig M, Cuss F. Inhibition of interleukin-5 with a monoclonal antibody attenuates allergic inflammation. *Allergy* 52:787-94, 1993.
6. Kung TT, Stelts DM, Zurcher JA, Adams GK, Egan RW, Kreutner W et al. Involvement of IL-5 in a murine model of allergic pulmonary inflammation: prophylactic and therapeutic effect of an anti-IL-5 antibody. *Am J Respir Cell Mol Biol* 13:360-5, 1995.
7. Zia-Amirhosseini P, Minthorn E, Benincosa LJ, Hart TK, Hottenstein CS, Tobia LA et al. Pharmacokinetics and pharmacodynamics of SB-240563, a humanized monoclonal antibody directed to human interleukin-5 in monkeys. *J Pharmacol Exp Ther* 291:1060-7, 1999.
8. Van Oosterhout AJM, Ladenius ARC, Savelkoul HFJ, Van Ark I, Delsman KC, Nijikamp FP. Effect of anti-IL-5 and IL-5 on airway hyperreactivity and eosinophils in guinea pigs. *Am Rev Respir Dis* 147:548-52, 1993.
9. Heusser C, Jardieu P. Therapeutic potential of anti-IgE antibodies. *Curr Opin in Immunology* 9:805 1997.
10. Hide M, Tanaka T, Yamamura Y, Koro O, Yamamoto S. IgE-mediated hypersensitivity against human sweat antigen in patients with atopic dermatitis. *Acta Derm Venereol.* 82(5):335-40, 2002.
11. Ishizaka K. IgE binding factors and regulation of the IgE antibody response. *Annu Rev Immunol* 6:513, 1998.
12. Ying S, Humbert M, Barkans J, Corrigan CJ, Pfister R, Menz G, Larche M, Robinson DS, Durham SR, Kay AB. Expression of IL-4 and IL-5 mRNA and protein product by CD4+ and CD8+ T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. *J Immunol.* Apr 1;158(7):3539-44, 1997.
13. Kelly-Welch AE, Melo ME, Smith E, Ford AQ, Haudenschild C, Noben-Trauth N, Keegan AD. Complex role of the IL-4 receptor alpha in a murine model of airway inflammation: expression of the IL-4 receptor alpha on nonlymphoid cells of bonemarrow origin contributes to severity of inflammation. *J Immunol.* Apr 1;172(7):4545-55, 2004.
14. Kita H, Jorgensen RK, Reed CE, Dunnette SL, Swanson MC, Bartemes KR, Squillace D, Blomgren J, Bachman K, Gleich GJ. Mechanism of topical glucocorticoid treatment of hay fever: IL-5 and eosinophil activation during natural allergen exposure are suppressed, but IL-4, IL-6, and IgE antibody production are unaffected. *J Allergy Clin Immunol.* Sep;106(3):521-9, 2000.
15. Bradley BL, Azzawi M, Jacobson M, Assoufi B, Collins JV, Irani AM, Schwartz LB, Durham SR, Jeffery PK, Kay AB. Eosinophils, T-lymphocytes, mast cells, neutrophils and macrophages in bronchial biopsy specimens from atopic subjects with asthma; comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J Allergy Clin Immunol* 88:661-74, 1991.
16. Maggi E, Parronchi P, Manetti R et al. Reciprocal regulatory effects of IFN-gamma and IL-4 on the in vitro development of human Th1 and Th2 clones. *J. Immunol* 148:2142-7, 1992.
17. Corry DB, Kheradmand F. Biology and therapeutic potential of the interleukin-4/interleukin-13 signaling pathway in asthma. *Am J Respir Med.* 1(3):185-93, 2002.
18. Pene J, Rousset F, Briere F, Chretien I, Bonnefoy JY, Spits H, Yokota T, Arai N, Arai KI, Banchereau J, de Vries JE. IgE production by normal human lymphocytes is induced by interleukin-4 and suppressed by interferons-alpha and -gamma and prostaglandin E2. *Proc. Natl. Acad. Sci. U.S.A.* 85:6880, 1998.
19. Grunig G, Ford JG, Donaldson DD, Venkayya R, McArthur C, Hansell E, Kurup VA, Warnock M, Rennick D. Roles of interleukin-13 and interferon-gamma in lung inflammation. *Chest.* Mar;121(3 Suppl):88S, 2002.
20. Look DC, Rapp SR, Keller BT, Holtzman MJ. Selective

- induction of intercellular adhesion molecule-1 by interferon- $\gamma$  in human airway epithelial cells. *Am. J. Physiol.* 263:L79, 1992.
21. Gajewski TF, Fitch FW. Anti-proliferative effect of IFN- $\gamma$  in immune regulation. I. IFN- $\gamma$  inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *J. Immunol.* 140:4245, 1988.
  22. Kang H, Byun DG, Kim JW. Effects of substance P and vasoactive intestinal peptide on interferon- $\gamma$  and interleukin-4 production in severe atopic dermatitis. *Ann Allergy Asthma Immunol.* Sep;85(3):227-32, 2000.
  23. Peebles RS Jr, Dworski R, Collins RD, Jarzecka K, Mitchell DB, Graham BS, Sheller JR. Cyclooxygenase inhibition increases interleukin 5 and interleukin 13 production and airway hyperresponsiveness in allergic mice. *Am J Respir Crit Care Med.* Aug;162(2 Pt 1):676-81, 2000.
  24. Loza MJ, Peters SP, Zangrilli JG, Perussia B. Distinction between IL-13<sup>+</sup> and IFN- $\gamma$ <sup>+</sup> natural killer cells and regulation of their pool size by IL-4. *Eur J Immunol.* Feb;32(2):413-23, 2002.
  25. Lack G, Bradely KL, Hamelmann E, Renz H, Loader J, Leung DYM, Larsen G, Gelfand EW. Nebulized IFN- $\gamma$  inhibits the development of secondary allergic responses in mice. *J Immunol* 157:1432, 1996.
  26. Robinson DS, Ying S, Bentley AM, Meng Q, North J, Durham SR, Kay AB, Hamid Q. Relationships among numbers of bronchoalveolar lavage cells expressing mRNA for cytokines, asthma symptoms, and airway methacholine responsiveness in atopic asthma. *J Allergy Clin Immunol* 92:397-403, 2002.
  27. Ekerfelt C, Ernerudh J, Jenmalm MC. Detection of spontaneous and antigen-induced human interleukin-4 responses invitro: comparison of ELISPOT, a novel ELISA and real-time RT-PCR. *J Immunol Methods.* Feb 1;260(1-2):55-67, 2002.
  28. Tang MLK, Kemp AS, Thorburn J, Hill DJ. Reduced interferon- $\gamma$  secretion in neonates and subsequent atopy. *Lancet* 344:983, 1994.
  29. Amrani Y, Moore PE, Hoffman R, Shore SA, Panettieri RA Jr. Interferon- $\gamma$  modulates cysteinyl leukotriene receptor-1 expression and function in human airway myocytes. *Am J Respir Crit Care Med.* Dec 1;164(11):2098-101, 2001.
  30. Wang CY, Walfield AM, Fang X, Hammerberg B, Ye J, Li ML, Shen F, Shen M, Alexander V, MacGlashan DW. Synthetic IgE peptide vaccine for immunotherapy of allergy. *Vaccine.* Apr 2;21(15):1580-90, 2003.
  31. Wierenga EA, Messer G. Regulation of interleukin 4 gene transcription: alterations in atopic disease? *Am J Respir Crit Care Med.* Sep;162(3 Pt 2):S81-5, 2000.
  32. Egan RW, Umland SP, Cuss FM, Chapman RW. Biology of interleukin-5 and its relevance to allergic disease. *Allergy* 51:71-81, 1996.
  33. Leckie MJ, Brinke A, Jamey K, Zuzana D, Brian C, Christine M et al. Effects of interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet.* 356:2144-8, 2000.
  34. Kang BS. Inhibitory effects of anti-asthmatic material on the proinflammatory cytokine. A thesis for the degree of master of science. Chungbuk National University, 1999.
  35. Min BK, Oh SR, Lee HK, Takatsu K, Chang IM, Min KR et al. Sophoricoside analogs as the IL-5 inhibitors from *Sophora japonica*. *Planta Med* 65:408-12, 1999.
  36. Li XM, Huang CK, Zhang TF, Teper AA, Srivastava K, Shofield BH et al. The Chinese herbal medicine formula MSSM-002 suppresses allergic airway hyperreactivity and modulates Th1/Th2 response in a murine model of allergic asthma. *J Allergy Clin Immunol* 106:660-8, 2000.
  37. Garbor M, Razga Z. Effect of benzopyrone derivatives on simultaneously induced croton oil ear and carrageenin paw edema in rats. *Acta Physiol Hung* 77:197-207, 1991.
  38. Barton HJ, Boot BJR, Brandon DR, O'Brien A. The relationship between development of lung inflammation and changes in bone marrow populations in guinea pigs following inhaled antigen challenge. *Int Arch Allergy Appl Immunol* 96:1-11, 1991.
  39. Nabe T, Shinoda N, Yamashita K, Yamamura H, Kohno S. Leukocyte kinesis in blood, bronchoalveoli and nasal cavities during late asthmatic responses in guinea-pigs. *Eur Respir J* 11:636-42, 1998.
  40. Budde IK, Aalberse RC. Histamine-releasing factors, a heterogeneous group of different activities. *Clin Exp Allergy.* Sep;33(9):1175-82, 2003.
  41. Bober LA, Grace MJ, Pugliese-Sivo C, Waters TA, Sullivan LM, Narula SK. Human IL-10 reduces the number of IL-4-induced IgE B cells in cultures of atopic mononuclear cells. *Int Arch Allergy Immunol.* Sep;105(1):26-31, 1994.
  42. de Waal Malefyt, R., J. Haanen, H. Spits, M. G. Roncarolo, A. te Velde, C. Figdor, K. Johnson, R. Kastelein, H. Yssel, and J. E. de Vries. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J. Exp. Med.* 174: 915-924, 1991.
  43. Enk, A. H., V. L. Angeloni, M. C. Udey, and S. I. Katz. .

- Inhibition of Langerhans cell antigen-presenting function by IL-10: a role for IL-10 in induction of tolerance. *J. Immunol.* 151: 2390-2398, 1993.
44. Borish, L., A. Aarons, J. Rumbyrt, P. Cvietusa, J. Negri, and S. Wenzel. Interleukin-10 regulation in normal and asthmatic subjects. *J. Allergy Clin. Immunol.* 97: 1288-1296, 1996.
45. Becker, J. C., C. Czerny, and E.-B. Brocker. Maintenance of clonal anergy by endogenously produced IL-10. *Int. Immunol.* 6: 1605-1612, 1994.
46. Bonfield, T. L., M. W. Konstan, P. Burfeind, J. R. Panuska, J. B. Hilliard, and M. Berger.. Normal bronchial epithelial cells constitutively produce the anti-inflammatory cytokine interleukin-10, which is downregulated in cystic fibrosis. *Am. J. Respir. Cell Mol. Biol.* 13: 257-261, 1995.
47. Takanashi, S., R. Nonaka, Z. Xing, P. O'Byrne, J. Dolovich, and M. Jordana.. Interleukin 10 inhibits lipopoly- saccharide induced survival and cytokine production by human peripheral blood eosinophils. *J. Exp. Med.* 180: 711-715, 1994.
48. Ohmori, H., T. Kanda, T. Takai, and M. Hikida. Induction of antigen-specific IgE response in murine lymphocytes by IL-10. *Immunol. Lett.* 47: 127-132, 1995.
49. Ohmen, J. D., J. M. Hanifin, and B. J. Nickoloff. Overexpression of IL-10 in atopic dermatitis: contrasting cytokine patterns with delayed-type hypersensitivity reactions. *J. Immunol.* 154: 1956-1963, 1995.
50. Punnonen, J., R. de Waal, Malefyt, P. Van Vlasselae, J.-F. Gauchat, and J. E. de Vries. IL-10 and viral IL-10 prevent IL-4-induced IgE synthesis by inhibiting the accessory cell function of monocytes. *J. Immunol.* 151: 1280-1289, 1993.
51. Martinez, J. A. B., T. E. King Jr., K. Brown, C. A. Jennings, L. Borish, R. L. Mortenson, T. Z. Khan, T. W. Bost, and D. W. H. Riches. . Increased expression of the interleukin 10 gene in alveolar macrophages in interstitial lung diseases. *Lung Cell. Mol. Physiol.* 17: L676-683, 1997.
52. Del Prete, G., M. De Carli, F. Almerigogna, M. G. Giudizi, R. Biagiotti, and S. Romagnani. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J. Immunol.* 150: 353-360, 1993.
53. Grunig, G., D. B. Corry, M. W. Leach, B. W. P. Seymour, V. P. Kurup, and D. M. Rennick. Interleukin-10 is a natural suppressor of cytokine production and inflammation in a murine model of allergic bronchopulmonary aspergillosis. *J. Exp. Med.* 185: 1089-1099, 1997.
54. Rousset, F., E. Garcia, T. Defrance, C. Peronne, N. Vezzio, D. H. Hsu, R. Kastelein, K. W. Moore, and J. Banchereau. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* 89: 1890-1893, 1992.
55. Briere, F., C. Servet-Delprat, J.-M. Bridon, J.-M. Saint-Remy, and J. Banchereau. Human interleukin 10 induces naive surface immunoglobulin D+ (sIgD+) B cells to secrete IgG1 and IgG3. *J. Exp. Med.* 179: 757-762, 1994.