Neutrophil Apoptosis associated with the Pathogenesis of Asthma

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This certifies that the dissertation of Eun Ju Yang is approved.

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Abbreviations

BAL fluid	: Bronchoalveolar fluid
CCLs	: CC chemokine ligands
CCRs	: CC chemokine recpetors
CI	: Chemotactis index
CXCR	: CXC chemokine receptor
ELISA	: Enzyme-linked immunosorbent assay
FBS	: Fetal bovine serum
FITC	: Fluorescein isothiocyanate
FEV1	: Forced expiratory volume in 1 second
FVC	: Forced vital capacity
G-CSF	: Granulocyte colony-stimulating factor
GINA	: Global initiative for asthma
GM-CSF	: Granulocyte macrophage colony-stimulating factor
IgE	: Immunoglobulin E
IL-5	: Interleukin-5
Lkn-1	: Leukotactin-1
MCP-1	: Monocyte chemoattractant protein-1
MIP-1α	: Macrophage inflammatory protein-1α
PI	: Propidium iodide
RANTES	: Regulated on activation normal T expressed and secreted
RT-PCR	: Reverse transcription-polymerase chain reaction
TARC	: Thymus- and activation-regulated chemokine

Abstract

Asthma is an inflammatory airway disease and is characterized by the releases of inflammatory mediators including chemokines. Chemokines are mainly associated with the recruitment, activation and dysregulation of specific inflammatory cells, especially mast cells, monocytes, T cells, eosinophils, and neutrophils in asthma. In various inflammatory cells, neutrophils play an important role in the pathogenesis of severe asthma. In this study, the effects of CC chemokine ligand 2 (CCL2) on constitutive apoptosis of neutrophils isolated from the peripheral blood of healthy subjects were investigated. CCL2 blocked the constitutive apoptosis of neutrophils through CC chemokine receptor 2 (CCR2). CCL2 also induced elevation of the cytosolic Ca^{2+} concentration, but had no effect on normal neutrophil chemotaxis. Anti-apoptotic signaling mediated by CCL2 was found to be associated with the PI3K/Akt/ERK/NF-κB cascade in neutrophils. The supernatant collected from CCL2-treated normal neutrophils inhibited the constitutive apoptosis of neutrophils. Both the cleavage of procaspase 3 and procaspase 9, and the decrease in Mcl-1 expression were delayed by CCL2 stimulation. For confirmation of the anti-apoptotic effect induced by CCL2 on asthma pathogenesis, these effects of CCL2 on normal neutrophils were compared to the effect of CCL2 on neutrophils of asthmatic patients. Although asthmatic neutrophils were not affected by constitutive apoptosis, calcium influx or cell migration following CCL2 stimulation, and the inhibition of NF-κB blocked

constitutive apoptosis of neutrophils from asthmatic patients via inhibition of the cleavage of procaspase 3 and procaspase 9. The anti-apoptotic effect of BAY 11-7985 on neutrophils of severe asthma was stronger than that on neutrophils of mild or moderate asthma. NF-κB was involved in CCL2-induced anti-apoptotic signaling in normal neutrophils, whereas it functioned as a basal pro-apoptotic factor in asthmatic neutrophils. A better understanding of the difference in the regulation of neutrophil apoptosis mediated by CCL2 between normal subjects and asthmatics will enable to elucidate the role of CC chemokine in neutrophils and build a framework for understanding the pathogenesis of asthma.

Keywords: Asthma, Neutrophils, CCL2, NF-κB, Apoptosis, Anti-asthmatic effect

1. **Introduction**

Asthma is an inflammatory disease of the lungs characterized by increased infiltration of leukocytes, especially eosinophils, into the airways and reduced respiratory function (Robinson et al., 1999). The inflammation leads to bronchoconstriction, increased airway hyperresponsiveness, and mucus production (Djukanovic et al., 1990). The prevalence of asthma is rapidly increasing around the world, especially in young children, and it has become a significant cause of morbidity and mortality in developed countries (Braman, 2006). Both eosinophils and T helper (Th) 2 lymphocytes play pathogenic roles in asthma (Elsner and Kapp, 1999; Busse and Rosenwasser, 2003). Eosinophils are commonly associated with allergic inflammation, and act as effector cells in the pathogenesis of this disease by releasing cytotoxic granule proteins (Kay, 2001). An imbalance between Th1 and Th2 leads to the clinical expression of allergic disease (Romagnani et al., 2001). Th2 cells produce interleukin (IL)-4, IL-5, IL-10, and IL-13, known as Th2 cytokines, and which function as important factors in pathogenesis of allergic diseases (Lucey et al., 1996; Heijink and Van Oosterhout, 2005; Homey et al., 2006). The number of Th2 cytokines typically increases in allergic diseases and has important effects on airway infiltration, eosinophil activation, induction of IgE production, mucus secretion and the release of a variety of inflammatory mediators (Kips, 2001; Woodfolk, 2006; Gould and Sutton, 2008).

The severity of asthma is classified as intermittent, mild persistent, moderate persistent or severe persistent. Asthma is clinically classified according to the frequency of symptoms, forced expiratory volume in 1 second (FEV_1), and peak expiratory flow rate (Yawn, 2008). In the GINA (Global Initiative for Asthma) guidelines, the severity of asthma is based on lung function and asthma control, respectively (Table 1). Severe and corticosteroid-resistant asthmatics raise neutrophil counts in their airways. Previous studies have shown that increased sputum neutrophils are involved in irreversible loss in lung function of asthmatic patients. In addition, airway neutrophilia is a recognized feature of chronic severe asthma, but the mechanisms that underlie this phenomenon are unknown. Evidence for factors present in airway secretions that prolong neutrophil survival has been sought and it has been hypothesized that these might be augmented in neutrophilic asthma (Maianski et al., 2004). The previous reports have led to the suggestion that neutrophils might be an important pathogenic factor and a valuable target in the treatment of asthma (Douwes et al., 2002; Louis and Djukanovic 2006).

Human neutrophils play a key role in innate immune response, responding to inflammatory mediators and chemotactic agents (Witko-Sarsat et al., 2000). Neutrophils, which are the most abundant leukocytes in the peripheral blood, have a very short half-life of 6 to 18 h during circulation and between 1 and 4 days in the tissue due to their constitutive apoptosis by exogenous ligands (Savill et al., 1989). Constitutive apoptosis in neutrophils is an important mechanism for

maintaining their number in pathogenic lesions, but persistent accumulation of neutrophils in inflammatory diseases may lead to the release of cytotoxic molecules and the development of tissue injury (Scheel-Toellner et al., 2004). Neutrophil apoptosis is essential for the resolution of inflammation and prevention of tissue damage; accordingly, delayed neutrophil apoptosis has been reported in association with the production of cytokines and chemokines (Colotta et al., 1993; Dibbert et al., 1999). Anti-apoptotic cytokines such as granulocyte macrophage colony-stimulating factor (GM-CSF) delay neutrophil apoptosis by increasing Mcl-1 stability and by suppressing caspase 3 activation (Derouet et al., 2004; van Raam et al., 2008). The anti-apoptotic effect of GM-CSF may depend on the expression of Mcl-1 and Bax (Fulop et al., 2002).

Chemokine and the chemokine receptor regulate a variety of pathophysiological responses including leukocyte recruitment in inflammatory responses and cell differentiation in hematological malignancy (Ben-Baruch et al., 1995; Murdoch and finn, 2000; Bisset and Schmid-Grendelmeier, 2005; Laurence, 2006). Chemokine receptors (CCRs) binding to CC chemokines include CCR1 through CCR11 while CXC chemokine receptors (CXCRs) interacting to CXC chemokine, CXCR1 though CXCR5 (Laurence, 2006). In various chemokines, eotaxin is a potent chemoattractant for eosinophils and it is generally elevated after asthma induction. Among various chemoattractants, the CC(β) chemokine family have been reported to increase the chemotactic activity of the monocyte lineage, while CXC(α) chemokines induce neutrophil migration (Taub and

Oppenheim, 1994). These CC chemokines include leukotactin-1 (Lkn-1), macrophage inflammatory protein 1α (MIP-1α), regulated on activation normal T expressed and secreted (RANTES), and thymus- and activation-regulated chemokine (TARC), and they are involved in allergic disease (Murphy et al., 2000). Increased level of monocyte chemoattractant protein-1 (MCP-1)/CCL2 in serum is also associated with asthma (Burn et al., 1994; Rose et al., 2003).

CCL2 is a prototype of CC chemokine that is produced by a variety of cells including monocytes, fibroblasts, vascular endothelial cells, and smooth muscle cells (Deshmane et al., 2009). CCL2 interacts with the CCR2 receptor and induces intracellular signal transduction. CCR2 is a G-protein-coupled receptor that is expressed in monocytes, natural killer cells, T cells and B cells. CCL2 signaling through CCR2 is involved in a pertussis toxin (PTX)-sensitive increase of intracellular calcium and activation of the protein tyrosine kinases, phosphatidylinositol 3-kinase (PI3K), and extracellular signal-regulated kinase (ERK) (Yamasaki et al, 2001; Jiménez-Sainz et al., 2003). The increase in CCL2 expression causes cardiac myocyte apoptosis, but CCL2 helps the survival of central memory $CD8^+$ cells (Zhou et al., 2006; Wang et al., 2008). The role of CCL2 in resting neutrophils is not well understood because of the low CCR2 expression in neutrophils (Johnston et al., 1999; Murphy et al., 2000).

To investigate the novel mechanism of neutrophil in asthma pathogenesis, the role of CCL2 in the human neutrophils was investigated. In this study, I examined the expression levels of CCRs and effects of CCL2 on the neutrophil apoptosis.

Also, different regulation induced by CCL2 between normal and asthmatic neutrophils was investigated via the determination of intracellular signaling pathway.

Table 1. Global Initiative For Asthma Guidelines

Severity	Intermittent	Persistent		
		Mild	Moderate	Severe
GINA grade	1	2	3	4
Daytime symptoms	< 1x a week	≥ 1x/wk	Daily Affects daily activities	Daily Limits daily activities
Nighttime symptoms	≤ 2x/month	> 2x/month	> 1x/week	Frequent
Peak expiratory flow (PEF)	≥ 80% predicted	≥ 80% predicted	60 ~ 79% predicted	< 60% predicted
PEF Variability	≤ 20% variability	20 ~ 30% variability	> 30% variability	> 30% variability
Forced expiratory volume in 1 second (FEV1)	≥ 80%	≥ 80%	60 ~ 70%	< 60%

(Ref.: URL: http://www.ginasthma.org 2009)

2. Materials and methods

Normal subjects and asthmatic patients

Sixty-four asthmatic patients (average age: 38.8 years) were recruited from the Konyang University Hospital. The asthma group included 29 female (average age: 56.5 years) and 35 male (average age: 50.1 years) patients with allergic asthma and who had mild to severe symptoms of the disease, which were measured based on the criteria stated in the Global Initiative for Asthma (GINA) (URL: http://www.ginasthma.org 2009) (Table 1). Fifty-four normal subjects (average age: 30.0 years) were recruited as controls. The normal subjects included 45 females (average age: 23.6 years) and 9 males (average age: 26.6 years), and had normal lung function, no history of asthma, and did not require medication. This study was approved by the Institutional Review Board of Eulji University for normal volunteers and by the Institutional Review Board of Konyang University for asthma patients. All participants in this study gave their written informed consent.

Neutrophil isolation and cell culture

Human neutrophils were isolated from the heparinized peripheral blood of healthy persons and asthmatics using Ficoll-Hypaque (density: 1.077 g/ml) (GE

Healthcare, Piscataway, USA) gradient centrifugation and CD16 microbeads magnetic cell sorting kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were washed after hypotonic lysis to remove erythrocytes and were resuspended at 3 x 10^6/ml in an RPMI 1640 medium (Invitrogen, Paisley, UK) with 1% penicillin-streptomycin (Invitrogen) and 10% FBS (Invitrogen). This method routinely yielded above 97% neutrophil purity as assessed by counting the cells on cytospin.

Reverse transcription-polymerase chain reaction for the mRNA expressions of CC chemokine receptors

Reverse transcription-polymerase chain reaction (RT-PCT) was performed to evaluate relative quantities of mRNAs for CCR1, CCR2, CCR3, CCR4 and CCR5 in human neutrophils. Total RNA was extracted from the cells using Trizol reagent (Invitrogen). For cDNA preparation, total RNA (2 μg) was incubated at 37℃ for 90 min using first-strand cDNA synthesis kit (Promega, Madison, USA). The cDNA products were denatured at 94℃ for 5 min, followed by 30 cycles of denaturation at 94℃ for 1 min, annealing at 60℃ for 1 min, extension at 72℃ for 1 min and a final extension at 72℃ for 10 min. Primer sequences were described in Table 2 (Sato et al., 2003; Oba et al., 2005). GAPDH was used as an internal control for each PCR reaction. The PCR products were analyzed via 1% agarose gel electrophoresis, and then visualized by ethidium bromide (Promega) staining.

Data were expressed as a representative of six individual experiments.

Table 2. Primers of human gene used for RT-PCR

Target mRNA	Forward primer (5' → 3') Reverse primer (5' → 3')
CCR1	CTC TTC CTG TTC ACG CTT CC CCA AAT GTC TGC TCT GCT CA
CCR2	AAC TCC TGC CTC CGC TCT AC TCA CTG CCC TAT GCC TCT TC
CCR3	TCT TCC TCG TCA CCC TTC CA GCT TCG TCC GCT CAC AGT CA
CCR4	CTT CCT GCC CCC ACT GTA TT TCT TCA CCG CCT TGT TCT TC
CCR5	TCC TGC CTC CGC TCT ACT GAA CTT CTC CCC GAC AAA
GAPDH	ACC ACA GTC CAT GCC ATC AC TCC ACC ACC CTG TTG CTG TA

Flow cytometry for the protein expressions of CC chemokine receptors

To detect the surface expressions of CC chemokine receptors (CCR1-5), neutrophils were incubated with the anti-CCRs antibodies (R & D systems, Minneapolis, USA) or control IgG antibodies (SantaCruz Biotechnology, Santa Cruz, USA) for 30 min, followed by anti-mouse or anti-rat IgG FITC-conjugated antibodies (Molecular Probes, Eugene, USA). The samples were analyzed using CellQuest software on a FACSCalibur (BD bioscience, San Diego, USA). Ten thousand events were collected for each experiment. Data were expressed as a representative of six individual experiments.

Neutrophil migration

The activity of cell migration was estimated using a 48-well microchamber (Neuroprobe, Gaithersburg, USA). The lower wells were filled with 28 μl phosphate buffered saline (PBS) alone or with PBS containing chemokine. In case of neutrophil chemotaxis assay, a polyvinylpyrrolidone (PVP)-free filter (Neuroprobe) with 5 μm pore size was placed over each lower well. Cell suspension was added with 50 μl of neutrophils at 2 x 10^6 cells/ml to the upper wells. The assembled chamber was incubated for 90 min at 37℃ in a humidified incubator. Non-migrated cells adhering to the upper surface of polycarbonate filter

were removed with a filter wiper. The filter was dried, fixed, and stained with Diff-Quick (Baxter, McGaw Park, USA). The cells from four randomly selected fields per well were counted, and the chemotactic index (CI) was calculated from the number of cells that migrated to the control. Significant chemotaxis was defined as CI > 2. A single experiment included six replicate measurements.

Neutrophil apoptosis

An annexin V–fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences) was used for the detection of neutrophil apoptosis. Isolated neutrophils were incubated with an FITC-labeled annexin V and propidium iodide (PI) for 15 min at room temperature. Apoptotic neutrophils were analyzed using a FACSCalibur with CellQuest software (BD bioscience) and were determined as the percentage of cells showing annexin V+/PI- and annexin V+/PI+. For the morphological estimation of neutrophil apoptosis, neutrophils were cytocentrifuged and stained with Wright staining solution (Merck, Darmstadt, Germany).

Calcium influx in neutrophils

Intracellular calcium concentration was measured in neutrophils as previously described (Ko et al., 2007). The cells were loaded with the Ca^{2+}

indicator 1 μM fluo-3-AM ester (Sigma, ST. Louis, USA) and 0.01% pluronic F-127 (Sigma) for 30 min at 37℃. Dye-loaded cells were resuspended in Ca^{2+} assay buffer and were incubated for 30 min at 37℃. Chemokines (100 ng/ml) (R & D systems) were added, and intracellular Ca^{2+} concentration was subsequently analyzed at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACSCalibur. The increased fluorescence was measured every second for 120 sec. Baseline fluorescence was monitored for 120 sec before chemokines were added.

Western blotting of apoptotic molecules and signal proteins in neutrophils

After being treated with CCL2 (R & D systems), the cells were harvested and lysed in a cytosolic extraction buffer (Invitrogen). The homogenate (100 μl) was centrifuged at 10,000 g for 1 min at 4℃. The supernatant was collected as a cytosolic fraction. The pellet was resuspended in 50 μl of nuclear extraction buffer and was centrifuged at 12,000 g for 15 min at 4℃. The supernatant was collected as a nuclear fraction. The protein samples (50 μg/lane) were separated by 12% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Millipore, Billerica, USA). The transferred membranes were incubated with anti-phospho Akt, anti-phospho ERK, anti-Iκ-Bα, anti-Mcl-1, anti-Bax, and anti-procaspase 3 or anti-procaspase 9 antibodies (SantaCruz

biotechnology) and were developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech., Piscataway, USA). The same blot was stripped and reprobed with anti-ERK2 (SantaCruz biotechnology) antibodies for use as an internal control.

NF-κB p65 transcription factor assay

The DNA-binding activity of NF-κB was assessed using EZ-Detect^{TM} transcription factor kits for NF-κB p65 (PIERCE, Rockford, USA), according to the manufacturer's instructions. DNA binding specificity was assessed using wild type or mutant NF-κB oligonucleotides. chemiluminescent detection was performed using a luminometer (Berthold Technologies, Bad Wildbad, Germany).

Enzyme-linked immunosorbent assay of IL-5, IL-6, IL-8, GM-CSF and CCL2

The concentrations of IL-5, IL-6, IL-8, GM-CSF and CCL2 in the cell supernatants were measured with a sandwich enzyme-linked immunosorbent assay (ELISA) using OptEIA^{TM} Set human IL-4, IL-5, IL-6, IL-8, GM-CSF and CCL2 (BD Biosciences, San Diego, USA) and G-CSF (Abfrontier, Seoul, Korea) according to the manufacturer's instructions using an ELISA reader, BIO-TEK EL

x 808 (Bio-Tek, Winooski, USA). In brief, 100 μl/well of capture antibody diluted in coating buffer (0.1 M Sodium Carbonate, pH 9.5) was loaded on a 96-well plate. The plate was sealed and was incubated overnight at 4 ℃. The 96-well plate was washed with 300 μl/well wash buffer (PBS with 0.05% Tween-20), blocked with 200 μl/well assay diluents (PBS with 10% FBS, pH 7.0) at room temperature (RT) for 1 h. 100 μl/well of standard, sample, and control were added to the plate. After the plate was incubated for 1 h at RT, it was washed and filled with 100 μl/well of detection antibody with strepavidin-HRP reagents. After 1 h incubation of the plate, it was washed and was added with 100 μl of substrate solution (TMB, tetramethylbenzidine) with hydrogen peroxide to each well for 30 min at RT in the dark. And then 50 μl of stop solution (2N H_2SO_4) was added to each well in the plate, and signal of the plate was read absorbance at a wavelength of 450 nm within 30 min of stopping reaction.

Measurement of phosphorylated Akt was also performed with human phospho-Akt (S473) DuoSet® IC ELISA kits (R&D Systems) as described above. All assays were performed in triplicate. The concentration of each protein was calculated from the standard curve.

Statistical analysis

Data were analyzed by one-way ANOVA or Student's *t* test using the SPSS statistical software package, Version 10.0 (SPSS Inc., Chicago, USA). All data

were expressed as the means ± S.E.M. A p value less than 0.05 was considered significant.

3. Results

Severe asthma induces the increase of neutrophils in peripheral blood and BAL fluid

As shown in Table 3, various parameters-associated with asthma pathogenesis significantly alternated among asthmatic patients from GINA 1 to GINA 4. In asthmatic patients, patients with severe asthma (GINA 4 grade) showed a markedly decreased forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV1). Especially, patients with severe asthma had the highest level of neutrophils of peripheral blood and BAL fluid in asthma patients (Table 3). As the disease worsened, the rate of bacterial infection in the lung tissues increased. These results indicate that neutrophils may play an important role in the pathogenesis of severe asthma.

Table 3. Characteristics of healthy individuals and asthmatic patients

		Healthy individual	Asthma patient			
			G 1	G 2	G 3	G4
Age (years)		24.1	51.0	51.7	52.3	62.6
Gender (F/M)		45/9	10/6	9/14	9/8	1/7
FVC (L)		4.5	3.0	2.9	3.1	2.3
FVC (% predicted)		95.2	94.6	95.2	92.7	75.1
FEV1 (L)		4.1	2.4	2.3	2.2	1.3
FEV1 (% predicted)		101.6	92.2	88.0	79.2	55.2
FEV1/FVC (%)		91.6	80.5	76.2	71.2	58.1
Serum IgE (IU/ml)		140.4	311.4	515.6	435.1	297.7
Bacterial infection (%)		0	12.5	13.0	6.0	62.5
ESR (mm)		11.0	37.8	17.6	28.6	33.0
Corticosteroid treatment (%)		0.0	12.5	13	6.0	62.5
Blood	Total cells (cells/mm^3)	4950.0	8243.8	8626.7	9894.1	8737.5
	Neutrophils (%)	48.3	57.7	61.3	61.2	54.7
	Eosinophils (%)	1.2	5.7	7.6	5.3	2.3
	Basophils (%)	0.3	0.5	0.4	0.5	0.5
	Lymphocytes (%)	39.9	28.0	24.3	25.2	23.5
	Monocytes (%)	10.4	7.6	6.3	7.5	7.8
BAL fluid	Total cells (x10^6)	4.7	7.0	14.3	8.9	11.6
	Neutrophils (%)	2.0	13.3	18.2	20.5	41.1
	Eosinophils (%)	0.3	3.9	4.4	3.3	2.3
	Basophils (%)	0.0	0.0	0.1	0.0	0.0
	Lymphocytes (%)	10.7	13.4	17.4	15.2	9.1
	Monocytes (%)	86.2	67.1	55.2	59.5	42.7
	Epithelial cells (%)	0.8	1.8	4.6	1.4	4.8

Expression of CC chemokine receptors and chemotactic effect of chemokines on human neutrophils

Human neutrophils in asthmatic patients are migrated by various chemokines from the blood into inflamed tissues, and continuously induce inflammatory responses. To provide the molecular mechanism of neutrophil migration induced by chemokines and chemokine receptors into the site of inflammation, mRNA and surface protein expression of CCRs, CCR1 through CCR5, in neutrophils was examined first by performing RT-PCR and flow cytometry. As shown in Figure 1 and Figure 2, both CCR1 and CCR3 mRNAs were strongly expressed, but protein expressions of the receptors were not detected on the cell surface. Protein expression of CCR2 was weakly detected (Fig. 2), surface protein expression of other chemokine receptors, CCR4 and CCR5 was not detected. These results indicate that the difference between CCR mRNA and protein expression may be due to a different translation process of individual CCR mRNA.

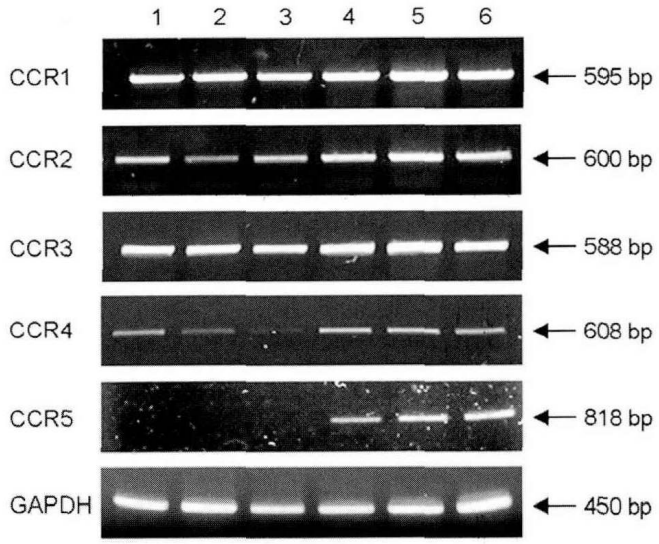

Figure 1. mRNA levels of CC chemokine receptors in human neutrophils. Total RNA was extracted from isolated neutrophils. mRNA levels of CC chemokine receptors were analyzed by RT-PCR using CCR1, CCR2, CCR3, CCR4 or CCR5 primer described in Table 2. GAPDH was used as an internal control.

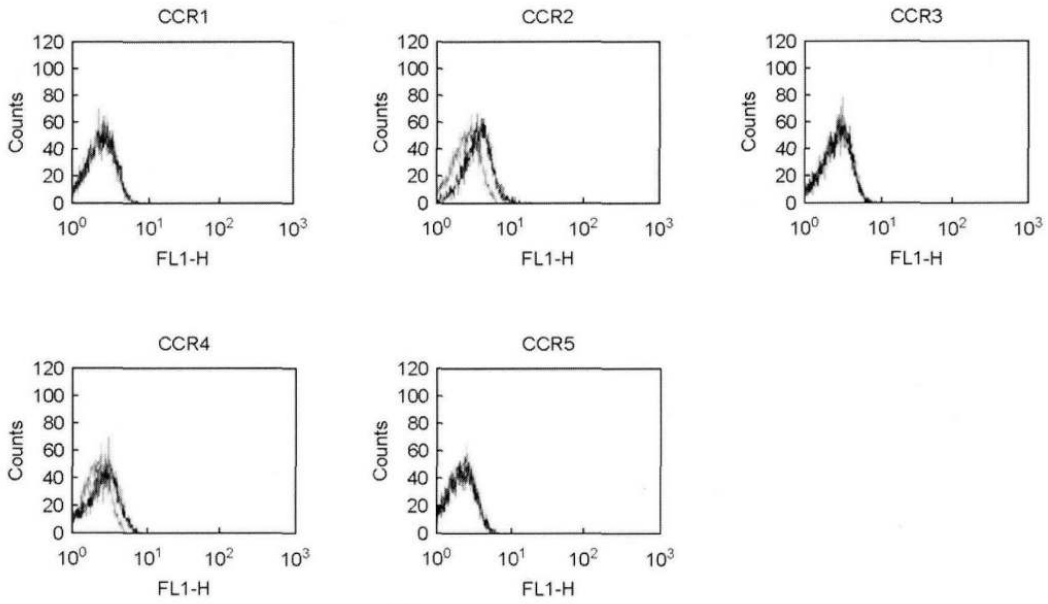

Figure 2. Expression of CC chemokine receptors in the surface of human neutrophils. Isolated neutrophils were analyzed by fluorescence-activated cells sorter using anti-CCR1, anti-CCR2, anti-CCR3, anti-CCR4 or anti-CCR5 antibodies (thick line). Baseline fluorescence was obtained by incubating control mouse IgG instead of anti-CCR antibodies (thin line).

CCL2 delays the apoptosis of human neutrophils

A chemotaxis assay to detect the functional activity of CCRs was conducted. CC chemokines did not induce a significant migration of neutrophils from the normal group (Fig. 3). IL-8 was used as a positive control, a chemotactic factor on neutrophils (Kunket et al., 1991; Baggiolini et al., 1994).

Since neutrophils showed weak CCR2 expression, I evaluated whether CC chemokines such as CCL2 are associated with the regulation of neutrophil apoptosis. The levels of apoptotic cells of human neutrophil at 24 h after neutrophil isolation were 72.1% (Fig. 4A). The addition of CCL2 led to a significant reduction in the apoptosis of neutrophils, compared to control, only medium-treated group. CCL2 had a similar anti-apoptotic effect on early stage (annnexin Ⅴ +, PI -) and late stage (annexin Ⅴ +, PI +) at neutrophils (Fig. 4B). Other CC chemokines, such as CCL3, CCL5, CCL11, CCL15, CCL16 and CCL17, binding to CCR1 through CCR5 had no effects on neutrophil apoptosis. GM-CSF, a well defined anti-apoptotic agent, was used as a positive control in this experiment. It was confirmed that CCL2 inhibits neutrophil apoptosis in a time- and a dose-dependent manner (Fig. 5A and B).

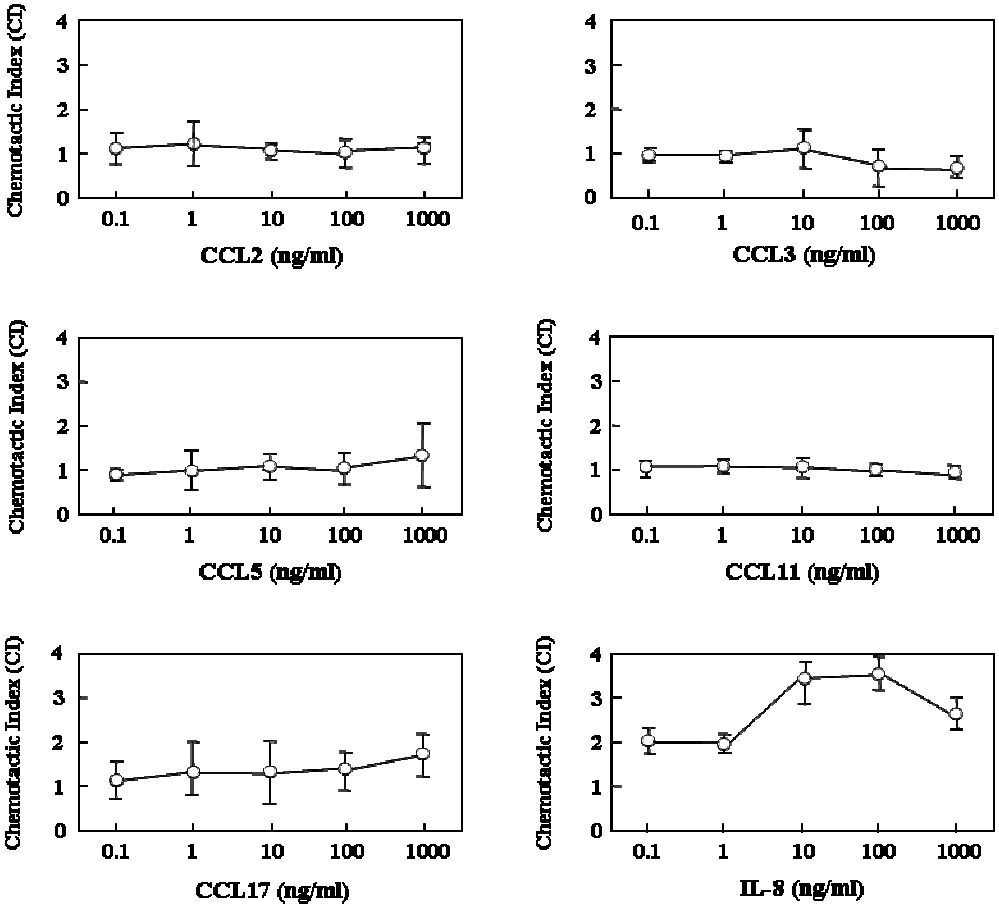

Figure 3. The chemotactic effect of CC chemokines on neutrophils. Neutrophils from peripheral blood of normal subjects (n=3) were incubated with CCL2, CCL3, CCL5, CCL17 and IL-8 in the indicated concentrations and chemotactic activity was analyzed using a chemotaxis assay.

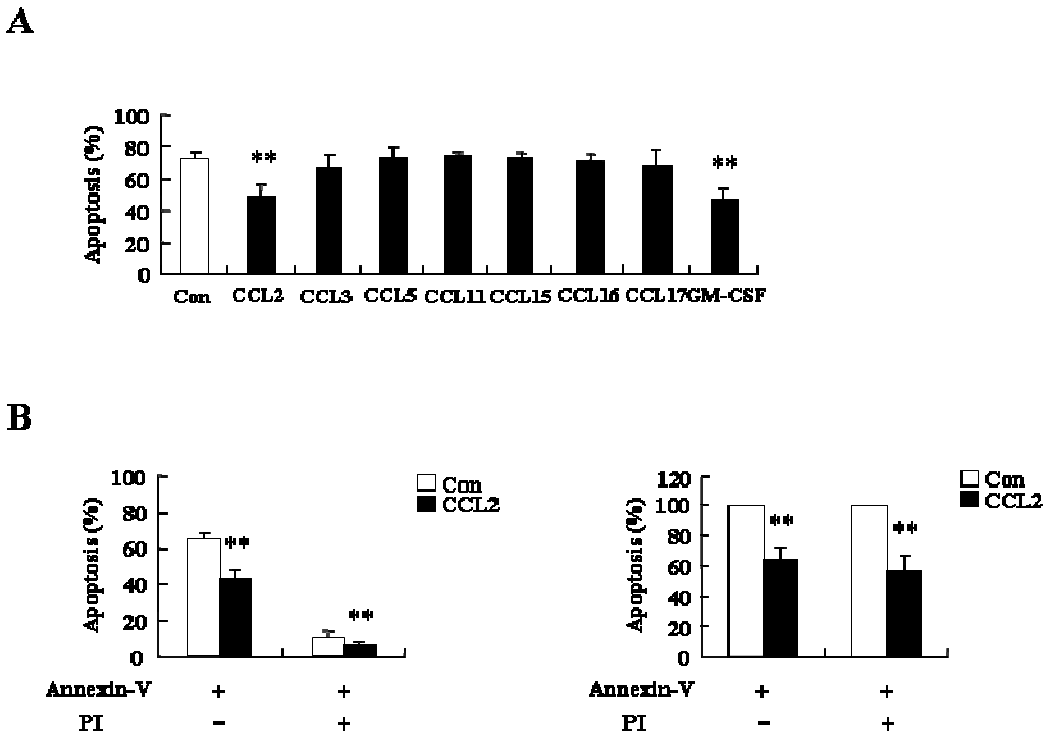

Figure 4. **CCL2 delays constitutive apoptosis of peripheral blood neutrophils.** (A) Neutrophils were isolated from peripheral blood of normal subjects (at least n=18). The cells were incubated for 24 h in the absence (Con) and presence of CCL2, CCL3, CCL5, CCL11, CCL15 (100 ng/mL), CCL16, CCL17 (1000 ng/ml) or GM-CSF (100 ng/ml). Apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. (B) CCL2-treated group in normal subjects (n=22) was divided into the early stage apoptosis (annnexin+, PI-) and the late stage apoptosis (annexin+, PI+). Data are presented as absolute apoptotic degree (left panel) and relation apoptosis compared to the control, which was set at 100% (right panel). Data are presented in relation to the control, which was set at 100%. Data are

expressed as the means ± S.E.M. $^{**}p < 0.01$ indicates a highly significant difference between the control and CCL2-treated groups.

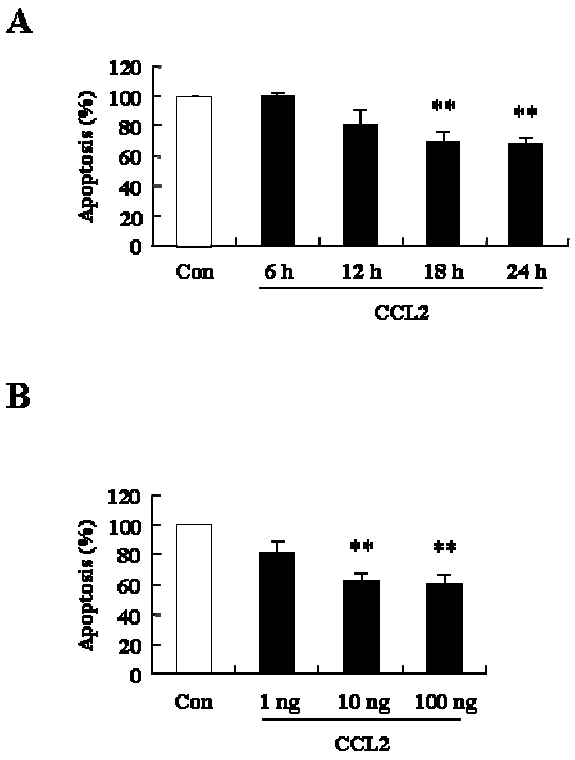

Figure 5. CCL2 delays constitutive apoptosis of neutrophils in a time- and dose-dependent manner. Normal blood neutrophils were incubated for the indicated time with or without CCL2 (100 ng/ml) (n=6) (A) or for 24 h in the presence and absence of CCL2 in the indicated concentration (n=4). (B). Neutrophil apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. Data are presented in relation to the control, which was set at 100%. Data are expressed as the means ± S.E.M. $^{**}p < 0.01$ indicates a highly significant difference between the control and CCL2-treated groups.

CCL2 induces anti-apoptotic effects through CCR2 in neutrophils

To determine the functional activity of CCR2, the cytosolic calcium influx was evaluated. CCL2 led to a transient increase of the cytosolic Ca^{2+} concentration in neutrophils (Fig. 6). Furthermore, RS102895, a CCR2 antagonist, and anti-CCR2 blocking antibody recovered the constitutive apoptosis reduced by CCL2 (Fig. 7). These results indicate that CCL2 induces the alteration of calcium concentration in cytoplasm and anti-apoptosis through CCR2, but no cell migration in neutrophils (Fig. 3).

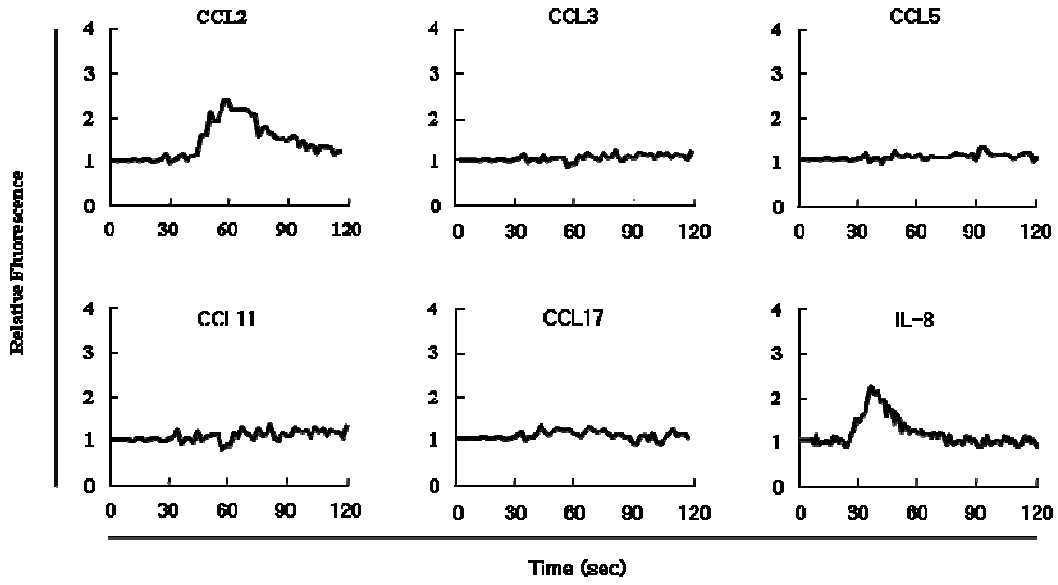

Figure 6. CCL2 induces Ca^{2+} influx into the cytoplasm of neutrophils. The alteration of cytosolic Ca^{2+} concentration was measured in neutrophils from peripheral blood of normal (n=3) using 1 μM fluo-3-AM ester, the Ca^{2+} indicator.

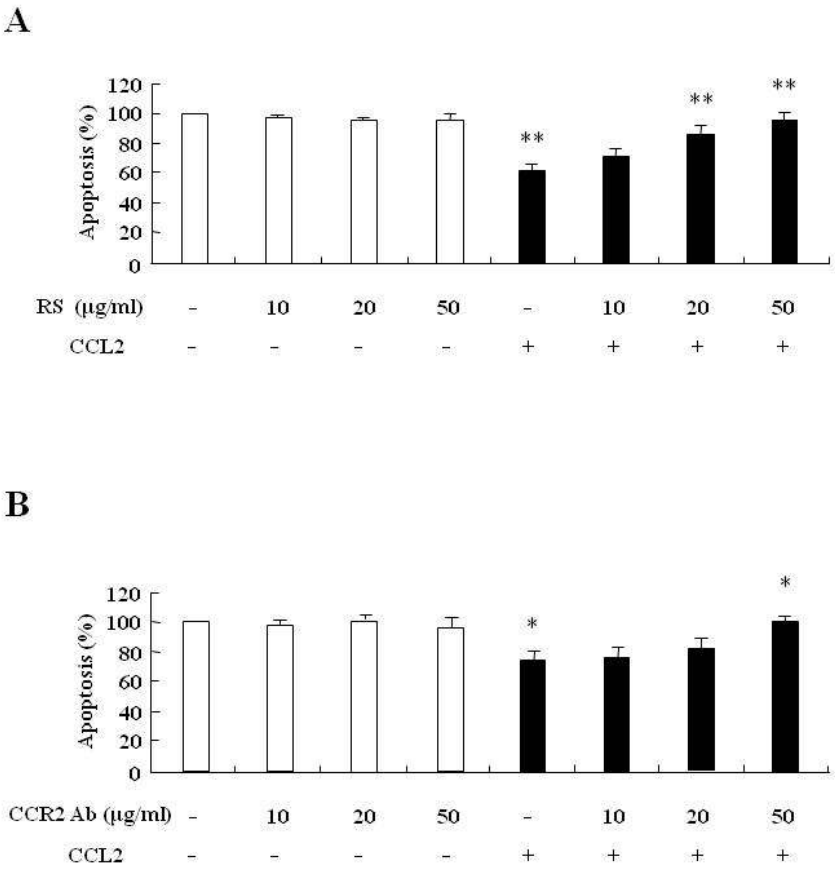

Figure 7. CCL2 induces anti-apoptotic effect in neutrophils through CCR2. Normal blood neutrophils were pretreated with RS102895 (RS) (A) or anti-CCR2 antibody (CCR2 Ab) (B) at the indicated concentrations (n=7). The cells were incubated for 24 h in the presence and absence of CCL2 (100 ng/ml). Apoptosis was then analyzed by measuring the binding of annexin V-FITC and PI. Data are expressed as the means ± S.E.M. and are presented in relation to the negative control, which was set at 100%. $^{**}p < 0.01$ indicates a highly significant difference between the control and the CCL2-treated groups or between the CCL2-treated and the RS102895-treated group.

CCL2 induces the activation of PI3K, Akt, ERK and NF-κB for inhibition of neutrophil apoptosis

To evaluate the role of intracellular signaling molecules in the apoptosis delay induced by CCL2, normal neutrophils were pre-treated with specific inhibitors for PI3K, ERK and NF-κB for 1 h and subsequently treated them with CCL2. Ly294002 (an inhibitor of PI3K), AKTi (an inhibitor of Akt), PD98059 (an inhibitor of MEK), and BAY 11-7085 (an inhibitor of NF-κB) blocked the suppressive effect on neutrophil apoptosis induced by CCL2 in a concentration-dependent manner (Fig. 8A and B).

To confirm the activation of signal molecules in the presence of CCL2, the alteration of phosphorylation of the associated signal proteins was investigated. As shown in Figure 9A and B, CCL2 induced the activation of Akt in a time-dependent manner. The phosphorylated Akt induced by CCL2 was 17.5 ng per 1×10^6 cells of neutrophils. CCL2 also induced the activation of ERK in a time-dependent manner (Fig. 10) and the ERK activation was inhibited by Ly294002 and AKTi (Fig. 11). CCL2 induced Iκ-Bα degradation mediated by phosphorylation of Iκ-Bα in a time-dependent fashion (Fig. 12). NF-κB activity was increased by CCL2 in a time-dependent manner (Fig. 13A), and the activity was blocked by treatment with Ly294002 and AKTi and PD98059 (Fig. 13B). These data indicate that the apoptosis delay of neutrophils following CCL2 treatment requires the PI3K/Akt/ERK/NF-κB pathway.

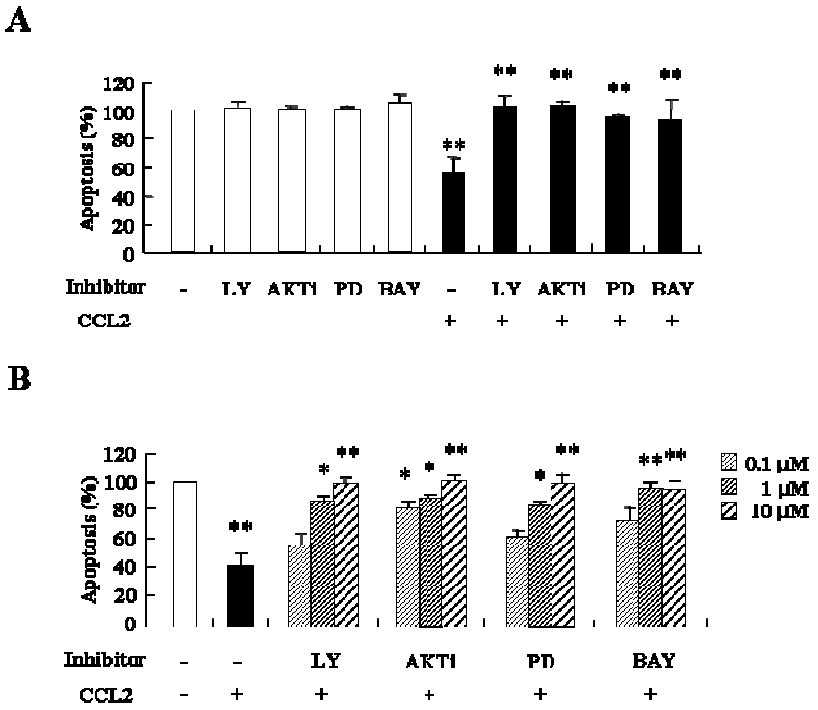

Figure 8. The anti-apoptotic effect of CCL2 is suppressed by specific inhibitors against PI3K, Akt, ERK, and NF-κB. Normal blood neutrophils were pre-treated for 1 h with and without 10 μM LY294002 (LY), 10 μM Akt inhibitor (AKTi), 10 μM PD98059 (PD), and 10 μM BAY-11-7085 (BAY) (n=6) (A), or with and without the indicated concentration of LY294002, AKTi, PD98059, and BAY-11-7085 (n=6) (B). The cells were incubated for 24 h in the presence or absence of CCL2 (100 ng/ml). Apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. Data are expressed as the means ± S.E.M. *$p < 0.05$ and **$p < 0.01$ indicate a significant and a highly significant difference between the control and the CCL2-treated group, between the CCL2-treated and the inhibitor-treated group or between the media group and the supernatant-treated group.

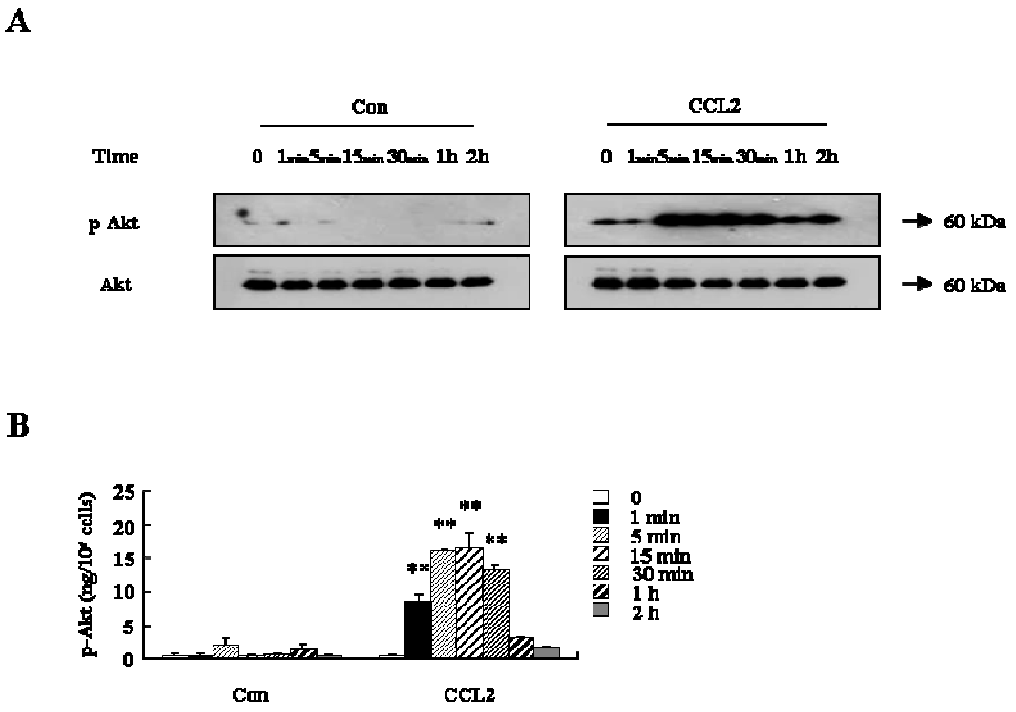

Figure 9. CCL2 requires the activation of PI3K/Akt for the inhibition of neutrophil apoptosis. Normal blood neutrophils were incubated with 100 ng/ml of CCL2 for the indicated time. Akt phosphorylation in the lysates was detected by Western blotting (A) and by using human phospho-Akt ELISA kit (B). $^{**}p < 0.01$ indicates a significant difference between the control and the CCL2-treated group.

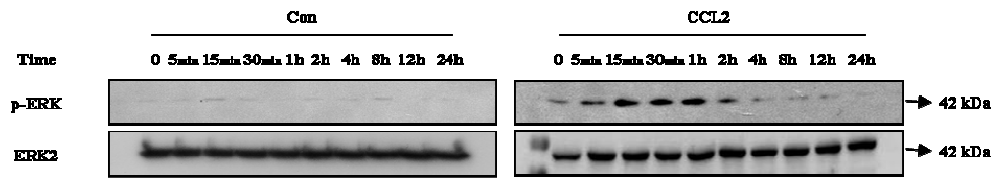

Figure 10. CCL2 induces ERK activation for the inhibition of neutrophil apoptosis. Normal blood neutrophils were incubated with 100 ng/ml of CCL2 for the indicated time. Phospho-ERK (p-ERK) in the lysates was detected by Western blotting.

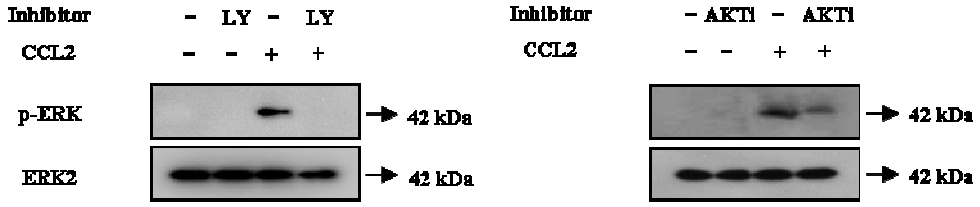

Figure 11. CCL2 inhibits neutrophil apoptosis through PI3K/Akt/ERK cascade for the inhibition of neutrophil apoptosis. Normal blood neutrophils were incubated with 100 ng/ml of CCL2 for the indicated time. Phospho-ERK (p-ERK) in the lysates was detected by Western blotting. Normal blood neutrophils were incubated with 100 ng/ml of CCL2 for 1 h after pretreatment with 10 μM LY294002 (LY) (left panel) or 10 μM Akt inhibitor (AKTi) (right panel). Harvested cells were lysed, and the phospho-ERK (p-ERK) in the lysates was detected by Western blotting.

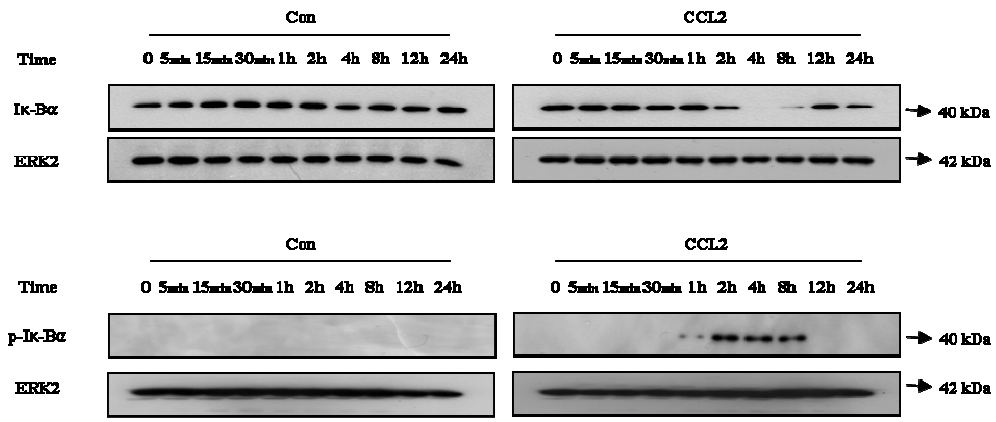

Figure 12. CCL2 induces the phosphorylation of Iκ-Bα for the inhibition of neutrophil apoptosis. Normal blood neutrophils were incubated with 100 ng/mL of CCL2 for the indicated time (n=4). The alteration of expression level of Iκ-Bα (upper panel) and phosphorylation of Iκ-Bα (lower panel) were detected by Western blotting.

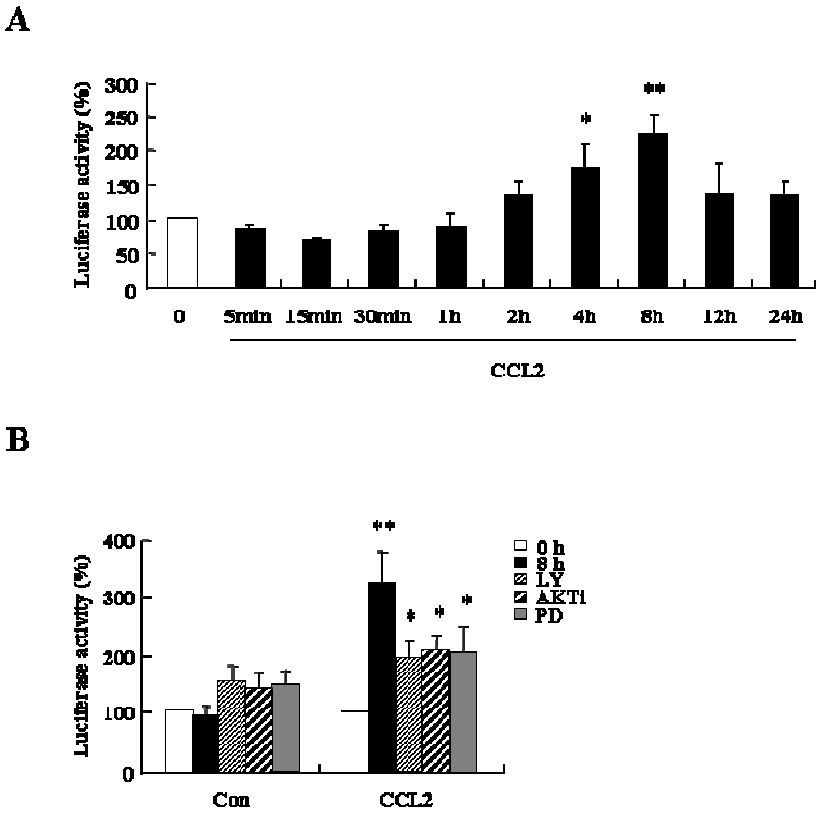

Figure 13. CCL2 requires the activation of NF-κB for the inhibition of neutrophil apoptosis. Normal blood neutrophils were incubated with 100 ng/mL of CCL2 for the indicated time (n=4) (A) or with 100 ng/ml of CCL2 for 8 h after pre-treatment with 10 μM LY294002 (LY), 10 μM Akt inhibitor (AKTi), or 10 μM PD98059 (PD) (n=3) (B). The nuclear fraction was extracted, and the NF-κB DNA binding activity was assessed using an EZ-DetectTM transcription factor kit. Data are expressed as the means ± S.E.M. and are presented in relation to the control, which was set at 100%. *$p < 0.05$ and **$p < 0.01$ indicates a significant

and a highly significant difference between the control and CCL2-treated group, or between CCL2-treated group and the inhibitor-treated group.

Inhibition of normal neutrophil apoptosis by CCL2 is associated with molecules released by CCL2

The release of cytokines from neutrophils was examined because their cytokine secretion by extracellular survival factors is an important step in the regulation of constitutive apoptosis (Kennedy and DeLeo, 2009). The expression of IL-6, IL-8, and GM-CSF weakly increased after CCL2 treatment to neutrophils (Fig. 14A). Following the exposure of neutrophils to CCL2, the collected cell-free supernatants were added to fresh neutrophils. The supernatant from the CCL2-treated neutrophils led to a significant decrease in the apoptosis of fresh neutrophils (p <0.01) (Fig. 14B). These results indicate that unknown secretion factors play a role in the regulation of neutrophil apoptosis via CCL2.

CCL2 delays the decrease of procaspase 3, procaspase 9, and Mcl-1 expression during neurophil apoptosis

Both caspase 3 and caspase 9, which are important molecules in constitutive apoptosis, were cleaved after the constitutive apoptosis began, and the cleaved proteins act as an active molecules. The levels of procaspase 3 and procaspase 9 decreased after beginning constitutive apoptosis, indicating that cleavage of both procaspases occurred. However, CCL2 inhibited the cleavage of the procaspase forms (Fig. 15A) and delayed the decrease of Mcl-1 expression (Fig. 15B).

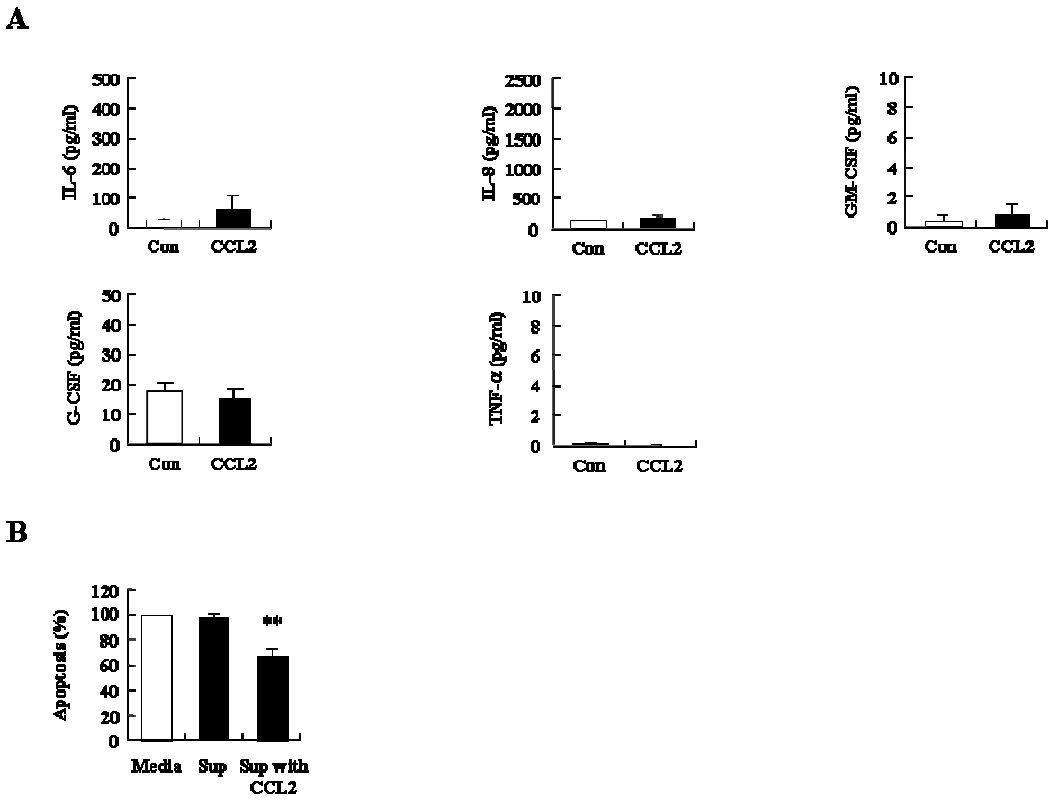

Figure 14. Enhanced cytokine release is involved in CCL2-induced apoptosis delay. (A) Neutrophils from peripheral blood of normal (n=10) were incubated with 100 ng/ml of CCL2 for 24 h. The supernatant was collected and analyzed by ELISA. Data are expressed as the means ± S.E.M. (B) Neutrophils were incubated with or without 100 ng/ml of CCL2 for 24 h. The supernatant (Sup) was collected and added to the fresh neutrophils obtained from the peripheral blood of normal subjects (n=6). Apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. Data are expressed as the means ± S.E.M. and are presented in relation to the media, which was set at 100%. $^{**}p < 0.01$ indicates a highly significant difference between the control and the supernatant-treated group.

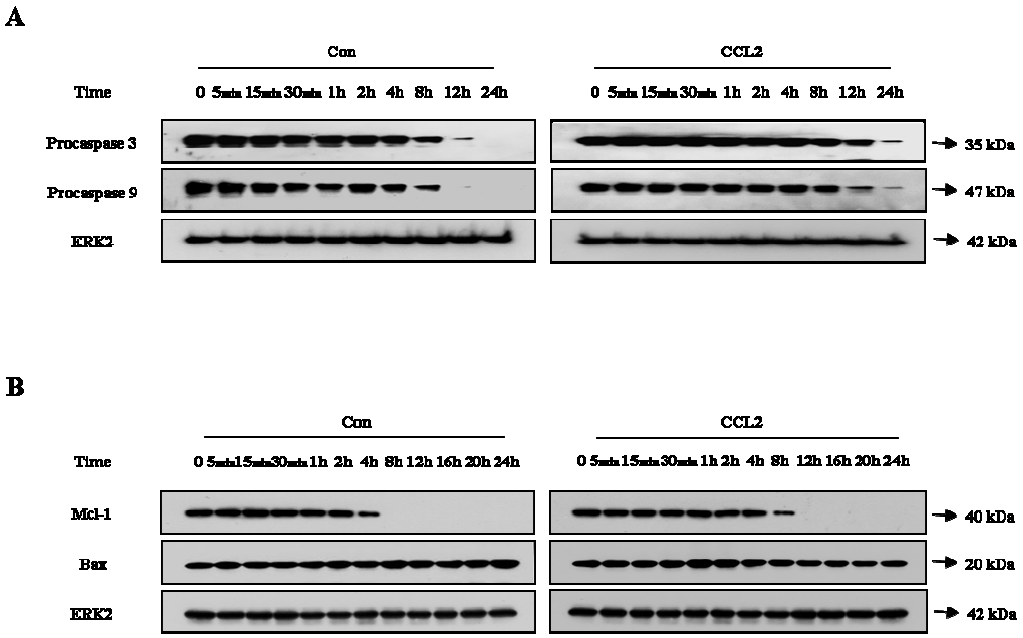

Figure 15. CCL2 inhibits the cleavage of caspase 3 and caspase 9 and delayed Mcl-1 degradation. Normal blood neutrophils were incubated with 100 ng/ml of CCL2 for the indicated time. Procaspase 3 and procaspase 9 (A) or Mcl-1 and Bax proteins (B) were detected by Western blotting. The membrane was stripped and reprobed with anti-ERK2 antibodies as an internal control.

CCL2 delays constitutive neutrophil apoptosis in normal subjects, but not in asthmatic patients

To confirm the anti-apoptotic effect of CCL2 on asthma pathogenesis, effects of CCL2 on normal neutrophil apoptosis were compared to these effects of CCL2 on apoptosis of neutrophils from asthmatic patients. First of all, it was confirmed that asthmatic neutrophils express the similar level of CCR subtypes to normal neutrophils. The protein levels of CCR subtype were different between the normal neutrophils and asthmatic neutrophils (Fig. 16). In addition, CCL2 had no effect on the constitutive apoptosis and cytoplasmic Ca^{2+} influx of asthmatic neutrophils (Fig. 17). Other CC chemokines binding to CCR1 through CCR5 such as CCL3, CCL5, CCL11, CCL15, CCL16 and CCL17 also had no effect on the neutrophil apoptosis of the asthmatic groups (Fig. 17A). These results indicate that only CCL2 has the different apoptotic regulation between normal neutrophils and asthmatic neutrophils.

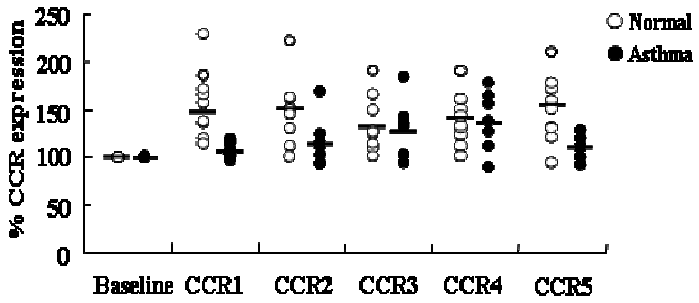

Figure 16. Surface protein expression of CCRs is different between normal and asthmatic neutrophils. (A) Neutrophils were isolated from peripheral blood of normal (n=9) and asthmatic patients (n=7), and the expression of CCR subtypes on the cell surface was analyzed by flow cytometry. An isotype control (Baseline) was analyzed with control mouse IgG. Data are presented in relation to the isotype control, which was set at 100%.

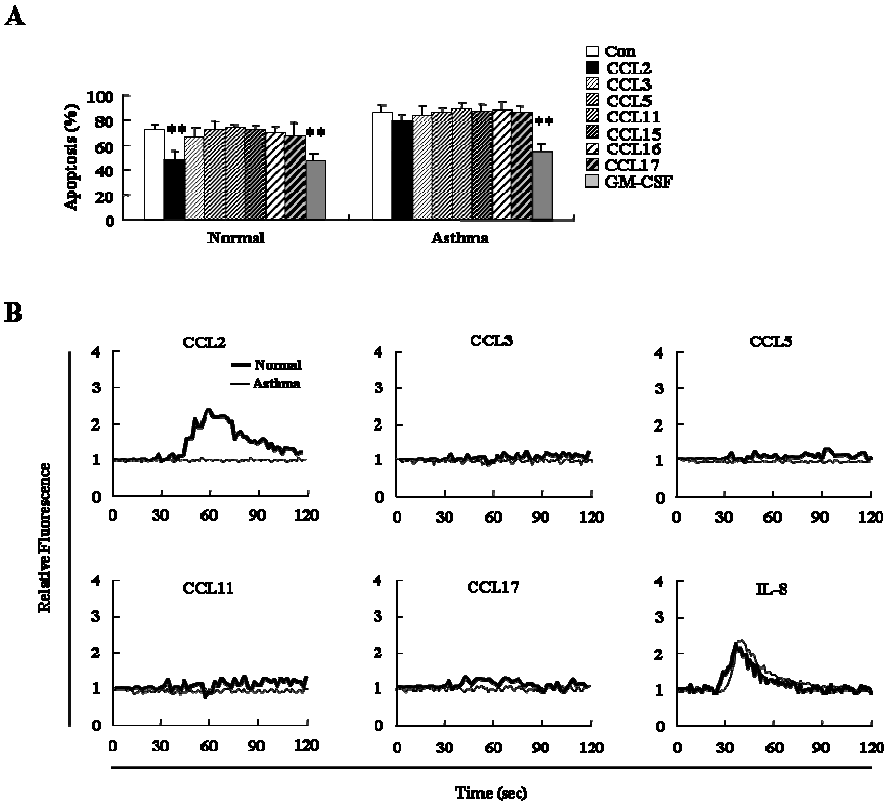

Figure 17. CCL2 induces the different effect on apoptosis and cytoplasmic Ca^{2+} influx of normal and asthmatic neutrophils. (A) Neutrophils were isolated from peripheral blood of normal (at least n=18) and asthmatic patients (n=13). The cells were incubated for 24 h in the absence (Con) and presence of CCL2, CCL3, CCL5, CCL11, CCL15 (100 ng/mL), CCL16, CCL17 (1000 ng/ml) or GM-CSF (100 ng/ml). Apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. Data are presented in relation to the control, which was set at 100%. Data are expressed as the means ± S.E.M. $^{**}p < 0.01$ indicates a highly significant difference between the control and CCL2-treated groups. (B) The alteration of cytosolic Ca^{2+} concentration was also measured in neutrophils

from peripheral blood of normal subjects (n=3) and asthmatic patients (n=3) using 1 μM fluo-3-AM ester, the Ca^{2+} indicator.

The role of NF-κB is different between normal and asthmatic neutrophils in regulation of constitutive apoptosis

Since CCL2 is not associated with its anti-apoptotic effect on asthmatic neutrophils in contrast to normal neutrophils, different functioning mechanism of CCL2 in normal and asthmatic neutrophils was further determined. The roles of PI3K, ERK and NF-κB of asthmatic neutrophils were investigated because these signal proteins were involved in the CCL2-associated signal of normal neutrophils (Fig. 8). BAY-11-7085 unexpectedly inhibited the constitutive apoptosis of asthmatic neutrophils, but not that of normal neutrophils (Fig. 18). However, the NF-κB activity of asthmatic neutrophils did not differ from that of normal neutrophils (Fig. 19). These results led to investigate whether NF-κB inhibition is involved in cytokine secretion. In fact, inhibition of NF-κB led to a significant increase in IL-8 secretion, but weak or no effect on the release of CCL2, IL-6, GM-CSF, and TNF-α (Fig. 20). The culture supernatants from normal and asthmatic neutrophils after NF-κB inhibitor treatment had no effect on neutrophil apoptosis (Fig. 21). Because NF-κB inhibitor blocked the degradation of procaspase 3 and procaspase 9, NF-κB might be associated with the basal pro-apoptotic pathway in asthmatic neutrophils (Fig. 22).

Neutrophils are usually increased in the peripheral blood of severe asthmatic patients. The anti-apoptotic effect of BAY 11-7085 was determined on neutrophils of asthma-classified with the level of total IgE or with GINA grade. As shown in

Fig. 23, the anti-apoptotic effect of BAY 11-7085 on neutrophils of asthmatic patients with a high level of IgE was stronger than that on neutrophils of asthmatic patients with a low level of IgE. Also, the anti-apoptotic effect of BAY 11-7985 on neutrophils of severe asthma (GINA 4 grade asthma) was stronger than that on neutrophils of mild or moderate asthma (Fig. 24).

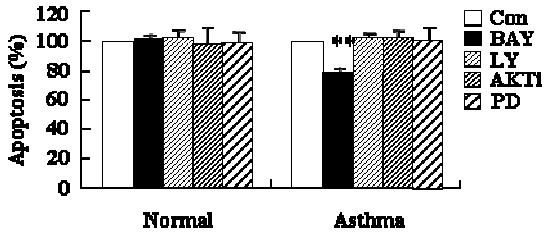

Figure 18. BAY 11-7085 delays constitutive apoptosis of neutrophils from asthmatic patients. Normal neutrophils and asthmatic neutrophils were treated without (Con) and with 20 μM LY294002 (LY) (n=8), 20 μM PD98059 (PD) (n=8), and 10 μM BAY-11-7085 (BAY) (n=34). Apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. Data are are presented in relation to the negative control, which was set at 100%. $^{**}p < 0.01$ indicates a highly significant difference between the control and inhibitor-treated group.

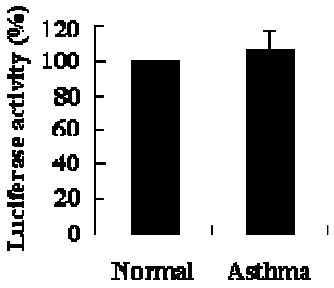

Figure 19. Different basal levels of NF-κB between normal and asthmatic neutrophils. Neutrophils were collected from normal subjects and asthmatic patients (n=5), and the nuclear fraction was extracted from the cells. The DNA binding activity of NF-κB was assessed using an EZ-DetectTM kit. Data are presented in relation to the normal group, which was set at 100%.

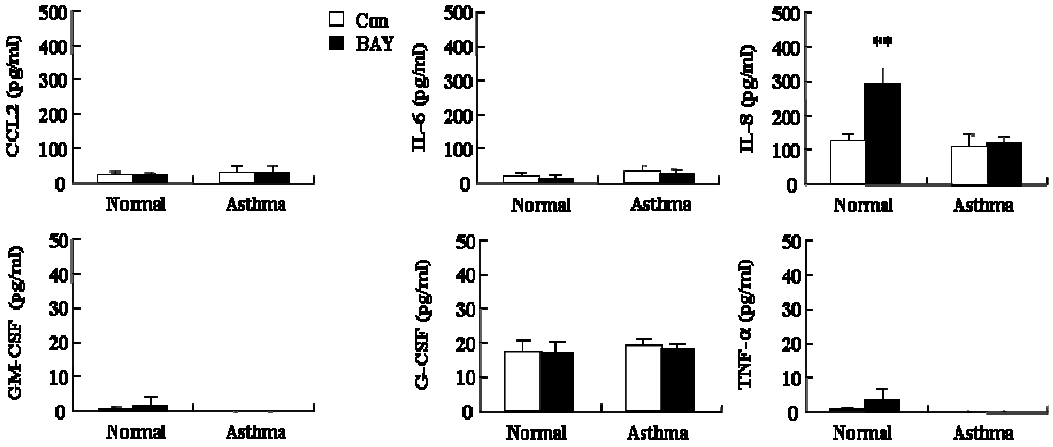

Figure 20. **Cytokine release mediated by BAY 11-7085 in normal and asthmatic neutrophils.** Neutrophils from the normal subjects (n=13) and asthmatic patients (n=21) were incubated with 10 μM BAY-11-7085 (BAY). The supernatant was collected and analyzed by ELISA. $^{**}p < 0.01$ indicates a highly significant difference between the control group and BAY-11-7085-treated group.

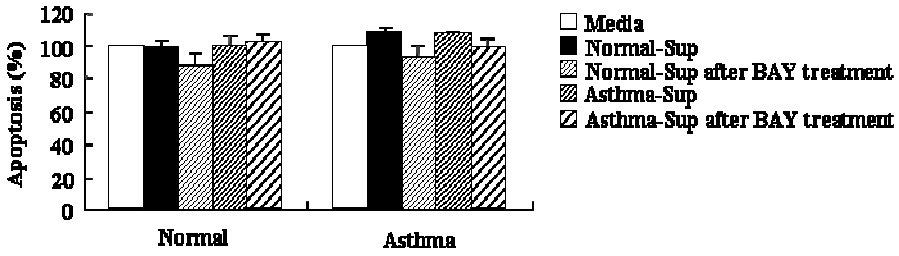

Figure 21. Increased cytokine release is not involved in BAY 11-7085-induced apoptosis delay. Neutrophils from the peripheral blood of normal subjects and asthmatics were incubated with 10 μM BAY-11-7085 for 24 h. The supernatant was collected and added to the fresh neutrophils obtained from the peripheral blood of normal and asthmatic patients (n=3). Apoptosis was analyzed by measuring binding of annexin V-FITC and PI. Data are presented in relation to the negative control, which was set at 100%.

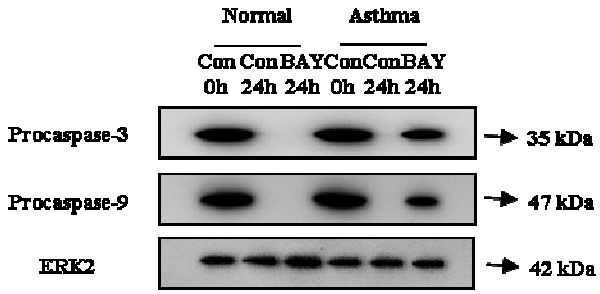

Figure 22. BAY 11-7085 inhibits the cleavage of caspase 3 and caspase 9. Normal neutrophils and asthmatic neutrophils were incubated with 10 μM of BAY 11-7085 for the indicated time. Procaspase 3 and procaspase 9 were detected by Western blotting. The membrane was stripped and reprobed with anti-ERK2 antibodies as an internal control.

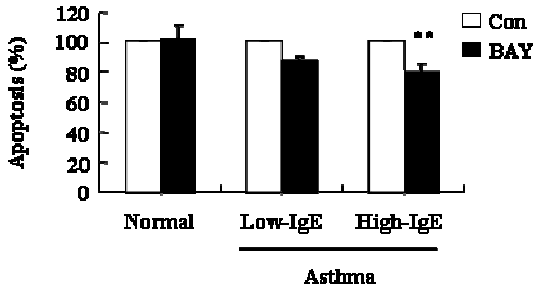

Figure 23. Comparison of anti-apoptotic effect induced by BAY 11-7085 in asthmatic patients classified by the level of IgE. Neutrophils of normal subjects (n=15) and neutrophils of asthma patients with a low level (n=16) or a high level (n=18) of total IgE were treated without (Con) and with 10 μM BAY-11-7085 (BAY). Apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. Data are are presented in relation to the negative control, which was set at 100%. $^{**}p < 0.01$ indicates a highly significant difference between the control and BAY-treated group.

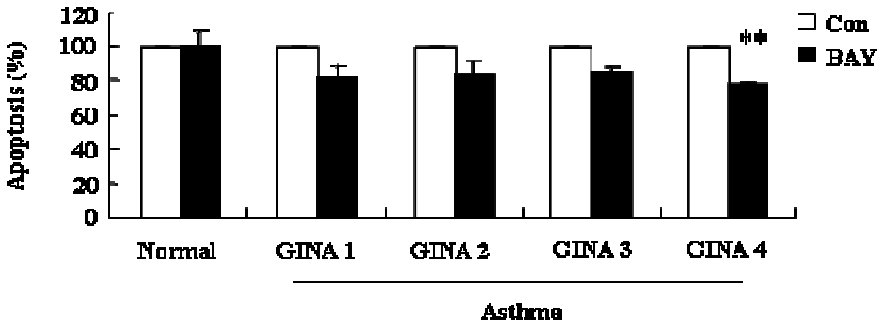

Figure 24. Comparison of anti-apoptotic effect induced by BAY 11-7085 among asthmatic patients divided by GINA grades. Neutrophils of normal subjects (n=15) and neutrophils of asthma patients of GINA 1 (n=8), GINA 2 (n=10), GINA 3 (n=9), and GINA 4 (n=7) were treated without (Con) and with 10 μM BAY-11-7085 (BAY). Apoptosis was analyzed by measuring the binding of annexin V-FITC and PI. Data are presented in relation to the negative control, which was set at 100%. $^{**}p < 0.01$ indicates a highly significant difference between the control and BAY-treated group.

4. Discussion

Asthma is an inflammatory disease of the airways characterized by infiltration of various inflammatory cells, reversible airway obstruction and bronchial hyperresponsiveness (Busse and Rosenwasser, 2003). As the prevalence of asthma increases around the world, investigation of a novel mechanism in asthma pathogenesis, especially severe asthma, and development of an effective therapeutic drug for asthma are needed. In the present study, the novel mechanism of neutrophil associated with pathogenesis of severe asthma was investigated and demonstrated that CCL2 induces a delay in neutrophil apoptosis through CCR2 in normal neutrophils, despite weak CCR2 expression. Anti-apoptotic signaling due to CCL2 also involves the activation of PI3K, ERK and NF-κB, and is associated with the inhibition of procaspase 3 and procaspase 9 cleavages, as well as with the delay of Mcl-1 degradation. Constitutive apoptosis of asthmatic neutrophils was not affected by CCL2. NF-κB functions as a proapoptotic factor in asthmatic neutrophils when compared with normal neutrophils. This difference between normal and asthmatic neutrophils may be caused by different apoptotic or anti-apoptotic pathways and the associated molecules.

Although it has been reported that CCR2 expression is not detected in resting neutrophils, the role of CCL2 in neutrophils is still controversial due to the results of previous studies conducted to evaluate the association of CCR2 and neutrophils. Both CCR2 knockout mice and wild type mice showed the

suppression of neutrophil infiltration into the lung in response to CCL2 and LPS after treatment with CCR2 blocking antibodies (Maus et al., 2002). CCR2 in chronic inflammation and sepsis could also induce neutrophil migration in response to CCL2 (Johnston et al., 1999; Speyer et al., 2004). Balamayooran et al. recently reported that CCL2 induces neutrophil migration after LPS treatment and *E. coli* infection in mice (Balamayooran et al., 2011). Although these reports indicate that CCL2 plays an important role in neutrophils, there is no report that CCL2 directly stimulates the function of neutrophils in the peripheral blood of normal subjects. As shown in Figure 1, 2, and 16, CCR2 is weakly expressed in normal and asthmatic neutrophils. Based on the results of a previous report on the strong interaction of CCL2 with CCR2 (Kim et al., 2004), the interaction between two molecules in neutrophils was investigated. CCL2 was found to induce the elevation of cytosolic Ca^{2+} concentration through CCR2, but not chemotactic activity (Fig. 3 and 7), and spontaneous apoptosis was inhibited by CCL2 in a time and dose-dependent manner (Fig. 4 and 5). Therefore, it can be suggested that CCL2 triggers an anti-apoptotic effect through CCR2 in resting normal neutrophils and has a greater influence on apoptosis delay. However, CCL2 has no effect on cell migration.

Constitutive apoptosis is a critical process involved in regulating neutrophil function and the associated inflammation. Survival factors such as GM-CSF, G-CSF, IL-8 and TNF-α transduce the anti-apoptotic signals through a variety of signaling pathways that include the PI3K pathway, the MAPK pathway and the

PKC pathway, and the regulation of caspase and Bcl-2 family proteins (Webb et al., 2000; Simon, 2003; Duan et al., 2004; Luo and Loison, 2008). The experiment results revealed that pretreatment with Ly294002, AKTi, PD98059 and BAY 11-7085 recovered the constitutive apoptosis of CCL2-treated normal neutrophils to the apoptotic level of untreated cells (Fig. 8). Therefore, the activation of inhibitor-associated proteins was confirmed. CCL2 induced NF-κB activation through the PI3K/Akt/ERK pathway (Fig. 8-13). In addition, CCL2 regulates key proteins involved in an intrinsic pathway of neutrophil apoptosis (Fig. 14B). Both procaspase 3 and procaspase 9 cleavage are delayed after CCL2 stimulation in a time-dependent manner. Degradation of the anti-apoptotic protein, Mcl-1, was also inhibited by CCL2, while the proapoptotic protein, Bax, was not altered by CCL2. Overall, the novel approach taken in this study provided better insight into the CCL2-associated anti-apoptotic mechanism in neutrophils.

The cytokine secretion induced by survival stimulators in neutrophils inhibits apoptosis when the released cytokine functions as an autoregulator (Cowburn et al., 2004; Ocaña et al., 2008). The results of the present study indicate that the measured cytokine cannot induce anti-apoptotic processes even though CCL2 increased the release of IL-6, IL-8 and GM-CSF in normal neutrophils (Fig. 14A). It was also found that the levels of the measured cytokines were too low to induce an anti-apoptotic effect alone or cooperatively. Although IL-8 is known as a direct survival factor in neutrophils based on previous reports, IL-8 was not induced from normal neutrophils after CCL2 treatment (Fig. 14A)

and a previous report (Yang et al., 2011). In this study, IL-8 had no anti-apoptotic effect on CCL2-induced apoptosis delay of neutrophils. Next, the effects of other unmeasured factors on apoptosis was evaluated. Apoptosis of the fresh normal neutrophils was inhibited by incubation with the culture supernatants from the normal neutrophils after CCL2 treatment. However, the apoptosis was not affected by treatment with the culture supernatants from the asthmatic neutrophils after CCL2 treatment, even though CCL2 led to an increase in IL-8 secretion in asthmatic neutrophils. Taken together, these results indicate that the anti-apoptotic effect induced by CCL2 is dependent on the increased factor release and that the underlying mechanism involves the activation of PI3K, Akt, ERK, and NF-κB, However, the exact factor involved in this process was not identified in this study. Accordingly, further studies are needed to examine more complex mechanisms involved in this process.

Asthma is associated with CC chemokines including CCL2 (Borish and Steinke, 2003). Neutrophils have long been considered as an important factor in the pathogenesis or severity of asthma (Douwes et al., 2002; Louis and Djukanovic, 2006). As shown in Table 3, both blood and bronchoalveolar lavage fluid (BALF) neutrophils in asthma were significantly elevated when compared with normal neutrophils, and the increase of neutrophils in BALF was related to the severity of asthma. CCL2 induces calcium influx in normal neutrophils when compared to asthmatic neutrophils. In contrast to the anti-apoptotic effect of CCL2 in normal neutrophils, neutrophil apoptosis of asthmatic patients was not altered

by CCL2 stimulation (Fig. 17A). These results led to determine if the effect of CCL2 on constitutive neutrophil apoptosis differed between normal subjects and asthmatics. First, CCL2-induced apoptosis was evaluated to determine if it required alteration of CCR2 expression or increased CCR2 expression in this study. In this study, a little difference in CCR2 expression between normal and asthmatic neutrophils was found (Fig. 16). Infiltrated neutrophils in BALF increased according to the asthma severity, with the greatest levels being observed in subjects with severe asthma (GINA 4). However, the effect of CCL2 and other CC chemokines on constitutive apoptosis was not dependent on asthma subtype based on GINA classification (GINA1: n=9, GINA2: n=18, GINA: n=12, GINA: n=4) (data not shown). Asthmatic neutrophils were evaluated to determine if they differed from normal neutrophils based on intracellular signaling and protein expression. Basal apoptosis of asthmatic neutrophils was not affected by LY294002 and PD98059, but by BAY-11-7085 (Fig. 18). NF-κB can be considered as a pro-apoptotic protein in asthmatic neutrophils in contrast to the role of NF-κB in normal neutrophils in this study. In other reports, NF-κB has been known to be an anti-apoptotic or pro-apoptotic signaling protein in normal neutrophils (Choi et al., 2003; Edwards et al., 2009; Langereis et al., 2010). The anti-apoptotic effect of NF-κB inhibitor was not found to be associated with secreted molecules, but was associated with the inhibition of caspase 3 and caspase 9 activity (Fig. 22). A further study is now undertaken to investigate more

detailed mechanism, associated with the different effects of CCL2 between normal subjects and asthma patients.

In summary, CCL2 delays the constitutive apoptosis of normal neutrophils and the CCL2-mediated signaling is involved in the PI3K/Akt/ERK/NF-κB cascade in normal neutrophils (Fig. 25). In contrast to normal neutrophils, apoptosis of asthmatic neutrophils is not affected by CCL2 and the constitutive apoptosis is supported by basal NF-κB activation. NF-κB activation in neutrophils of asthmatic patients induces activation of caspase9/caspase3, subsequently resulting in apoptosis (Fig. 25). The results of this study may provide important information for the role of CCL2 in different regulation of constitutive neutrophil apoptosis between normal subjects and asthmatics.

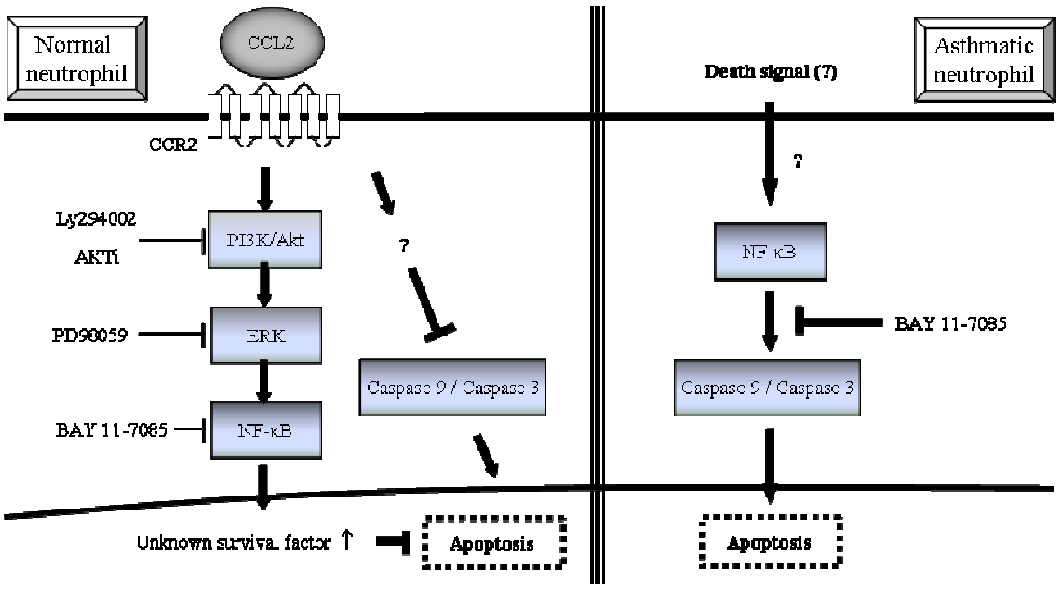

Figure 25. The proposed signaling pathway of survival or apoptosis in normal and asthmatic neutrophils. Anti-apoptotic signaling via CCL2 involves the activation of PI3K, Akt, ERK and NF-κB, and it is associated with inhibition of procaspase 3 and procaspase 9 cleavages. In contrast to normal neutrophils, apoptosis of asthmatic neutrophils is delayed by the inhibition of NF-κB.

Conclusions

Neutrophils of blood and BAL fluid increased in asthmatic patients of GINA 4 grade. In the present study, the novel mechanism of neutrophil associated with pathogenesis of severe asthma was investigated. CCL2 delays the constitutive apoptosis of normal neutrophils and the CCL2-induced signal is involved in the PI3K/Akt/ERK/NF-κB cascade in normal neutrophils. NF-κB functions as an anti-apoptotic molecule in normal neutrophils and pro-apoptotic molecule in severe asthmatic neutrophils. On the other hand, the inhibition of NF-κB blocks the constitutive apoptosis of neutrophils from patients with severe asthma. In conclusion, neutrophils have different signaling activation patterns between normal subjects and asthma patients.

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국문요약

천식질환의 발병에 관여하는 호중구의 세포고사 기전

천식은 매년 발병률이 급증하고 있는 기도에서 발생하는 만성 염증질환으로, 많은 수의 염증세포가 기관이나 기관지로 이동하고 침윤한 세포는 과도한 면역반응의 활성을 유발하여 기도 과민반응 및 점액분비의 증가로 기침, 천명, 호흡곤란 등의 천식증상을 일으킨다. 천식질환에 관여하는 세포는 Th2 세포, 비만세포, 호산구, 대식세포가 대표적이고, 최근 호중구성 천식질환의 심각성에 대한 인식도 증가하고 있다. 천식 질환 중, 중증의 증상을 동반하는 단계인 GINA 4의 천식환자에서 호중구의 증가가 관찰되고 관련 염증인자들의 작용에 대한 연구들이 보고되고 있으나 정확한 발병기전을 파악하기에 여전히 부족한 실정이다.

이에 본 연구에서는 다양한 염증인자들에 대한 호중구의 작용기전을 파악하고자 정상인에서 호중구를 분리하여 실험에 이용하고자 하였다. 그 결과, CCL2가 CCR2에 결합하여 세포 내 Ca^{2+} 유입을 증가시킨 다음 호중구의 세포고사가 억제됨을 관찰하였다. CCL2의 억제효과는 세포 내 신호전달 과정에서 PI3K/Akt/ERK/NF-κB cascade를 통해 발생하여 결국 procaspase 3와 procaspase 9의 활성을 막고 MCL-1의 감소를 지연시켰다. 이러한 CCL2의 작용을 명확히 파악하기 위해 천식환자에서 분리한 호중구에서 CCL2의 작용을 확인하였다. 그러나 정상인의 호중구와는 다르게 CCL2가 아무런 작용을 하지 않는 반면, BAY 11-7085를 처리하여 NF-κB를 억제시켰더니 procaspase 3와 procaspase 9의 활성을 막아 세포고사가 억제되었다. 중증의 천식환자에서 분리한 호중구 일수록 BAY 11-7085의 세포고사 억제효과가 증가하였다. 이를 통해, 동일한 호중구임에도 불구하고 질병의 발병 정도에 따라 세포

내 신호전달 단백질들의 작용이 상이하게 나타날 수 있음을 알 수 있었다. 또한 본 연구를 통해 호중구에서 CCL2의 작용 및 강력한 전사인자인 NF-κB의 새로운 작용기전을 파악함으로써 천식의 발병기전의 이해를 도와 질병의 치료기전 개발에 기여하고자 한다.

핵심어: 천식, 호중구, CCL2, 세포고사, 항천식 효과