Regulation of Cellar Cytokine Production and NF-κB Pathway by Ginseng and Ginsenosides

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to quality for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

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Publications

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Summary of Thesis

Chronic obstructive pulmonary disease (COPD) is characterised by persistent inflammation in the airway and the lung which results in airway obstruction. Various inflammatory cells and mediators have been involved in the pathogenesis of COPD, in particular TNF-α through NF-κB signalling pathway. Current treatments for COPD are still not satisfactory and there is a need to develop new therapies targeting inflammatory mechanisms of COPD. Ginseng is a well-known medicinal herb and has been used in the treatment of COPD. Understanding the anti-inflammatory mechanism of ginseng and ginseng based formulas will facilitate the development of novel agents for treating COPD and other inflammatory diseases. The main objective of this project is to study the actions and mechanisms of ginseng and ginseng related products on the regulation of TNF-α and NF-κB pathway in inflammatory cell models related to COPD. The key hypothesis is that ginseng and ginsenosides may target TNF-α, cAMP and NF-κB signalling pathways to exert their anti-inflammatory actions.

In order to test this hypothesis, the inflammatory cell models were firstly set up using macrophage-like U937 cells induced by Lipopolysaccharide(LPS) or cigarette smoke extract(CSE), which then were used to evaluate the effects of ginseng and ginsenosides on cellular release of cytokines and activation of NF-κB and cAMP pathways. U937 cells treated with phorbol ester 12-O-tetradecanoylphorbol-13-acetate (PMA) were incubated with different concentrations of ginseng, ginseng formula extracts or ginsenosides for 2 hours, then treated with LPS (1 μg/ml) for different time durations to measure the level of cytokines in the cell media by ELISA, the expression of proteins by Western blot, the transcriptional activity by Dual-Glo Luciferase Reporter Assay System, the level of cAMP by EIA and the activity of PDE4 by scintillation counting. LPS caused a significant increase in the cellular release of TNF-α, IL-1β and IL-6 and the expression of the key NF-κB proteins (p-IKK, p-

IκBα and p-p65), as well as the transcriptional activity of NF-κB. The level of cAMP, PDE4 activity and p-CREB expression were also elevated by LPS. CSE also significantly induced the release of TNF-α. In LPS inducing cell model, BAY11-7082, the NF-κB pathway inhibitor, significantly inhibited the expression of IKK, p-IKK, IκBα, p-IκBα, p65 and p-p65,transcriptional activity of NF-κB and the release of TNF-α; the cAMP pathway activator, Foskolin, increased the production of cellular cAMP and the expression of p-CREB, but inhibited the release of TNF-α and the transcriptional activity of NF-κB, without affecting the expression of IKK, p-IKK, IκBα, p-IκBα, p65 and p-p65; the PDE4 inhibitor, Rolipram, also significantly inhibited the release of TNF-α . These results indicate that the cell model establishment was successful and also suggest a possible interaction between NF-κB and cAMP pathways on the regulation of TNF-α induced by LPS.

Using the established cell model, effects of ginseng (G115) and several ginseng formulas (GHMFs) as well as ginsenosides on the release of cytokines were investigated. It was found that the release of TNF-α was significantly inhibited by G115, GHMF-III, Rb1, Rg1, Rg3, CK and Rh1, but not Rh2 in LPS-induced cell model and by G115 and GHMF-III in CSE-induced cell model. GHMF-III, G115, Rb1 and Rg1 also inhibited the release of IL-1β and IL-6 induced by LPS. Among the GHMFs tested, GHMF-III seemed to be the most potent. In addition, G115, GHMF-III, Rb1, Rg1, Rh1 and CK, but not Rh2 significantly inhibited the expression of IKK, p-IKK, IκBα, p-IκBα, p65 and p-p65 and decreased the transcriptional activity of NF-κB induced by LPS. G115, GHMF-III and Rg1, but not Rb1, Rh1, CK and Rh2 significantly increased the cellular level of cAMP and the expression of p-CREB, but inhibited the activity of PDE4 induced by LPS. These findings indicate that ginseng and ginseng related products have a significant inhibition of cytokine release and activation of NF-κB pathway in LPS-induced macrophage-like U937 cells. In addition, they

may also act as PDE4 inhibitor to regulate cAMP pathway. Such actions of ginseng and ginseng related products may contribute to its therapeutic efficacy against COPD.

A separate research objective in the present study was to evaluate the clinical efficacy of TNF-α inhibitors in the treatment of the progression of joint damage (JD) in active rheumatoid Arthritis (RA). The rational of this research is that there is a wide use TNF-α inhibitors in the management of JD in active RA, although it is not clear if there are differences between these inhibitors when used alone or in combination with Methotrexate (MTX), and which factors may affect their efficacy. A meta-analysis was conducted to compare the effects of TNF-α inhibitors on the radiological progression (RP) of active RA when used alone or combined with MTX, and to study the correlation between the degree of activity of RA and the efficacy of TNF-α inhibitors on the progression of JD. It was found that TNF-α inhibitors showed a better efficacy than that of MTX, and TNF-α inhibitors in combination with MTX produced a better efficacy than TNF-α inhibitors used alone. Among different types of TNF-α inhibitors, infliximab in combination with MTX exhibited a better efficacy than other types of TNF-α inhibitors. CRP, ESR and DAS28 were factors affecting the efficacy of TNF-α inhibitors on the progression of JD in active RA. These findings may help to guide clinical use of TNF-α inhibitors to control the progression of JD in active RA.

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Abbreviations

AC	Aspiratory capacity
ACR	American College of Rheumatology
ADA	Adalimumab
AEC	Air epithelial cells
AM	Alveolar macrophage
AR20	American College of Rheumatology core criteria 20
ARA	American Rheumatism Association
ASEs	Air smooth muscle cells
BAL	Bronchoalveolar lavage
BCG	Bacillus Calmette
cAMP	Cyclic adenosine monophosphate
CAPE	Caffeic acid phenethyl ester
CBP	CREB-binding protein
CD11b	AlphaMbeta(2)-integrin(Mac-1) Cumulative Index to Nursing and Allied Health Literature
CINAHL	Cochrane Central Register of Controlled Trials
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CREB	cAMP response element-binding
CRP	C-reactive protein
CS	Cigarette smoking
CSE	Cigarette smoke extract
CTGF	Collagen tissue growth factor
DARE	Database of Abstracts of Reviews of Effects
DAS	Disease Activity Score
db-Camp	Dibutyryl cAMP
DEEP	Tp0751 recombinant protein
DMARD	Disease modifying anti-rheumatic drugs
DS	Dyspnea score
EBC	Exhaled breath condensate
EC	European commission
EC	European commission

ENA-78	Epithelial cell-derived neutrophil-activating peptide-78
ERK1/2	Mitogen-activated protein kinase 1/2
ERS	Erythrocyte sedimentation rate
ESHI	Exacerbation strains of H. influenzae
ET-1	Endothelin-1
ETA	Etanercept
FDA	Food and Drug Administration
FEV	Forced expiratory volume
FMLP/B	Formyl-met-leu-phe plus cytochalasin B
FVC	Forced vital capacity
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GRO- α	Growth-related oncogene-α (CXCL1)
HAQ	Health Assessment Questionnaire
HBEC	Human bronchial epithelial cell line
HDAC2	Histone deacetylases 2
HMSM	Human myometrial smooth muscle
HNP	Human neutrophil peptides
IFN-induced protein 10	Inducible CXC chemokine ligand 10(IP-10)
IFN-γ	interferon gamma
IKK	IκB kinase
IL-10	Interleukin-10
IL-1β	Interleukin-1 beta
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
INF	Infliximab
IP-10	Interferon-γ inducible protein of 10 kDa (CXCL10)
IP-10	IFN-inducible CXC chemokine ligand (CXCL) 10(IFN-induced protein 10)
I-TAC	interferon-γ inducible T cell chemoattractant (CXCL11)
JD	Joint destruction

LPS	Lipopolysaccharide
LT	Lymphotoxin
LTB4	Leukotriene B4
LTC	Leukotriene C(4)
MAPK	Mitogen-activated protein kinase
MC	Mean change
MCP-1	Monocyte chemoattractant protein 1
MeSH	Medical subject headings
MHC-II	Major histocompatibility complex class II molecules
MIP	Maximum Inspiratory Pressure
MIP-1	Macrophage inflammatory protein-1
MMP	Matrix metalloproteinase
MTX	Methotrexate
MVV	Maximum Voluntary Ventilation
NE	Neutrophil elastase
NE	Neutrophil elastase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHI	Bronchial epithelial cell line
NSAID	Non-steroidal anti-inflammatory drug
P38 MAPK	P38 mitogen-activated protein kinases
PBLs	Peripheral blood lynphocytes
PBS	Pharmaceutical Benefits Scheme
PDEs	Phosphodiesterases
PFTs	Pulmonary Function Tests
PGP	Proline-glycine-proline
PI3Ks	Phosphatidylinositol 3-kinases
PKA	Protein kinase A (cAMP-dependent protein kinase)
PMA	Phorbol ester 12-O-tetradecanoylphorbol-13-acetate
RA	Rheumatoid arthritis
RANTES	Released by activated normal T cells expressed and secreted (CCL5)
RE	Response elements
ROS	Reactive oxygen species
RP	Radiological progression

RR	Risk ratio
SDD	Smallest detectable difference
SGRQ	St George Respiratory Questionnaire
SMAD3	Mothers against decapentaplegic homolog 3
TACE	TNF-α converting enzyme
TGF-β	Transforming growth factor beta
Th1	T helper cells type 1
Th1	T helper cells type 1
TIMPs	Endogenous tissue inhibitors
TNF-a	Tumor necrosis factor a
TRP	Tp0751 recombinant protein
TSLP	Thymic stromal lymphopoietin
VEGF	Vascular-Endothelial Growth Factor
VO_2 max	Maximal Oxygen Consumption

Chapter 1. General Introduction

1.1 Chronic Obstructive Pulmonary Disease

1.1.1 Introduction

Chronic obstructive pulmonary disease (COPD) is the major and growing health problem leading to increase in morbidity, disability, mortality and medical costs worldwide. A population-based prevalence study showed that the prevalence of Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage II or higher COPD had reached 10.1% for man and 8.5% for women in 2007[1]. It was the fourth death cause in 2008[2], and is expected to become the third death cause within the next decade in Europe and the United States[3], and the fifth most common cause of disability in the world by 2020 [4]. In Australia, COPD affects almost 13% or one in seven Australians 40 or over, and Australia has one of the highest rates of COPD deaths in the developed world. This will result in the escalating health care costs worldwide[5].

COPD is a disease characterized by a progressive and non- reversible airway limitation which is associated with a persistent inflammatory response of the airway and lungs to bacteria, viruses, noxious particles and gases[6-8]. The pathogenesis of COPD is still not fully clear, although the persistent inflammatory response is a specific aspect of COPD and cigarette smoking(CS), as the primary risk factor[9], increases the susceptibility of COPD patients to pathogens[10,11]. Currently, there are still no satisfactory therapies that can block the inflammatory progression of COPD including corticosteroid[12]. Since the inflammatory cells and mediators play an important role in the persistent inflammation in COPD [13], it is also important to understand the relevant signalling pathways in these cells.

1.1.2 Inflammation-Related Cells in COPD

A variety of inflammatory cells are involved in COPD[14], including neutrophils, T lymphocytes, macrophages, air epithelial cells (AEC), air smooth muscle cells (ASEs) and eosinophils. These cells play different roles in the inflammatory mechanism of COPD.

1.1.2.1 Neutrophil

There is evidence that the number of neutropils was significantly increased in sputum and bronchoalveolar lavage (BAL) fluid from patients with COPD, the increase is also correlated with the severity of COPD and the decline in lung function of COPD patients[15-18]. The abnormal increase of neutrophils in COPD patients was likely to relate to CS, as it has been showed to stimulate the release of granulocyte from the bone marrow which was mediated by granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) released by alveolar macrophages (AM)[19]. Additionally, CS also increased the retention of neutrophils in the lung[20].

Chemokines may play a key role in the abnormal distribution of neutrophils in COPD. Many kinds of chemokines can recruit neutrophils to the inflammatory site, such as interleukin-8 (IL-8), interferon gamma (IFN-γ), leukotriene B4(LTB4) and growth-related oncogene-α (GRO- α; CXCL1)[21-24], and all of these chemokines were increased in the patients with COPD[16,25-27]. As neutrophil is a major source of IL-8[28], it in turn recruits neutrophil itself and other inflammatory cells to the site to amplify the inflammatory response. In addition, neutrophils also produced LTB4[29]. These chemokines links may be a vital reason for the abnormal distribution of neutrophils and release of IL-8 in COPD patients.

Neutrophils also secrete proteases, including neutrophil elastase (NE), matrix metalloproteinase (MMP)-8 and MMP-9[30-32], as well as released oxygen radicals such as superoxide anion, hydrogen peroxide[33,34], which may contribute to lung tissue damage and the decline of lung function[35,36].

1.1.2.2 T Lymphocyte

Histopathologic studies showed that the total number of T lymphocytes was increased in lung parenchyma, peripheral and central airways of patients with COPD[37-40], and there is a correlation among the number of T cells, the amount of alveolar destruction, and the severity of airflow obstruction[40] . It has also been shown that the extent of lymphocytes infiltrates in airway was correlated with the severity of COPD[37].

T cells are divided into five categories according to their functions, including helper T cells (CD^+4), cytotoxic T cells (CD^+8), memory T cells, regulatory T cells and natural killer T cells. Among these cells, CD^+4 and CD^+8 have been shown to be involved in the pathogenesis of COPD and CD^+8 are likely to play more important roles than CD^+4[41-44]. The numbers of CD^+8 cells was elevated in the circulation of COPD patients who did not smoke[41,42]. The number of CD^+4 cells was also increased in the small airway of smokers with severe COPD[43]. It was reported that the increase in CD^+8 cells was greater than CD^+4 cells in COPD[44]. T helper cells type 1 (Th1) seemed to contribute to the unbalance between CD^+4 and CD^+8, since there was an unbalance between Th1 and Th2 in COPD patients[45]. Th1 cells secret Interleukin-2 (IL-2) which is an inductor for the differentiation of CD^+8 cells[46,47].

The tissue damage mediated by CD^+8 cells in COPD may involve two mechanisms. First, CD^+8 cells may contribute to the modulation on apoptosis. It has been reported that there

was a correlation between the number of CD^+8 and apoptosis of alveolar cells in emphysema[40], which seemed to be related with the perforin andgranzyme-B released by CD^+8[48]. Secondly, CD^+8 cells may modulate the release of MMPs. The study about CD^+8 cells - deficient mice exposed in CS demonstrated that CD^+8 cells contributed to the lung destruction through the release of IFN-inducible CXC chemokine ligand (CXCL) 10 (IFN-induced protein 10, IP-10) which induced the production of MMP-12[49].

1.1.2.3 Macrophage

It has been shown that the number of macrophages was elevated (5–10 folds) in the airways, lung parenchyma, BLA fluid and sputum of smokers and patients with COPD[37], and the increase was associated with the severity of COPD[17]. The increase in the number of macrophages in inflammatory sites of COPD patients may be related to the increased release of monocyte-selective chemokines and the reduced apoptosis of macrophages. Monocyte chemotactic protein-1(MCP-1) can recruit monocytes from the circulation to tissues[50,51] , which is the premise of monocytes differentiation to macrophages. The level of MCP-1 was increased in BAL fluid of COPD patients[22,52,53]. In addition, the expression of the anti-apoptotic protein Bcl-XL was increased in macrophages from smokers[54].

1.1.2.3.1 Macrophage and the Immune Response

Macrophage plays a key role in the defence against airway pathogens[55]. Alveolar macrophage(AM) was considered as the sentinel phagocytic cell of the innate immune system in the lung[56]. AMs have a unique localization in the airway and act as the first line of defence against the airway pathogens, such as inhaled bacteria, viruses, noxious particles and gases. During this defensive process, macrophages are not only served as scavenger to clear the pathogens in the airway, but also as pathogen to induce the immunity response.

Macrophages play an antigenic role and induce the cellular immunity response in the abnormal inflammation of COPD. It has been shown that macrophages present the antigen characteristic of major histocompatibility complex class II molecules (MHCII) after digesting a pathogen to activate $CD4^+$ T [57,58]. So the antigen presentation of infected macrophage may be a part of pathogenesis of COPD.

1.1.2.3.2 Macrophage and Inflammatory Mediators

Macrophage can produce a wide array of powerful inflammatory mediators which is directly involved in the inflammatory response in COPD. The chemokines produced by macrophages include Growth-Related Oncogene-α (GRO-α; CXCL1), interferon-γ inducible protein of 10 kDa (IP-10; CXCL10), MCP-1 and IL-8[22,26,52,59-61]. These mediators in turn recruit different inflammatory cells, such as IP-10 for CD^+8[62], MCP-1 for monocytes, neutrophils and T lymphocyte[63, 64,65] , Gro-α and IL-8 for neutrophils[21,22].

Macrophages also produce various cytokines. For example, TNF-α was produced by AMs from patients with COPD induced LPS[46,47]. Other cytokines associated with COPD and produced by macrophages included Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), Interleukin-10 (IL-10), granulocyte-macrophage colony-stimulating factor (G-CSF) and IFN-γ[13,66-68]. Macrophages synthesize these cytokines through activating many intracellular inflammatory pathways, especially nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway[50,69]. There is strong evidence that NF-κB is the key transcriptional factor which regulated the synthesis of inflammatory gene in macrophages from patients with COPD[70,71].

In addition, macrophages can regulate the cellular production of reactive oxygen species (ROS) in COPD. Many studies have shown that the level of oxidative stress was increased in

COPD[34,72,73], and the increase is derived from ROS produced by activated macrophages in airway of patients with COPD[73].

1.1.2.3.3 Macrophage and Emphysema

Emphysema is an important pathological stage of COPD. The elastolysis of the lung is the primary cause of emphysema[74]. Studies have shown that the number of AMs was significantly increased in BAL fluid of smokers with emphysema comparing with those healthy smoker without emphysema, and this increase was correlated to the extent of emphysema[75]. AMs also expressed cathepsins (L and S), MMP-2, MMP-9 and MMP-12[76-78], and all of them involved in the pathogenesis mechanism of emphysems[79]. Cathepsins L and S are elastolytic cysteine proteases. MMP-2, MMP-9 and MMP-12 are the predominant MMP capable of elastolysis in COPD patients[80]. It has been demonstrated that a MMP inhibitor, Marimastat decreased the elastolytic activity derived from MMPs in macrophages from patients with COPD[80].

1.1.2.4 Airway Epithelia Cell

Airway epithelia cell (AEC) play an important role in the innate defence system of airway. AEC is the pseudostratified ciliated columnar epithelial cells (from tracheatobronchioles), and their ciliary movement can protect the airway against the invasion of pathogens[81]. The goblet epithelia cells, as secretary cells, can also produce mucus to trap the foreign substances entering in the respiratory tract[82]. In addition, AEC can release certain mediators and cationic peptides with antimicrobial effects to fight against the pathogens entering into the respiratory tract[83]. ASE also contributes to the adaptive defence, as they transported IgA to the airway lumen[84]. Studies have shown that cigarette smoke weakened the innate and

adaptive immune functions of airway epithelic cells, resulting in the increase of susceptibility of COPD patients to infection[10,11].

Similar to the role of macrophages, AEC has a capacity of recruitment of inflammatory cells, and regulating lung tissue damage through the release of chemokines and MMPs. For example, AECs increased the release of IL-8 , Pro-α, GM-CSF, CXCR3, IP-10, interferon-γ inducible T cell chemoattractant (I-TAC; CXCL11)[26,85-88], and CXCR3 in BALF of COPD patients[86,87]. The expression of activated normal T cells expressed and secreted (RANTES; CCL5) and the level of another chemoattractant for eosinophils (eotaxin) were significantly increased in ASEs from patients with COPD[89,90]. The expression of MMP-9 was at a low level in ASEs from the non-smoking subjects, but it was significantly increased in ASEs from COPD patients[91].

1.1.2.5 Eosinophil

The role of eosinophil in COPD is still uncertain. A study showed that the number of eosinophils increased in the induced sputum in COPD patients, but in stable COPD patients, the number of eosinophils remained unchanged[92,93]. Other studies reported that the number of eosinophils was elevated in sputum, BAL fluid, airway and lung in the acuate exacerbations of chronic bronchitis with obstruction patients[15,94,95]. The increased number of eosinophils in the inflammatory site may be related to IL-8 and RANTES[51,90,96,97]. It has been shown that RANTES was strongly expressed in ACEs from patients with chronic bronchitis exacerbations[90]. The concentration of IL-8 was significantly increased in sputum of patients with COPD[16], and further increased during acute exacerbations[98]. The level of another chemoattractant for eosinophils (eotaxin) was also significantly increased in ASEs from patients with COPD[89]. In addition, eosinophils maybe related to therapeutic outcome of

certain drugs as it has been shown that the presence of eosinophils in COPD patients displayed a good clinical response to corticosteroids treatment[99].

1.1.2.6 Airway Smooth Muscle

Studies have shown a significant increase of the number of airway smooth muscle (ASM) in small airways of COPD patients [58,100,101], especially in patients with severe COPD[102], and this increase in the number was correlated with the FEV[58]. The proliferation of ASM is the main feature of fibrosis in COPD patients[103]. ASM released many inflammatory mediators, including IL-8, MCP-1, GRO-a, IP-10 and GM-CSF in inflammatory conditions[104-108].

1.1.2.7 Inflammatory Cells and Fibrosis in COPD

Three types of inflammatory cells have been shown to participate in the regulation of fibrosis in COPD, including macrophages, AECs and ASMs, involving two main mechanisms. The first mechanism is through transforming growth factor beta (TGF-β)/mothers against decapentaplegic homolog 3(SMAD3)/ASMs pathway to modulate the chronic fibrosis in small airways[109]. The second mechanism is through TGF-β/ET-1/collagen tissue growth factor (CTGF, an important downstream factor of TGF-β)/fibroblasts pathway to promote the activation of fibroblasts[110]. ASM is the main structure cell in chronic fibrosis. The proliferation of ASM was up-regulated by TGF-β/SMADs[111]. There was a self-regulation of ASM on the proliferation, as ASM also released the TGF-β[112] , which may explain why ASM continuously proliferated leading to the airflow limitation in COPD. ASM also contributed to the regulation on TGF-β/ET-1/CTGF/fibroblasts pathway, as it released and expressed the CTGF to induce the activation of fibroblasts[113]. Similar to ASM, AEC expressed both TGF-β and CTGF [114], indicating it may regulate the fibrosis through both

mechanisms. The expression of TGF-β was increased in AMs and AECs from patients with COPD[74,114,115], thus AMs may mainly regulate chronic fibrosis through the release of TGF-β.

1.1.3 The Inflammatory Mediators in COPD

The inflammatory cells release various inflammatory mediators which can be divided into different types according to their functions in inflammatory responses, including chemokines, cytokines and proteases. Table 1-1 lists all inflammatory mediators which have been shown to be increased in COPD patients. Among these, some mediators have been investigated as drug targets in experimental studies and clinical trials.

1.1.3.1 IL-8

IL-8 is a powerful neutrophil and lymphocyte chemoattractant. It can be secreted by macrophages, neutrophils, and AECs[118,140]. CS was a powerful inducer of the synthesis of IL-8 *in vivo*[141]. It has been shown that TNF-α, LPS, IL-1β, bacterial products, certain viruses, oxidative stress and CSE were stimulators of IL-8 secretion *in vitro*[142-148]. Further studies showed the synthesis of IL-8 was related to the activation of transcription factors, among which NF-κB is predominant[149,150], and the protein and mRNA expression of IL-8 was significantly inhibited by IKKβ inhibitors [151].

Table 1-1 Expression of inflammatory mediators in COPD patients

Mediator	Target	Source	Reference
IL-8	Protein	Induced sputum, BAL fluid, serum	16,116,117
	mRNA	Lung tissue	116,117
	Protein	AMs, AEC, Neutrophils	118
Gro-α	Protein	Sputum, lung tissue	116
	Protein	AECs	26
IP-10	Protein	Lung tissue	116,119
ENA-78	Protein	BAL fluid	21
	mRNA	AECs	120
MCP-1	Protein	Sputum, BAL fluid, lung	52
	mRNA	Lung tissue	116,117
CCR2	Protein	Lung tissue	117
	mRNA	Lung tissue	116
CCL3	Protein	Lung tissue	117
	mRNA	Lung tissue	116
CCL4	Protein	BAL fluid	116
CXCR2	Protein	PBLs	116
	mRNA	BAL fluid	121
TNF-α	Protein	Sputum, serum, leg muscle	16,116,119
	mRNA	Serum, sputum	116,119
	Protein	AMs, AEC	122,123
IL-6	Protein	Sputum, BAL fluid, EBC, plasma	124-126
	Protein	Monocytes	127
IL-9	Protein	T lymphocytes	128
GM-CSF	Protein	BAL fluid	129
IL-10	Protein	Sputum, serum	130
IFN-γ	Protein	Bronchial biopsies	128,131
TGF-β	Protein	AECs, AMs, PBLs	115,132
		Airway biopsies	133,134
VEGF	mRNA	Pulmonary vascular smooth muscle	135
Cathepsin L	Protein	BAL fluid	136
MMP-1	Protein	Bronchoalveolar lavage	137
MMP-9	Protein	BLA fluid ,lung parenchyma	137,138
		AMs	139

ENA-78=Epithelial cell-derived neutrophil-activating peptide-78 (CXCL5)
PBLs=Peripheral blood lynphocytes
EBC=Exhaled breath condensate
VEGF=Vascular-Endothelial Growth Factor

IL-8 has two receptors, CXCR1 and CXCR2, located on the surface of neutrophil[140]. CXCR1 is a specific receptor for IL-8 and blocking its activity leads to blocking inflammatory signalling from IL-8 to neutrophils. CXCR2 is non-specific receptor of IL-8, which binds not only to IL-8, but also to ENA-78 and Gro-α[141,142]. In addition, collagen fragment (the product of lung tissue), especially proline-glycine-proline (PGP) derived from the breakdown of extracellular matrix, also bound to CXCR2[143]. In patients with COPD, the levels of ENA-78 and GRO-α were significantly increased in BAL fluid [21].The concentration of PGP which was broken down by MMP1 and MMP9 from collagen was also increased in the sputum and serum of COPD patients[144]. CXCR2 seems to play a more important role than CXCR1 in neutrophils related inflammatory response in COPD, as CXCR2, but not CXCR1, was expressed by bronchial epithelial cells from COPD patients[145]. Qiuet et al. also found that the expression of CXCR2 was significantly increased in airway neutrophil during acute exacerbations of COPD, and this increase is correlated with the numbers of neutrophil in the airway[120]. Previous studies on blocking IL-8 activity had limited success in eliminating the neutrophil related inflammation[146]. It indicates that CXCR2 may be a better drug target than IL-8 itself.

Various IL-8 antagonists have been developed. ABX-IL-8 is an antibody against IL-8. It has been evaluated in clinical trial phase II for COPD[147]. Although the dyspnea was improved, there were no improvements on the lung function, quality of life and the six minute walking test. So, in 2003, ABX-IL-8 was withdrawn from further studies[148]. As alternatives, the antagonists (SB656933) which prevent Gro-α& IL-8 binding to CXCR2 have also been evaluated in clinical trial for COPD. SB656933, as an oral CXCR2 antagonist was reported to inhibit the binding of Gro-α and IL-8 to CXCR2 but not CXCR1 in human neutrophils[148]. CD11b (Mac-1, alphaMbeta(2)-integrin), as both a complement receptor (CR3) and a cell adhesion molecule present on the surface of cells, maybe involved in the regulation of

SB656933 in COPD patients. CD11b can trigger chemotaxis of neutrophils and shape change of lung tissue[149]. Neutrophils over-expressed CXCR1 and CXCR2 was reported to respond to IL-8 with the up-regulation of CD11b in smokers with COPD, and SB-656933 was found to inhibit the expression of CD11b in neutrophils and shape-changes of lung tissue in COPD patients[149]. This result was also confirmed in an animal model of COPD[150].

Another type of IL-8 antagonist (SB225002 and SB265610) has been reported to interfere with the chemokine-binding sites[116]. SB265610 significantly inhibited the repair and neutrophil recruitment in a skin-wound healing model in mice[151]. Other novel CXCR2 antagonists are also under development. Studies showed that highly selective N,N'-diarylurea CXCR2 antagonist, significantly reduced the chemotaxis of neutrophil *in vitro*, and decreased the number of neutrophil in rabbit models for ear swelling and neutropenia[152].

1.1.3.2 TNF-α

In 1968, Dr. Gale A Granger named a cytotoxic factor produced by lymphocytes as lymphotoxin (LT)[153]. In 1975, Dr. Lloyd J. Old found that the mice injected with LPS after vaccinated by Bacillus Calmette (BCG) produced the factor which killed some tumor cells or induced the necrosis of tumor tissue *in vivo*, and named it as tumor necrosis factor (TNF)[154]. In 1985, Shalaby named TNF produced by macrophages as TNF-α，and lymphotoxin(LT) produced by lynphotocyes as TNF-β[155]. TNF-α plays a key role in the inflammatory response of COPD. Previous studies showed that the levels of TNF-α were increased in peripheral blood, sputum and BLA fluid of patients with COPD[16,156]. The concentration of TNF-α was also increased in the serum from patients with stable COPD[16], as well as in the serum from COPD patients with acute exacerbation[157,158]. The concentration of soluble TNF-α receptors was also increased in sputum of patients with COPD[159]. TNF-α can be

produced by various cells, such as epithelial cells, macrophages and monocytes, but mainly in macrophages[61,160,161]. CS and LPS are frequently used as inductors to stimulate the release of TNF-α in the experimental model of COPD[162,163]. CS induced TNF-α release maybe dependent on MMP12, as the release of TNF-α stimulated by CS was abolished in MMP12 knockout mice[122].

TNF-α is a maintainer of abnormal inflammation of COPD. It regulates a broad spectrum of inflammatory mediators through activating various inflammation-related pathways. These pathways include NF-κB, mitogen-activated protein kinase 1/2(ERK1/2), p38 mitogen-activated protein kinases (P38 MAPK) and phosphatidylinositol 3-kinases (PI3Ks) pathways[164-167]. For example, TNF-α can activate these pathways to release IL-8, IL-6 and MMP-9[168-170].

TNF-α is related to emphysema which is the end stage of COPD progression.The previous study showed that a lesser degree emphysema was generated by TNF-α knockout mice induced by CS than wild-type animals, and TNF-α was accounted for 70% of smoke-induced emphysema in the mice [171]. There is also evidence for a role of TNF-α in emphysema in TNFR−knockout mice, which may involve MMPs[171]. TNF-α may also be involved in weight loss during late stage of COPD, as the release of TNF-α was increased in peripheral blood monocytes from weight-losing COPD patients[123,156]. The effect of TNF-α on muscle wasting may contribute to the weight loss in COPD patients, as studies showed the expression of skeletal muscle proteins was inhibited by TNF-α through activating NF-κB[172].

It is well known that TNF-α promote the inflammatory response, which in turn causes many the clinical problems such as RA, Ankylosing Spondylitis, Juvenile Idiopathic Arthritis,

Psoriasis, Psoriatic Arthritis, Crohn's disease, Behçet's syndrome, Hidradenitis Suppurativa, Acute Ischemic Stroke, asthma and COPD[173]. In TNF-α gene knockout mouse, blocking the bind between TNF-α and TNF-α receptor attenuated the pulmonary inflammation induced by cigarette smoking[174]. Therefore, TNF-α is a potential drug target for COPD, and TNF-α inhibitors have been used in clinical trial or preclinical to adjust inflammatory disorders.

TNF-α antagonists include non-human/chimeric antibodies (infliximab, afelimomab and CytoTab), humanized antibodies (adalimumab, CDP-571 and CDP-870), human soluble TNFR/ TNFR fusion protein (Onercept and Etanercept), small molecules (ISIS-104838), and inhibitors of TNF-α converting enzyme (TACE)[148,175]. Three TNF-α inhibitors have currently been approved by the Food and Drug Administration (FDA) and the European commission(EC) for disease-specific application, infliximab (Remicade) for RA and Crohn, etanercept (Enbrel) for RA and AS, adalimumab (Humira) for RA. They are also listed by the Pharmaceutical Benefits Scheme (PBS) for use in Australia. The application of TNF-α antagonist (Infliximab) in COPD is currently developing in II clinical trials, and clinical studies demonstrated that COPD patients treated by infliximab over 6 to 24 weeks did not show improvement on lung function, body weight, quality of life scores, airway inflammation, or cytokine levels[176]. Another study on TNF-α inhibitor etanercept in a non-follow-up small clinical trial on COPD and asthma showed FEV1 and stable FEV1/forced vital capacity(FVC) were improved by etanercept plus withdrawal of β2-agonists in patients with severe asthma[177]. However, the uncertain effect of TNF-α inhibitors on COPD is still the primary factor which limits the application of these inhibitors. In addition, the cost of these agents was too expensive, which directly limited their widespread use. More importantly, these agents are associated with some serious side effects, such as lymphoma, congestive heart failure, opportunistic infections, and tuberculosis[178,179]. New efforts are needed to develop novel anti- TNF-α agents who are efficient, safe and cost effective to

replace the current ones[180,181]. Another potential development in this area is the use of inhalation formulation of TNF-α for treating the local airway inflammation. The local application of TNF-α inhibitors may not affect systemic pulmonary inflammation, but can be more directly and effectively inhibiting tissue remodelling and mucus hyper production in the inflammatory sites[148].

A separate approach is via inhibition of TNF-α biosynthesis. The biosynthesis of TNF-α is closely related with three biological processes which occur on the different parts of cell, including activating the inflammation-related pathways in cytoplasm, expressing mRNA through activating promoter in nucleus and protein synthesis in ribosome. Current studies of TNF-α synthesis focus on the inhibition on biological processes in cytoplasm and nucleus, especially the NF-κb pathway. In this aspect, a number of natural compounds such as ginseng, have been used in the treatment of COPD and have been shown to inhibit the release of TNF-α through decreasing the activity of NF-κB pathway. G115, a standardized extract of ginseng, has been used for the treatment of COPD, and was shown with significantly improvement of the Pulmonary Function Tests (PFTs), Maximum Voluntary Ventilation (MVV), Maximum Inspiratory Pressure (MIP) and Maximal Oxygen Consumption (VO2max) in patients with moderately-severe COPD[182]. Further study showed Rb1 and Rg1, as a major active components of G115 decreased the expression of activated NF-κB to inhibit the release of TNF-α[183,184].

1.1.3.3 MMPs

MMPs play an important role in the pathogenesis of emphysema. In the healthy adult lung, the expression of MMPs is in low quantities[185] . The concentrations and expressions of MMP-1 and MMP-9 were increased in macrophages from patients with COPD, and MMP1

has been shown to be involved in the formation of emphysema through degrading type III collagen[186]. The MMP2 and MMP9 were also expressed in emphysematous lung tissue[187], and their activities were inhibited by blocking the expression of ET-1 in CSE-induced emphysema rat[188]. MMP12 may be decisive factor of CS-induced emphysema, as MMP12 knockout mice did not develop emphysema to exposure in CS[189].

Many cells and cytokines involved in the elastolysis in COPD through regulating the release of MMP-9 and the modulations of these cells and cytokines on MMP-9 may be bidirectional. For example, both neutrophils and macrophages released MMP-9 contributing to alveolar destruction[32,79,190].IL-10 (anti-inflammatory cytokine) inhibited the release of MMP-9 in monocytes from patients with COPD[191]. MMP-9 also contributes to the repair (fibrosis) after elastolysis through activating fibrosis-inducing factor(TGF-β). MMP-9 promoted the transformation of TGF-β[192], which was achieved through activating TGF-binding protein-1[193]. Thus, MMP-9 may act as a bridge between the elastolysis and fibrosis.

MMPs have also been studied as a drug target of COPD. The existing antagonists of MMPs include TNF-α converting enzyme (TACE) MMP inhibitors, endogenous tissue inhibitors (TIMPs) and pharmacological inhibitors such as zinc chelatorsand doxycycline[175]. TACE is a protein which inhibits the pre-TNF-α cleavage into mature TNF-α, and also the release of MMPs. TACE is likely to have a potentially beneficial effect to inhibit the formation of mucus. There is evidence that the TACE-dependent mechanism was involved in the production of important constituents of mucus, mucins 5AC, induced by CS and LPS *in vitro*[194].

TIMPs are the endogenous tissue inhibitors of MMPs which comprise four proteases, including TIMP1, TIMP2, TIMP3 and TIMP4. All MMPs are inhibited by TIMPs once

TIMPs are activated, but the gelatinases (MMP-2 and MMP-9) can form a complex with TIMPs when they are in the latent form. The complex of latent MMP-2 (pro-MMP-2) with TIMP-2 serves to facilitate the activation of pro-MMP-2 at the cell surface by MT1-MMP (MMP-14)[195,196]. The role of pro-MMP-9/TIMP-1 complex is still unclear. TIMPs have been implicated in the pathogenesis of COPD. The concentration of TIMP1 and TIMP 4 were elevated in serum of COPD patients compared with that of control subjects[197]. TIMP3 knockout mice demonstrated a progressive airspace increase[198]. A polymorphism in the TIMP2 gene (G853A) was also associated with COPD[199].

The inhibitors of MMPs have been evaluated in clinical trial for many diseases, such as cancer, autoimmune disease, and cardiovascular disease[200,201]. However, only doxycycline is currently approved by the FDA for anthrax. MMPs inhibitors have certain side effects on the musculoskeletal system[175]. Further research may help to develop novel MMPs inhibitors with fewer side effects for the management of COPD.

1.1.4 Inflammation Related Pathways in COPD

It has been proven that several inflammation pathways contribute to the pathogenesis of COPD through the release of various inflammatory mediators (Table 1-2 Release of inflammatory mediators through the pathways related with COPD). These pathways include PI3K, ERK, P38 MAPK, NF-κB and cAMP, each of them plays different roles in COPD.

Table 1-2 Release of inflammatory mediators through the pathways related with COPD

Pathway	**Mediator**	**Compartment**	**Inducer**	**Reference**
PI3K	NE	Neutrophils	LPS	202
	IL-8	U937	HNP	203
	CCR1	U937	GM-CSF/IL-10	204
	MMP9	U937	TNF-α	205

ERK1/2	IL-6	HBECs	CSE	[206]
		human ACEs, BEAS-2B	CSE, NHI	[207,208]
		monocytes from COPD patients	Acetylcholine	[29]
P38	IL-8	HBECs,BEAS-2B	ESHI, CSE	[207,209]
		HBECs	DEEP, TRP	[210]
	TSLP	Human SMs	TNF-α,IL-1	[211]
	IL-6	HBECs,THP-1	DEEP	[210]
	COX-2	HBECs	DEEP	[210]
	IL-1	THP-1	TRP	[212]
	TNF-α	THP-1	TRP	[212]
NF-κB	IL-8	Human SMs, HBECs	CSE/IL-1β,ESHI	[209,211]
	Eotaxin	Human SMs	CSE, IL-1β	[211]
	VEGF-α	Human SMs	CSE, IL-1β	[211]
	MMP9	A549	TNF-α	[213]
	ICAM-1	Human SMs	TNF-α, IL-1β	[214]
	IL-6	Human SMs	TNF-α, IL-1β	[214]
	GM-CSF	Human SMs	TNF-α, IL-1β	[214]
	RANTES	Human SMs	TNF-α, IL-1β	[214]
	MCP-1	Human SMs	TNF-α, IL-1β	[214]
	Gro-α	Human SMs	TNF-α, IL-1β	[214]
	NAP-2	Human SMs	TNF-α, IL-1β	[214]
	ENA-78	Human SMs	TNF-α, IL-1β	[214]

HBEC= bronchial epithelial cell line
NHI:=bronchial epithelial cell line
ESHI:=Exacerbation strains of H. influenzae
DEEP= Tp0751 recombinant protein
TRP= Tp0751 recombinant protein
TSLP:=Thymic stromal lymphopoietin

1.1.4.1 Phosphatidylinositol 3-Kinases Dependent Pathway

PI3Ks are a family of related intracellular signal transducer enzymes. PI3Ks are involved in the regulation of various cellular functions through PI3k dependent pathway. Akt, as a downstream factor of PI3K pathway, can be phosphorylated by PI3K, and plays a key role in multiple intracellular processes such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking[215]. In macrophages from patients with COPD, the

expression of phosphorylated PI3K δ and Akt was increased compared with the control groups of age-matched smokers and non-smokers[216]. In addition, PI3K was involved in the regulation on the release of inflammatory mediators from macrophage and neutrophils, which are the key players in COPD inflammation(Table1-2).

It has been known that glucocorticoid insensitivity is the biggest problem in the treatment of COPD. COPD patients with glucocorticoid insensitivity often have a rapid development of COPD and eventually lead to the occurrence of airway limitation. There is evidence for a potential role of PI3K, especially PI3K δ, in the glucocorticoid insensitivity of COPD. Marwick et al. found that glucocorticoid insensitivity was restored in PI3K δ kinase dead knock-in smoke-exposed mice but not PI3K γ knockout mice, and this effect was correlated with the activity of histone deacetylases 2 (HDAC2)[217]. Knockdown of HDAC2 showed that HDAC2 was a prerequisite molecule for glucocorticoid-insensitivity and the over-expression of HDAC2 was able to restore the sensitivity of glucocorticoid[218]. In monocytes from COPD patients, the reduced sensitivity to dexamethasone was also reversed by inhibition of PI3kδ but not by PI3Kγ[216].

1.1.4.2 ERK1/2 Dependent Pathway

ERK1/2 is protein kinase intracellular signalling molecule. ERK1 is similar to ERK2 (85% sequence identity) [219]. Phosphorylation of ERK1/2 leads to the activation of their kinase activity. Phosphorylated ERK1/2 activates the downstream factors in its pathway, and finally involve in a wide variety of intracellular processes such as proliferation, differentiation, transcription regulation and development[220].

Activated ERK1/2 was elevated in non-smoking patients with COPD[221]. However, CSE significantly up-regulated the expression of p-ERK1/2 in human ASM cells and

macrophages[222,223], while the effect of CSE on the p-ERK1/2 was also proved *in vivo*[223]. ERK1/2 is likely to be related to muscle atrophy in COPD, as Lemireet et al. found that the ratios of phosphorylated to total expression of ERK 1/2 were significantly elevated in quadriceps from patients with COPD compared with healthy controls[224]. This effect of ERK1/2 may relate to its action on IL-6(Table1-2), as IL-6 induced the skeletal muscle atrophy[172]. Previous studies showed the concentration of IL-6 was increased in induced sputum, bronchoalveolar lavage, and exhaled breath condensate of COPD patients, particularly during exacerbations[124,225,226]. In the plasma of COPD patients, IL-6 was also increased [125,126,227], especially during exacerbations[228].

1.1.4.3 P38 MAPK Dependent Pathway

P38 mitogen-activated protein kinases (P38 MAPK) are a class of mitogen-activated protein kinase which participate in a signalling cascade controlling cellular responses to cytokines and stress. There was a strong induction of p38 phosphorylation in patients with COPD[221]. However, the expression of mRNA and protein of total/phosphorylated p38 MAPK was not up-regulated in biopsies obtained from stable out- patients with COPD[229].

Small molecule inhibitors of p38 MAPK have been used in the pre-clinical study of COPD. PH797804, a diarylpyridinone inhibitor of p38 MAPK, was reported to improve in dyspnea score(DS), aspiratory capacity(AC), and sustained decrease in serum CRP levels and lung function (FEV 1 ranging from 32% to 80%). It had no significant effects on liver enzymes in COPD patients. SB681323, as a specific inhibitor of p38 MAPK, down-regulated the level of TNF-α in whole blood from patient with COPD [230]. SB681323 also reduced the number of neutrophils in sputum and the concentration of fibrinogen in serum but not the level of CRP, IL-8, IL-1β , or IL-6 in serum of COPD patients without receiving inhaled

corticosteroid therapy, and this was accompanied by an improvement in FVC but not in FEV 1[231]. Another p38 inhibitor,GW856553(losmapimod), was shown to have no effect on the level of CRP, IL-8 and IL-6 in GOLD stage II COPD patients, but it improved hyperinflation [232]. However, there seems some different effect of p38 MAPKs inhibitor on the release of IL-8 *in vivo* and *in vitro*, as SB203580 (the inhibitor of p38 MAPK) down-regulated the release of IL-8 in CSEM-induced primary HBECs and BEAS-2B [207].

1.1.4.4 NF-κB Pathway

NF-κB is a protein complex and activated NF-κB that controls the transcription of DNA. While in an inactive state, NF-κB is located in the cytosol complexed with the inhibitory protein IκBα. Through the intermediacy of integral membrane receptors, a variety of extracellular signals can activate the enzyme IκB kinase (IKK). IKK is part of the upstream NF-κB signal transduction cascade. The IκBα (inhibitor of kappa B) protein inactivates the NF-κB transcription factor by masking the nuclear localization signals (NLS) of NF-κB proteins and keeping them sequestered in an inactive state in the cytoplasm. IKK specifically, phosphorylates the inhibitory IκBα protein. This phosphorylation results in the dissociation of IκBα from NF-κB and thereby activates NF-κB. The activated NF-κB is then translocated into the nucleus where it binds to specific sequences of DNA called response elements (RE). The DNA/NF-κB complex then recruits other proteins such as coactivators and RNA polymerase, which transcribe downstream DNA into mRNA, which, in turn, is translated into protein that results in a change of cell function. There are five proteins in the mammalian NF-κB family, including NF-κB1(p50), NF-κB2(p52), RelA(p65), RelB and c-Rel[233]. Among these proteins, p65 is the specific transcriptional factor for TNF-α[234].

NF-κB is the key transcriptional factor which controls the transcription of many proteins involved in the inflammation of COPD[235]. It regulates the release of many inflammatory mediators (Table1-2). The expression of activated NF-κB (p65) was up-regulated in the bronchial biopsies of COPD patients[70]. However, there was a down-regulating mechanism in COPD patients on the activity of NF-κB pathway, as the release of the inhibitory factor of NF-κB, IL-10, was also increased in COPD patients [236]. In addition, activated NF-κB is like to play an important role in muscle wasting in COPD, as it was required for the transition from inflammatory signalling to muscle atrophy signalling[237] and in pulmonary emphysema animal model with lower body weight the activity of NF-κB was exactly increased[238].

The inhibitors of NF-κB show a significant inhibition on the TNF-α synthesis and release. It has been shown that caffeic acid phenethyl ester(CAPE), inhibitor of IKK, could eliminate the synthesis of TNF-α which is induced by heat-inactivated S. aureus in primary astrocytes derived from C57BL/6 WT[239]. Similar results were also demonstrated *in vitro*[240]. It was also found that BAY 11-7082, another inhibitor of IKK, inhibited the synthesis of TNF-α induced by ET-1[241]. In addition, CAPE also inhibited the release of IL-8 and IL-6.mediators[242,243].

1.1.4.5 cAMP Pathway

Cyclic adenosine monophosphate (cAMP) is the second messenger that is important in many biological processes, and has been shown to modulate inflammatory response *in vivo* and *in vitro*. cAMP can be phosphorated by phosphodiesterases (PDEs), and then loses activity. In humans, cyclic AMP works by activating protein kinase A (PKA, cAMP-dependent protein kinase). PKA can activate a cAMP response element-binding (CREB). CREB is a cellular transcription factor, which can regulate the transcription of gene, causing increased

expression of specific genes. Different to other pathways, cAMP pathway is used to down-regulate the inflammatory response in COPD. The elevating agents of cAMP (forskolin, dibutyryl cAMP and PDE7A inhibitor) abrogated the release of TNF-α induced by LPS/PMA[244,245]. Foskoliin and dibutyryl cAMP also decreased the number of neutrophils and increased apoptosis in the pleural cavity in LPS-challenged mice[246].

The cyclic nucleotide PDEs comprise a group of enzymes that degrade the phosphodiesterase bond in the second messenger molecules cAMP and cGMP. PDEs are important regulators of cell signal transduction mediated by cAMP and cGMP. PDEs were initially isolated from rat brain in the early 1970s using polyacrylamide gel electrophoresis[247,248]. They have been shown widely distributed especially in the lung and many cell types[249,250]. Different PDEs have different substrate requirements. Some are cAMP-selective (PDE4, 7 and 8), some cGMP-selective (PDE5, 6, and 9) and others can hydrolyse both cAMP and cGMP (PDE1, 2, 3, 10, and 11). PDE4 is the most important drug target in COPD. PDE4 is composed by 4 gene families (A, B, C, D). All of CD^+4, CD^+8, Th1, Th2, Th17,eosinophils, neutrophils, monocyte, macrophages, dendritic cell and AECs express of PDE4A, 4B and 4D[249,251-259]. In peripheral blood neutrophils, CD^+4 and $CD8^+$T cells from patients with mild COPD, the expression of PDE4A, 4B and 4D were unchanged compared with the healthy subjects[260]. However, the expression of PDE4A4 (the subtype of PDE4A) was significantly increased in macrophages from BAL fluid of smokers with COPD compared with control smokers[250]. In addition, the distribution of SNP13 allele frequencies of the PDE4D gene was significantly different between the COPD and smoker in the Japanese population[261].

PDE inhibitors have been used as therapeutic agents for various diseases since 1977[262]. In 2011, the PDE4 inhibitor, roflumilast, was approved by the FDA for treating the

exacerbations of patients with severe COPD associated with chronic bronchitis and a history of exacerbations. There is a Cochrane Systemic Review that evaluated the efficacy and safety of roflumilast and cilomilast(another PDE4 inhibitor) in the management of stable COPD patients in 2011. This paper showed that PDE4 inhibitors significantly improved FEV1 regardless of COPD severity or concomitant COPD treatment; they also improved the quality of life (St George Respiratory Questionnaire [SGRQ]) and COPD-related symptoms, but could not improve exercise tolerance[263]. There was also an improvement in FEV1 in Asian COPD patients treated with roflumilast[264]. No serious adverse events were reported in the patients treated with PDE4 inhibitor compared with controls. However, roflumilast was associated with weight loss during the trial period[263]. There is another study on the efficacy of roflumilast used concomitantly with long-acting β(2)-agonists (LABAs) to reduce exacerbations of COPD patients, which reported that roflumilast reduced the rate of moderate or severe exacerbations, with LABA or without LABA [265] .

PDE4 inhibitors have the potential to target three main mechanisms of COPD: bronchoconstriction, mucus hypersecretion and inflammatory response[266-268]. Rolipram blocked secretions of LTC4 caused by eosinophils treated with formyl-met-leu-phe plus cytochalasin B (FMLP/B)[269]. LTC4 can trigger contractions in the smooth muscles lining the trachea, which is the main reason for bronchoconstriction[270]. Roflumilast was shown effectively in weakening EGF induced MUC5AC expression in human AECs to decrease the mucus hypersecretion[271]. There is evidence that PDE4 inhibitors regulate inflammatory cell chemotaxis to down-regulate the inflammatory response through inhibiting the release of chemokines. PDE4 inhibitor(RP73401) inhibited LTB4 production stimulated by LPS/ FMLP[272]. Roflumilast and its active metabolite (roflumilast N-oxide) concentration-dependently reduced the LPS-stimulated release of CCL2, CCL3, CCL4, CXCL10 from human lung macrophages, but without affecting that of CXCL1 or IL-8[273]. However, the

release of IL-8 was repressed by rolipram in primary human myometrial smooth muscle (HMSM) cells treated with IL-1β[274]. Rolipram also inhibited GM-CSF production in HMSM cells treated with IL-1β[273]. PDE4 inhibitors (rolipram, RPR-73401, RS-25344-000, cilomilast, and roflumilast) suppressed the release of IL-2, IL-4, IL-5, and TNF-α in a concentration-dependent manner in peripheral blood mononuclear cells[275].

1.1.4.6 Interactions among Inflammatory Pathways

NF-κB pathway is a core inflammatory pathway in COPD, which can be regulated by other pathways. Activated PI3K phosphorylated Akt, resulting in the activation of NF-κB via IKK[276]. There is also evidence that PI3k may activate NF-κB to express the inflammatory gene through regulating the release of ROS. It has been found that LPS-stimulated NCL-H292 cells secreted MMP-9 via EGFR/PI3K/Rac1/ROS pathway[205]. ROS directly induced the activation of NF-κB[277], and NF-κB is the main transcriptional factor of MMP9[278].

On the other hand, cAMP pathway also plays an important role in regulating the activity of NF-κB pathway. It has been shown that a down-regulation of NF-κB pathway at the transcriptional level is through the activation of CREB, a downstream factor of cAMP-dependent pathway[279,280]. CREB has been shown to competitively bind with CREB-binding protein (CBP) which is involved in the transcriptional co-activation of NF-κB. CBP can bind to both p- NF-κB and p-CREB in nuclear and then form a bridge to the basal transcription machinery to activate transcription[280]. Thus, cAMP pathway can regulate the transcriptional activity of NF-κB through competitively bind with CBP.

In addition, ERK1/2 and p38 MAPK dependent pathways also involve in the regulation of NF-κB pathway, however, the regulatory targets of them are different. Activated ERK1/2

phosphorylates IKK, but activated p38 pathway directly up-regulate the transcriptional activity of NF-κB to activate NF-κB pathway[281-283].

1.1.5 Experimental Models of COPD

Although patients are the most direct study subjects in disease research, the usage of them are widely limited by many factors, such as experimental methods, the research period, and medical ethics, etc. So instead researchers select adaptable disease model (animal or cell) to replace human as research subjects. Researchers have established wide varieties of disease models and even focus on the same disease, there are also many different models to express different pathological mechanisms. So the selection and establishment of the adaptable disease models are the first and important step in medical studies.

The existing disease models are divided into two broad categories: animal models and cell models of human diseases. These two types of model can satisfy different requirements in disease research. They are complementary to each other in their respective fields.

1.1.5.1 Animal Model of COPD

The animal model, using animal as the subject of study, simulates the performance and characteristics of human disease. According to the different reflecting degree of human disease, animal models are divided into three major types, including homologous, Isomorphic and predictive animal models. Homologous animal model is the most similar to human who bear the same diseases, have the same causes, symptoms and treatment options. However, as the cause of many human diseases is still uncertain, this kind animal model is very rare. Isomorphic animal model share the same symptoms and treatments with patients with same diseases, which is the most principle model in medical studies and is the most

common model. The last type of animal model is predictive animal model. It shares the treatment characteristics with patient with a disease. This kind of animal model is always used in drug screening.

The animal model of COPD is mainly divided into two types, according to the application of different inductors. Some studies use chemical substances, such as LPS and various enzymes to induce the COPD animal model[284]. All of them can be directly perused in airway to result in the local inflammation. These kinds of animal models belong to isomorphic animal model, and were used in the earlier studies of COPD. With the improvement of experimental technique, more and more researches use CS as inductor to establish the animal model of COPD. As this kind of model have the same cause, symptoms and treatment options with COPD patients, it belongs to homologous animal model. This is a specific animal model of COPD. There is a CS-induced acuate animal model (1~3 days[162,285]), which specifically displayed the abnormal inflammatory response in COPD[224]. This kind of animal model is widely used in the study about inflammation of COPD. The chronic animal model (six months or longer), which not only displayed the abnormal inflammation of COPD, but also showed the specific pathological manifestations of emphysema[286], was more suitable to the study about late COPD/emphysema.

There is a very important factor which can affect the result of the animal model induced by CS. It is the content of effective substances contained in smoke. As we all know, the main effective components of cigarette smoke are tar and nicotine and they are also used to control the quality of commercial cigarette. Although the contents of these effective components are different in the different brands of cigarette, they are fixed in the same brand cigarette. For example, each Marlboro Red contains 12 mg tar and 0.9 mg nicotine. Therefore, the selection of cigarette will affect the result of the preparation of CS induced

animal model. Besides these commercial cigarettes, there is a standard cigarette which is served as an international standard for research purposes and was approved by representatives of commercial manufacturers, which is made by the Kentucky Tobacco Research & Development Centre. There are more quality control on these reference cigarettes than commercial cigarette and provides a basis for comparing data that have been collected in different laboratories. The reference cigarette belongs three kinds: 1R3F (The reconstituted tobacco sheet portion of this blend was manufactured using the Schweitzer Process, 1974), 2R4F (a low nicotine cigarette, 2001) and 1R5F (an ultra low nicotine cigarette, 1989). Among these cigarettes, 1R3F is always used in the experimental work.

1.1.5.2 Cell Models of COPD

The cell model of human disease is a popular tool in the study about pathological mechanism and drug Intervention *in vitro*. Different with animal model, the cell model, using cell as the subject of study, simulates the performance and characteristics of human disease. Adaptable cell and corresponding inducer are the two major factors which affect the establishment of a cell model.

1.1.5.2.1 Selection of Adaptable Cell for Inflammatory Cell Models of COPD

As we all know, many kinds of inflammatory mediators are not expressed by cell under normal conditions, and even at inflammatory condition cells only express the specific inflammatory mediators themselves. Thus, it is very useful to collect the information that the expression of inflammatory mediator in the inflammation-related cell from COPD patients, which will be used as background information to help us select the adaptable cell for the inflammatory cell model of COPD. We collected relevant information from previous studies,

summarised the expression of various inflammatory mediators in cells collected from COPD patients (Table1-3).

According to the existing information from these studies, we found these studies used 5 types of cells as subjects (AM , monocytes ,airway epithelial cells, neutrophils and T cells) and 9 kinds of inflammatory mediators were involved in(IL-8, Gro-α, TNF-α,IL-9, TGF-β, MMP-1,MMP-9, ROS and NF-κb). Among these cells, macrophage and AEC showed more potent capability on the expression of various inflammatory factors. All of the expressions of these mediators were increased comparing with control group.

Table 1-3 Expression of inflammatory mediators in the cells collected from the patients with COPD

Product	**AMs**	**Monocytes**	**AECs**	**Neutrophils**	**T cells**
IL-8	↑[118]		↑[118]	↑[118]	
Gro-α			↑[26]		
TNF-α	↑[122]		↑[123]		
IL-9					↑[128]
TGF-β			↑[132]		
MMP-1	↑[137]				
MMP-9	↑[139]				
NF-κB	↑[70]				

The studies about inflammatory pathological mechanism of COPD *in vitro* mainly focued on 4 aspects of abnormal inflammation of COPD, including chemotaxis of inflammation related cells, release of inflammatory mediators, formation of tissue damage and repair (Fibrosis). The studies about the chemotaxis always use AM, AEC and neutrophils as subjects; the

studies about the release of inflammatory mediators always use AM, AEC and T cells as subjects, and among these cells, AM and AEC are more popular in previous studies; the studies about the formation of tissue damage always choose monocytes/AMs as subjects, especially using AMs as the representative; the studies about the fibrosis in COPD. We can use AEC, macrophage and ASC as subject. If researchers want to detect the regulation of NF-κB on the expression of inflammatory mediators, macrophage is a good choice.(Table3)

1.1.5.2.2 Inducers Involved in Inflammatory Cell Model of COPD

As we all know, CS and infected pathogens are two main risks in the pathogenesis of COPD. However, it is very difficult to simulate the process of CS and infection from human to cells. So researchers prepare the substitutes of CS and pathogenic microorganisms, cigarette smoke extract medium (CSEM) and LPS.

Lipopolysaccharide (LPS), a molecule consisting of a lipid and polysaccharide，elicits strong inflammatory responses in animals and cells. LPS is found in the outer membrane of gram-negative bacteria. Its Acyl chain embeds in the outer membrane of bacteria, and its sugar chains expose to the bacterial surface with the antigenic property. LPS is the most powerful inductor to prepare the cell model of COPD. It can induce various cells to express various inflammatory factors (Table4).

Although CS is the primary risk of COPD, it is very difficult to use cell as subjects to simulate CS process of human. As this method cannot be performed in cell-culture environment, it does not only increase the risk of cells infection, but also result in the increasing the ratio of cell death, so cigarette smoke extract (CSE) is applied gradually. The preparation of CSE simulates the process of cigarette smoking of human. Machine pump is used to simulate human smoking. Airtight pipes are used to connect the cigarette holder,

dissolution media and machinery pump. Actually, the dissolution media is cell culture media which is used to absorb the substance of smoke. Adjust the pump pressure and smoking frequency (typically 7 times per minute), so that the cigarette burn within the prescribed time (usually 3 minutes). The difference between commercial and homemade CSE is dissolution media. The dissolution media of commercial CSE is a filter, then use solution to dissolve the solute on the filter. The dissolution media of homemade CSE is cell culture medium. The medium containing CSE is named CSE medium (CSEM), which can be directly applied in the cell culture. However, as CSEM has poor stability, it is usually prepared just before it is to be used.

LPS and CSEM are used as potential inductors of inflammatory cell model of COPD. Besides them, there are other two inducers that are also used in the establishment of cell model of COPD. TNF-α and IL-β, as pro-inflammatory factors, not only are produced through the stimulation of LPS and CSEM, but are also used as inducers to stimulate cells to release the inflammatory mediators. Interestingly, TNF-α, as more powerfully pro-inflammatory factor, can also induce the release of IL-β (table4).

1.1.5.2.3 Inflammatory Cell Models of COPD

There are 16 kinds of inflammatory cell models commonly used for the studies of COPD. In these models, 5 types of cells are used as subjects (macrophage, airway epithelia, neutrophil, monocyte and lung fibroblast), all of them are the structure/functional cells of the lung. ; 4 kinds of inducers are involved (LPS, CSEM, TNF-α and IL-1β) and 12 kinds of inflammatory mediators ((IL-8, Gro-α, MCP-1, MIP-1. TNF-α, IL-1β, IL-6, IL10, GM-CSM, INF-γ, TGF-β and MMP-9) are produced by these models. Including IL-10, all of the expressions of these inflammatory mediators were increased in ICM of COPD. (Table4)

1.1.5.2.4 TNF-α Related Inflammatory Cell Model of COPD

TNF-α is a monokine which is mainly produced by monocytes, macrophages and AEC. LPS, CSEM and IL-1β are the main inductors of these cells to release TNF-α (Table1-4). There is a low level of TNF in normal condition. Once cells are stimulated by inducer, the release of TNF-α will boost at once. Thus, the establishment of TNF-α related to inflammatory cell model is the premise of the study about drug intervention effect on the synthesis of TNF-α. There are five inflammatory cell models which can release TNF-α, and LPS& CSEM induced macrophages are the main source of the production of TNF-α (Table 4).

The human macrophages are derived from differentiated monocytes. The monocyte is a mononuclear and a non-granular cell. These cells derive from bone marrow hematopoietic stem cells, and developed in the bone marrow, and finally released into the blood. Monocytes stay in the blood for 1-3 days, then migrating to different tissues as macrophages. Comparing with monocytes in blood, the volume of macrophages becomes greater; the number of intracellular lysosomal granules and mitochondria is increased; the phagocytic capacity strengthens. Primary macrophages are un-reproductive cells. Under suitable conditions, they survive for 2-3 weeks. Some cancerous mice provide the primary reproductive macrophages, such as P338D1 S774A.1, RAW309Cr, but these cells cannot develop in the cell lines. Currently reproductive macrophage line, such as RAW264.7, was established from a tumor induced by Abelson murine leukemia virus. However, if we want to use human macrophages as subjects, we can only obtain them through the differentiation of human monocytes.

There are two kinds human monocytes commonly used in related studies, THP-1 and U937. In 1984, Sundstrom and Nilsson isolated U937 from the histiocytic lymphoma of a 37 years old male patient. Previous studies showed that U937 was induced by human lymphocyte culture supernatant, vitamin D3, PMA, to differentiate into human macrophages[287-289]. Before differentiation, U937 is a suspension cell. After differentiation, differentiated mature macrophage-like u937 become adherent cell. The volume and intracellular granules are also increased. Both LPS and CSEM induced differentiated mature macrophage-like u937 to secrete TNF-α (Table1-4).

Table 1-4 Summary of the Inflammatory related cell models of COPD

Inducer	Cells	IL-8	Gro-α	MCP-1	CX3C	CXCL10	CCL5	TNF-α	IL-1β	IL-6	IL-10	IL-12	GM-CSF	INFγ	TGF-1	MMP-9
LPS	Macrophage	↑[290]		↑[291]					↑[291]			↑[292]				↑[293]
	AEC	↑[294]						↑[295]								
	Neutrophils	↑[296]														
	Monocyte							↑[292]		↑[127]	↑[292]	↑[292]				↑[297]
CSEM	AEC	↑[207]						↑[298]	↑[299]							
	Fibroblast	↑[300]														
	Macrophage	↑[301]														
	Neutrophils	↑[302]														
TNF-α	AEC		↑[26]						↑[303]							↑[170]
IL-1β	Fibroblast					↑[304]	↑[304]						↑[304]			
	Monocyte															↑[305]
	AEC	↑[306]														
H_2O_2	Neutrophils	↑[307]														
IL-17	Macrophage						↑[308]									
IFN-γ	AEC				↑[309]											
Cadmium	AEC	↑[310]								↑[310]						

1.2 Panax Ginseng

Ginseng, Panax ginseng C.A. Meyer, as a precious Chinese traditional medicinal herb, has been known for two thousand years. It was first documented in the *Shunqiuyundou volum of Weishu* in 6 B.C. *Shunqiuyundou* described ginseng as an herb only grow with the God's brilliance. In 200 A.D., another Chinese document, *Guangya*, defined ginseng as essence of *earth.*

1.2.1 The King of Herbs-Ginseng

Ginseng was first documented as herb in Divine Husbandman's *Classic of the Materia Medica* and its medicinal efficacy was also briefly introduced. Thereafter, ginseng was used in clinic. However, as people understood ginseng very little at that time, ginseng was just used as common herb. Until 1590, another pharmacy works, *Compendium of Materia Medica*, completely changed the clinical application status of ginseng. Subsequently, ginseng was worshiped as god herb and widely used in clinic. *Compendium of Materia Medica* was written by Shizhen Li in the Ming Dynasty. The book organised and supplemented the herbs knowledge which was gradually accumulated before the Ming Dynasty, and corrected the errors in them. The book was circulated in many countries after written and been praised as "Encyclopaedia in 1596" by Darwin. In this book, Ginseng's medicinal efficacy was more comprehensively introduced than before. Ginseng was described as a king of herbs which can cure various diseases, and can cure all the deficiency syndrome of men and women recorded. This book was also used as later clinical application guide of ginseng.

1.2.1.1 Ginseng and Deficiency Syndrome

Deficiency syndrome (DS) is a concept of Traditional Chinese Medicine (TCM). Whatever in TCM or modern medical science, DS plays a very crucial role. It promotes the process of diseases occurrence, development. Improving the DS can directly reverse the occurrence, development and even death of disease. Ginseng is such an herb which can be used in the treatment of all kinds of DS.

It is well known that there are over thousands of herbs were found, and hundreds of them were frequently used. These herbs were classified as different categories based on their different therapeutic properties, e.g. medicines that used to relieve superficies syndrome, or medicines used to strengthening body resistance. There are dozens of species of Chinese Herbs belongs to supplementing and boosting medicines, such as ginseng, astragalus, antler, rehmannia, polygonatum, etc. All these medicines have the effective supplementing and boosting functions and all can be used in DS. But why only ginseng can be used for both men and women to strengthen their deficiencies? Use the modern terminology, the DS in TCM concept cover the following two areas, the first is the Sub-health state before the onset of disease, and the other is the human body function decline in the process of disease development. In TCM theory, whatever which state in above, the key of treatment is tostrengthen and consolidate body resistance, and finally to get to the coordination of Yin and Yang. This can be analogy to restore the balance of human body ecosystem. However, the most important action of ginseng is to strengthen and consolidate body resistance. In TCM, this is the theory base that ginseng can be used to treat all kinds of DS.

There is another meaning that ginseng can treat DS. In all of the herbs, only ginseng was used as emergency medicine for saving the life which was threatened by severe deficiency syndrome. Ginseng can be used in saving the life of critically ill patients resulted by massive blood loss, severe vomit, diarrhea or chronic and serious illness. When only use

large numbers of concentrated decoction ginseng, ginseng can result in the strengthening of vitality and resurrection. In ancient China, Chinese always treasured ginseng and used it when family member near dying. Previous study showed that ginseng inhibited cardiomyocyte hypertrophy and heart failure[311]. The pharmacology studies showed that ginseng can be used to strengthen heart, boost blood pressure, regulates glucose metabolism and water-electrolyte metabolism[312-314]。

1.2.1.2 Ginseng Can Treat Many Kinds of Disease

As ginseng's special therapeutical effect on DS, which affects the occurrence and development of the disease, Ginseng was appraised as the panacea by later Chinese physicians. Ginseng was also used in the treatment of many kinds of modern diseases. The studies about modern pharmacology show that ginseng has many pharmacological actions such as anti-aging problems, anti-fatigue, antineoplastic, anti-inflammatory etc. [315-318]. As a result, ginseng has been widely used as a regulator of human multi-system such as nervous system, immune system, cardiovascular system, endocrine system, metabolic system and the endocrine system[319], and also has been used in the treatment of many diseases conditions, such as cardiovascular diseases ,certain metabolic conditions, wound-healing, respiratory diseases , chronic inflammatory diseases and cancers[182,311,320-322].

In summary, ginseng, as the king of herbs, has very prominent position and strong effect whatever in ancient China or in modern medical science. It has become the focus and hot spots of today pharmacological study. The Pharmacological mechanism of ginseng is very complex. Due to ginseng is the compound that composed by different active ingredients, in addition, there is a variety of forms on pharmaceutical use of ginseng, the premise on the

study of ginseng pharmacological mechanism is to research the main active component in ginseng and its application form.

1.2.2 The Application Forms of Ginseng

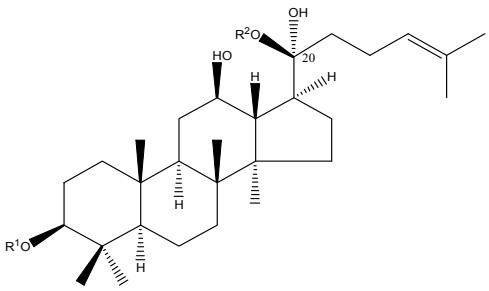
There is a variety of application forms of ginseng, and all of these forms can be divided into three categories：the active compounds of ginseng (gisenosides), the standardized extracts of ginseng and the orally administered ginseng formulas.

1.2.2.1 Chemistry of Ginsenosides

The main bioactive compounds in ginseng are triterpenoid saponins called ginsenosides, which can be classified into two groups, namely dammarane and oleanane (Ro) types, according to the difference of glycoside-based architecture[323]. Dammarane type includes two sub-types: protopanaxadiols (PDGs) (eg, Rb1, Rb2, Rc, Rd and Rg3) and protopanaxatriols (PTGs) (e.g. Rg1, Re and Rf) (Figure1) [324]. More than 150 ginsenosides have been identified so far[323]. The content of ginsenodies varies among different ginseng species. For example, *Panax ginseng* C.A. Mey contains Rf which is not present in *Panax quinquefolium* L, although the later contains 24- (*R*)-pseudoginsenoside F11, which has the same molecular weight as Rf[325] . Other ginsenosides, Rb1, Rb2, Rc, Rd, Rg3, Re and Rg1 are present in both species[325]. The mostly studied ginsenosides include Rb1, Rb2, Rc, Rd, Rg3, Re, Rf and Rg1[324]. It is important to point out that the actions of individual ginsenosides *in vivo* may depend on their bio-transformation or metabolism (e.g. hydrolysis) as it has been demonstrated that the bioavailability of ginsenosides is generally poor due to a low absorption through the gastrointestinal tract[326,327]. The major metabolites of PDGs are compound K (CK) and Rh2. The major metabolite of PTGs is Rh1[327].

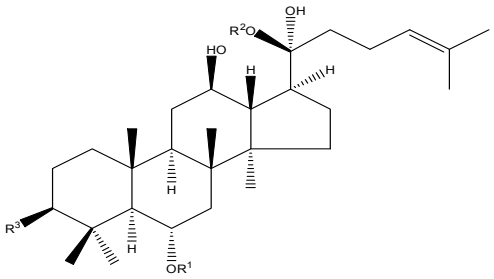
Dammarane type

Protopanaxadiol Class



	R^1	R^2
20(S)-Protopanaxadriol	H	H
Ginsenoside Rb1	glc(2─1)glc	glc(6─1)glc
Ginsenoside Rb2	glc(2─1)glc	glc(6─1)glc *p*
Ginsenoside Rc	glc(2─1)glc	glc(6─1)glc *f*
Ginsenoside Rd	glc(2─1)glc	glc
Ginsenoside Rg3	glc(2─1)glc	
Metabolites		
CK	H	H
Rh2	glc	H

Protopanaxatiol Class



	R^1	R^2
20(S)-Protopanaxatriol	H	H
Ginsenoside Re	glc(2─1)rha	glc
Ginsenoside Rf	glc(2─1)glc	H
Ginsenoside Rg1	glc	glc
Rh1	glc	H

glc= β-D-glucose
rha=α-L-rhamnose
ara=α-L-arabinose
p=pyranose
f=furanose

Oleanane types(Ro)

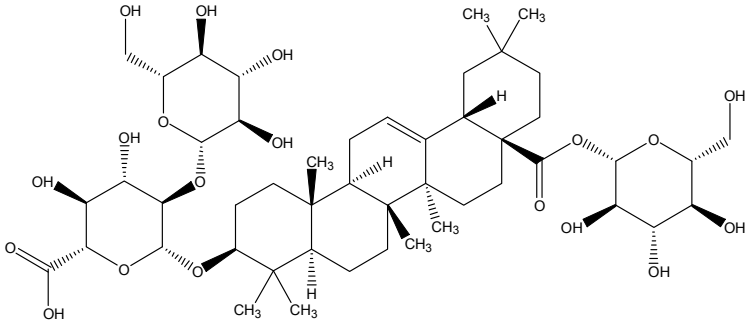


Figure 1-1 Structures of ginsenosides

1.2.2.2 Standardized Extracts of Herbs

Standardized Extracts of herbs (SEH) are referred to the extraction that satisfied certain quality level through standardized production procedures. They are deep processing products of herbs. SEH are widely used in China and other countries. In United States, the herb extracts are weighted as 95% in herb market, comparing with the raw materials and other product market shares that are less than 5%. In Germany, the herb extracts held 10% market shares in national medicine market. In China, as the major herb extracts exporter, supplies various herb extracts including Ginkgo Biloba, Hypericum Perforatum Linn, Acanthopanax, Angelica, Ginseng, etc. All herb extracts must comply with Chinese Herbal Medicine Production Quality Management Standards (GAP) and Pharmaceutical Production Quality Management Standard (GMP). At the same time, they should satisfy advanced technology and quality inspection standards, and use technology and equipment such as Macroporous Resin Separation Technology (MRS), High Performance Liquid Chromatography (HPLC, HPTLC), Gas Chromatography (GC), Gas Chromatography - Mass Spectrometry (GCMS), High Pressure Liquid Chromatography - Mass Spectrometry HPLC-MS）.

In China, the herb extracts have got some levels of scale of production. The enterprises specialized in producing herb extracts are over 200. However, due to the difference of purification technology and origin of herb planted, even the same herb extracts but with the different brand, the contents of active ingredient still have some difference, which results the pharmacology mechanism of these herb extracts has some slightly difference. The above reasons determined we need compose prescription from different brands of herbs extracts according to different requirement.

1.2.2.2.1 Standardized Extracts of Ginseng

Ginseng comprises a variety of active compounds. The pharmacological actions of ginseng were also determined by these compounds. Many factors can result in the change of the contents of these compounds in ginseng, such as different origin or harvest time of ginseng. Accompanied by the inevitably change of the content of these active compounds, the pharmacological actions of ginseng will also alter. In order to fix pharmacological actions of ginseng, we use ginseng standardized extracts to keep the content ratio of the different active compounds at a fixed range.

As we all know, different active compound of ginseng correspond to the specific pharmacological action. For example, polysaccharides of ginseng have the anti-tumour property, but ginsenosides have anti-inflammation property[328,329]. It means that we should choose a suitable preparation method to extract ginseng according to medicinal purposes. Actually, there are mainly two types of extracts of ginseng in clinical. One is a ginseng polysaccharides extracts, using ginseng as representation[19] another is ginsenosides extract, using G115 as representation[329,330].

G115 is a commercially available standardized ginseng extracts (Pharmaton SA, Switzerland). The total amount of ginsenosides in G115 is adjusted to 4% (w/w). Previous studies on G115 using high-performance liquid chromatography (HPLC) demonstrated that Rb1, Rb2, Rc, Rd, Re, Rg1 and Rg2 are the main ginsenosides which compose of G115[324]. To compare with other commercial extracts of Ginseng, G115 has higher Rg1 than others[324]. In the early application in clinical, G115 was always used for anti-aging purpose and previous studies show G115 improved the memory[331-333]. Later, G115 was used in the treatment of Parkinson's disease, and study showed G115 prevented various forms of neuronal cell loss including the nigrostriatal degeneration seen in Parkinson's disease[334]. G115 has anti- inflammatory properties[335]. As we all know, G115 is a ginsenosides-extract from Ginseng, so G115 reflect anti-inflammatory characteristic of ginsenosides.

1.2.2.2.2 The Orally Administered Ginseng Formulas

Orally administered herbal formula is the traditional administration method in Chinese medicine. To avoid variability of contents of active compounds of herbs among preparations of formulas, many researchers used commercial standardized extracts to replace raw herbs to compose the formula.

1.2.2.3 Ginseng Formula

From ancient time to current, ginseng is widely used as a main component in the formula of TCM. In TCM masterpiece, Treatise on Cold Pathogenic and Miscellaneous Diseases, there are 17 prescriptions include ginseng in all 112 prescriptions recorded. Treatise on Cold Pathogenic was written by *Zhongjing Zhang* in the Eastern Han Dynasty of China, who was regarded as the saint of Chinese Medicine. The book is a masterpiece that

discussed the treatment of various diseases, and was regarded as ancestor of Chinese prescriptions.

Some well known prescriptions using ginseng as main components including (1)*Pure Ginseng Decoction* (*Books about Cold Pathogenic*) (2) *Ginseng, Aconite Decoction* (*Annotation of Women Fang*) (3) *Ginseng, Aconite, Dragon Bone, and Oyster Shell Decoction (Ye ShiFang) (4) Ginseng-Ant Powder (Ji Sheng Fang) (5) Ginseng, Poria, and White Atractylodes & Powder (Ju Fang) (6) Ginseng Root-Securing Pill (Ye Shi Fang) (7) Shengmai Powder (Differentiating Internal and External Source of Sickness)*. In modern time, some physicians composed many prescriptions based on ancient prescriptions and experience of themselves, such as Supplemented *Ginseng Astragalus Decoction* that used to treat myasthenia gravis, *Ginseng, Curnu Cervi Pantotrichum and Qi Grass* used to treat cancer, *Cinnamon and Ginseng Decoction* used to treat chronic gastritis.

1.2.3 Ginseng and COPD

There is a long history of ginseng to be used in the treatment of COPD in China. Seven hundred years before, there is a famous Chinese medicine work in China, named *JishengFang*. In this book, ginseng and ant composed of *Shenyi Formula* was used to treat the chronic cough, shortness of breath, rapid breathing, wheezing after exercise or activity and low voice. Although there was no name of emphysema was shown in the work, all of these symptoms match the performance of emphysema which is among a group of COPD.

Recently, ginseng becomes a hot-point herb which is widely used in the treatment of various diseases, including COPD. G115 is a commercial standard extract of ginseng. Studies showed G115 improved PFTs, MVV, MIP and VO_2max in patients with

moderately-severe COPD[182]. Oral administration ginseng formula also improved the FEV1 and SGRQ in stable COPD patients[336].

Ginseng-Huangqi-Maidong Formula(GHMF) is one of the effective formula of COPD. It is provided by Guangdong Provincial Hospital and is composed by ginseng, Huangqi and Maidong. This formula is formed according to the clinical experiences from many famous Chinese medicinal doctors and has been used in the treatment of COPD for a long time. In order to improve the efficacy and standardize the quality control of GHMF, Guangdong Provincial Hospital provided four forms of GHMF for the secondary development. Among these forms, there are two forms which are composed by commercial extracts of Ginseng, Huangqi and Maidong from Guangdong and Jiangsu. We named them as GHMF-I (Guangdong) and GHMF-II (Jiangsu). The other two forms are directly extracted from raw herbs using different method. The difference between them is whether or not to contain polysaccharide in the final products. So we named them GHMF-III (excluding the polysaccharide) and GHMF-IV (including the polysaccharide).

Although ginseng (G115) and GHMF have a significant efficacy on COPD, the pharmacological mechanism of them is still uncertain. We are very interesting in the effect of G115, GHMF and ginsenosides on the inflammatory response, especially on the production of TNF-α and the activity of NF-κB pathway.

1.2.4 Effects of Ginseng /Ginsenosides on NF-κB Signalling Pathway

There is strong evidence that ginseng/ginsenosides can modulate multiple signalling components in NF-κB pathway, including NF-κB activation and related DNA binding and transcriptional activity, as well as the upstream regulating factors of NF-κB. The summary of multiple actions of ginsenosides is illustrated in Fig.1. In general, activation of NF-κB

leads to increase in its DNA binding activity and transcriptional activity, while inhibition of NF-κB activation leads to decrease in its DNA binding activity and transcriptional activity. The actions of ginseng and ginsenosides on NF-κB activation may explain their actions subsequent DNA binding and transcriptional activities. Thus detailed analysis is needed if identifying a direct action of ginseng or ginsenosides on particular components.

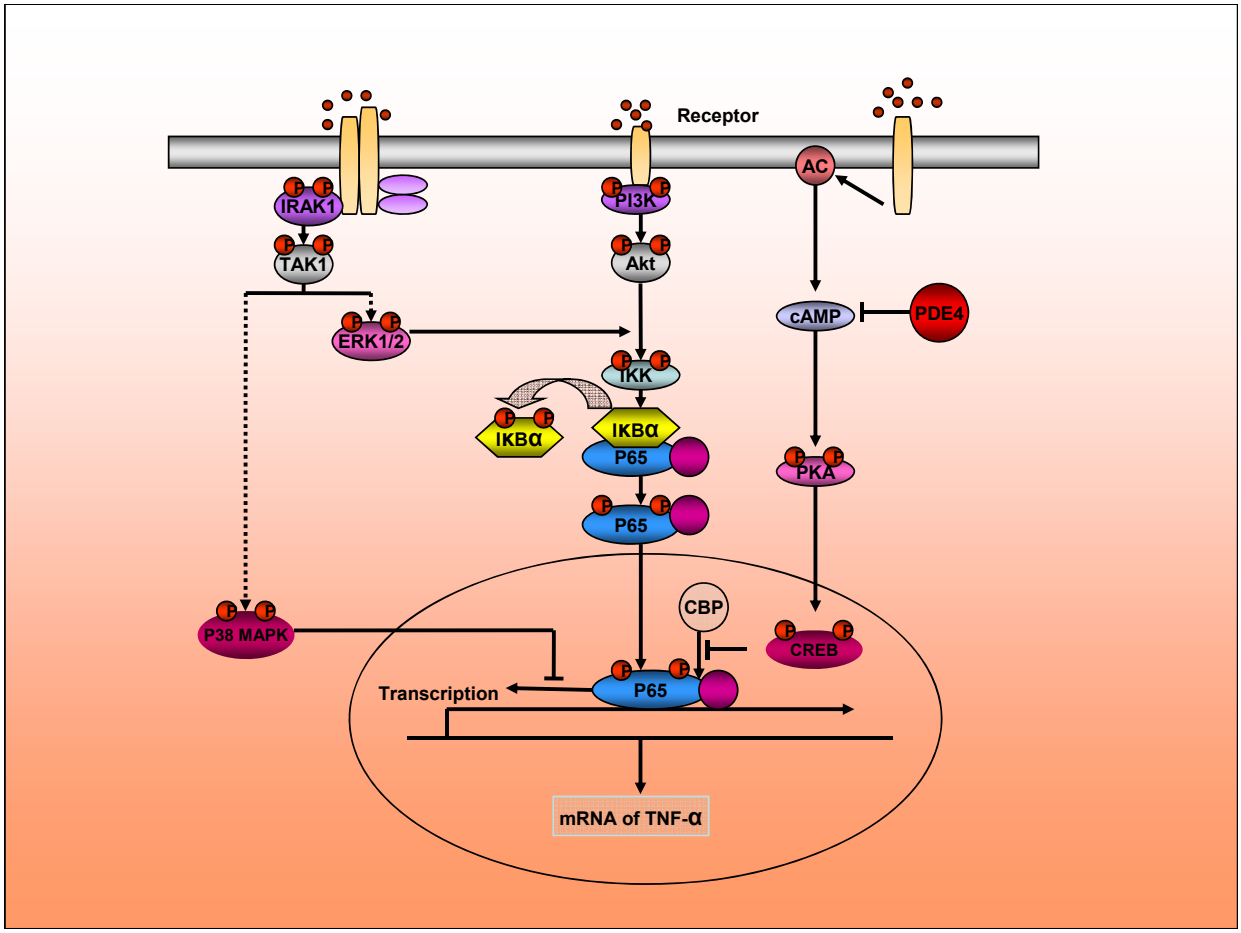


Figure 1-2 Cell signalling targets by ginsenosides on NF-κB signalling and related pathways.

Inactive NF-κB is located in the cytosol in a complex with the inhibitory protein I-κBα. A variety of extracellular stress-related signals can initiate phosphorylation of PI3K/Akt, IRAK1/TAK1/ERK1/2 to activate I-κB kinase (IKK). Activated IKK phosphorylates IκBα, which results in the dissociation of IκBα from NF-κB. Phosphorylated NF-κB is translocated into the nucleus and bind with specific sequences of DNA. The DNA/NF-κB complex then recruits CBP for co-activation, and then transcribes the mRNA expression of

TNF-α. cAMP degraded by phosphodiesterases (PDEs) works by activating protein kinase A (PKA). PKA can activate a cAMP response element-binding (CREB). Phosphorylated CREB can competitively bind with CBP to inhibit the transcriptional activity of NF-κB. Activated IRAK1/TAK1 also phosphorylates p38 MAPK pathway to up-regulate the transcriptional activity of NF-κB.

1.2.4.1 Regulation on NF-κB Activation

The key signalling components in regulating NF-κB activation include NF-κB, IKK, IκBα and phosphorylated products of them[337]. In different conditions, ginseng and ginsenosides displayed different regulation on the expression of these proteins. Under stressed conditions (e.g. treated with inducers such as LPS, H_2O_2, PMACI, TNBS, TNF-α and Streptozotocin), a number of ginseng, ginsenosides and their metabolites have been demonstrated to inhibit the expression of NF-κB, p65&p-p65, IKKa&p-IKKa and p-IκBα (Table1). In the basic condition (without any inducers), the transgenic ginseng (TG) and ginsenoside Rg3 showed the up-regulation on the expression of p-IκBα, and TG also up-regulated the expression of p-p65 in LLC-1cells[338,339](Table1). We collected the studies which involved in the inhibitions of more than one ginsenoside/its metabolite on these protein expressions, and tried to compare the distinction between them. We found all the papers just qualitatively determined the inhibition of ginsenoside and/or its metabolit on these protein expressions, but didn't do any quantitatively comparison between them[184,340,341]. Thus, the methodological perfection is necessary for the studies about ginseng and ginsenosides of the regulation on these protein expressions.

Table 1-5 Actions of ginseng and ginsenosides on protein expression related with activation of NF-κB

Protein	Test Agent	Source	Treatment	Effects
NF-κB	Rb2	N9 microglial cells	LPS	↓[184]
	Rd	N9 microglial cells	LPS	↓[184]
	Rg1	N9 microglial cells	LPS	↓[184]
		pheochromocytoma PC12 cells	H_2O_2	↓[342]
P-65	GSE	HMC-1 cell	PMACI	↓[343]
P-P65	Rb1	colon tissue	TNBS	↓[340]
		mouse peritoneal macrophages	LPS	↓[340]
	CK	colon tissue	TNBS	↓[340]
		mouse peritoneal macrophages	LPS	↓[340]
	Rg1	pheochromocytoma PC12 cells	H_2O_2	↓[342]
	GSE	LLC-1cells	--	↑[338]
IKKα	Re	3T3-L1 adipocytes cells	TNF-α	↓[344]
p-IKKα	CK	human astroglial cells	TNF-α	↓[341]
		u937	LPS	↓[341]
	Rh2	human astroglial cells	TNF-α	↓[341]
		u937	LPS	↓[341]
	Re	3T3-L1 cells	TNF-α	↓[344]
IKBα	Rg3	Raw264.7 cells	--	↓[339]
p-IKBα	Rb1	mouse peritoneal macrophages	LPS	↓[340]
	Rb2	N9 microglial cells	LPS	↓[184]
	Rd	N9 microglial cells	LPS	↓[184]
	Rg3	Raw264.7 cells	--	↑[339]
	CK	mouse peritoneal macrophages	LPS	↓[340]
		human astroglial cells	TNF-α	↓[341]
		u937	LPS	↓[341]
	Rh2	human astroglial cells	TNF-α	↓[341]
		u937	LPS	↓[341]
	Rg1	N9 microglial cells	LPS	↓[184]
	TGS	LLC-1cells	--	↑[338]
	FG	Rat pancreas , *in vivo*	Streptozotocin	↓[345]

LPS=Lipopolysaccharide; PMA+PMACI=12-myristate 13-acetate plus calcium ionophore; GSE= Ginseng extract; TGS=Transgenic ginseng; TNBS=2,4,6-trinitrobenzene sulfuric acid; LLC-1= Lewis lung carcinoma cells; FG=Fermented ginseng; STZ= Streptozotocin.

1.2.4.2 Regulation on NF-κB DNA Binding Activity

Many studies showed that ginseng and ginsenosides inhibited the NF-κB DNA binding activity *in vitro* and *in vivo*. All of the GSE, red ginseng extract (RGE), FG and ginsenosides including Rb1, Rc, Re, Rg1, Rg3 and the metabolite of Rg1 (Rh1) inhibited the NF-κB DNA binding activity[345-349]. Ginsenoside Rg3 used as a popular inhibitor of DNA binding of NF-κB activity was widely applied in many kinds of inflammatory cell models, such as TPA induced human breast epitelial cells (MCF-10A), docetaxel treated colon cancer cells and Aβ42 treated BV-2 cells[348,350,351]. It also inhibited the NF-κB DNA binding activity *in vivo*[347]. In addition, ginsenoside Rg1 seemed also displayed the more powerful inhibition on the DNA binding of NF-κB activity than other ginsenosides (Rb2, Rc, Re and Rg1)[347].

1.2.4.3 Regulation on NF-κB Transcriptional Activity

RGE, GTS and the PTG type ginsenoside Rg1 and its metabolite Rh1 significantly inhibited the transcriptional activity of NF-κB[347,350,352]. Jung et al. showed Rh1 just inhibited the transcriptional activity of NF-κB, but not the DNA binding activity of NF-κB in LPS induced BV-2 cells[352]. It meant that Rh1 inhibited the transcriptional activity of NF-κB through other way, but not NF-κB pathway.

Transcriptional activity of NF-κB, as representing terminal-response activity of NF-κB pathway, can be regulated by different pathway. It has been known that activation of cyclic adenosine monophosphatec (AMP)-dependent pathway can inhibit the activity of NF-κB pathway. One of the important cross links of cAMP and NF-κB pathways is phorsphorylated cAMP response element-binding (p-CREB), which is an activated transcriptional factor of cAMP-dependent pathway and can competitively bind to activated

NF-κB, and then inhibit the transcriptional activity of NF-κB[279,280]. P38 mitogen-activated protein kinases(p38 MAPK) pathway also involves in the regulation of transcriptional activity of NF-κB. Study showed that blocking p38 activity attenuated the transcriptional activity of NF-κB without altering its DNA-binding activity[283]. Ginseng and ginsenosides can also regulate the activity of both two pathways, so maybe they regulate the transcriptional activity of NF-κB through many pathways or specific pathway. However, to summarize the regulation of ginseng and ginsenosides on these pathways maybe help us to find out how to inhibit the transcriptional activity of NF-κB by Rh1.

1.2.4.4 Interaction with cAMP-dependent Pathway

Ginseng/ginsenosides can activate cAMP-dependent pathway, and ginsenoside Rg1 and Rb1 plays a crucial role in the regulation of ginseng on cAMP depend pathway. Both Rg1 and Rb1 increased the level of intracellular cAMP, and then increased the activity of PKA[353]. Rg1 increased the level of cAMP in hippocampus of both young and old rats, and Rb1 also increased the level of cAMP in rat liver compared with that in controls[354]. However, in the study of Jeong, ginseng total saponin (GTS) inhibited cAMP levels and protein expression of PKA in the tissue of locus coeruleus from morphin injected ICR mice[355]. It is possible that the regulation of cAMP by gisenosides depends on the nature of individual ginsenosides. As in this study, the content of Rg1 in GTS was very lower, only 6.42%[355]. The effect of Rg1 on cAMP maybe was eliminated by other potent ginsenosides.

It is likely that the effects of ginseng and its related product on cAMP may involve phosphodiesterases (PDEs), the key enzyme in cAMP degradation. An early study demonstrated that Rg1 inhibited the activity of cAMP-specific PDE in frontal cortex striatum, hypothalamus and hippocampus of young and aged rats, and Rg3 concentration-

dependently inhibited PDE resulting in increase of cAMP, as well as cyclic guanosine monophosphate (cGMP) contents in corporal smooth muscles[356].

Summary of existing evidences, we inferred that Rh1 inhibited the transcriptional activity of NF-κb through cAMP pathway, as the inhibited transcriptional activity by Rh1 accompanied with the increased p-CREB expression[352]. There are some conflicting evidences about the regulation of Rh1 on the activity. Jung et al. showed Rh1 just inhibited the transcriptional activity of NF-κB, but not the DNA binding activity of NF-κB in LPS induced BV-2 cells [352]. However, Jung, J. S et al. demonstrated Rh1 significantly inhibited the DNA binding activity of NF-κB in IFN-γ-stimulated BV2 microglial cells. Is Rh1 using p-CREB as a specific signalling component to modulate the transcription activity of NF-κB or p-CREB just a cofactor for Rh1 to modulate the transcription activity of NF-κB with NF-κB pathway? We need more experiment data to answer this question.

Many conclusive evidences showed that PTG type gisenoside Rg1 inhibited the transcriptional activity of NF-κB not only through down-regulating the up-steam activity of NF-κB pathway, but also down-regulating the activity of cAMP pathway[347,353]. Thus, as a more powerful inhibitor of NF- κB pathway, ginsenoside Rg1 can be more widely used in the related study.

1.2.4.5 Interaction with p38 MAPK Pathway

PDG type ginsenosides seems to play a key role in the inhibition of ginseng on the activity of p38 MAPK pathway. Studies demonstrated that PDG type ginsenoside Rb1, Rb2, Rg3 and CK, but not Rc or Rd, significantly inhibited the expression of p-p38[340,357,358]. Although many studies proved that ginsenoside Rb1, Rb2, Rg3 and CK down-regulated the activity of up-stream phosphorylation response and/or DNA binding activity of NF-

κB[340,184, 339, 341,347] , there are still lack of the direct evidence that ginsenoside Rb1, Rb2, Rg3 and CK inhibited the transcriptional activity of NF-κB through down-regulating the up-stream activity of NF-κB and the activity of p38 MAPK pathway. In addition, ginsenoside Rb1 displayed the inhibition on the up-stream activity of NF-κB and activity of p38 MAPK pathway and the up-regulation on the cAMP pathway[340, 44]. It may be the more powerful inhibitor on the activity of NF-κB pathway than Rg1, however, this hypothesis need more experimental results to verify.

1.2.4.6 Regulation on Upstream Pathway

It is possible that ginseng and ginsenosides may act on the upstream regulating factors of NF-κB pathway to modulate its activity, in particular TAK1/ extracellular signal-regulated kinase1/2(ERK1/2) and phosphoinositide 3-kinase(PI3K)/Akt pathways (Figure 2). As we all know, TAK1 can activate ERK1/2 pathway, and ERK1was used to activate IKK in NF-κB pathway[281,282]. Ginsenoside Rg1 and its metabolit (CK) have been shown to inhibit the p-IκB through inhibiting phorsphorylation of IRAK-1(p-IRAK1) which is known to activate TAK1, and this decreased expression of p-IRAK1 by Rb1 and CK accompanied with the down-regulation on the expression of phorsphorylated ERK1/2 (p-ERK1/2)[340]. There is a bidirectional regulation of ginseng and ginsenosides on the activity of ERK1/2 pathway. Under stress conditions, FG, GSE, TGS, Rb1, CK and Rg1significantly inhibited the expression of p-ERK1/2[340,343,345,359-361], except one study showing no effect of Re on ERK1/2 expression in TNF-α induced 3T3-L1 adipocytes[344] (Table3). On the other hand, under basal conditions, RG, Rg1 and CK were shown to enhance the expression of p-ERK1/2 under[362-364]. Choi compared the effects of the traditional GSE and novel TGS on the expression of P-ERK1/2 in human mast cell line cells, and found that EGS had more significant inhibition than GSE[343].

Table 1-6 Actions of Ginseng/Ginsenosides on the Expression of p-ERK1/2

Test agent	Source	Treatment	Effect
Rb1	mouse peritoneal macrophages	LPS	↓[340]
CK	human astroglioma cells	PMA	↓[360]
	mouse peritoneal macrophages	LPS	↓[340]
	mouse skin, *in vivo*	PMA	↓[361]
	MDA-MB-231 cells	--	↑[362]
Re	3T3-L1 adipocytes	TNF-α	--[344]
Rg1	human breast cancer cells	--	↑[363]
	rat basophilic leukemia-2H3 cells	SP+DNP-BSA	↓[365]
FG	pancreas tissue of Sprague Dawley rat	STZ	↓[345]
	RINm5F cells	STZ	↓[359]
RG	HUVECs	--	↑[364]
GSE	human mast cell line	PMA+PMACI	↓[343]
TGS	human mast cell line	PMA+PMACI	↓[343]

SP+DNP-BSA=Substance P+dinitrophenyl-bovine serum albumin; HUVECs= human umbilical vein endothelial cells.

Many studies have demonstrated that ginseng/ginsenosides may act on PI3K/Akt pathway. Zhou et al. found GSE significantly reduced infarct size in a dose-dependent manner, and this effect of GSE on infract was abolished by LY294002 (an inhibitor of PI3K) and Akt inhibitor IV (an inhibitor of Akt protein kinase)[366]. The difference in actions of ginsenosides on PI3K/Akt pathway has been reported. In mouse model of ischemia-reperfusion (I-R) injury, GSE, TGS, Rb1 and CK protected the myocardiumin in I-R injury through increasing the expression of phorsphorylated-PI3K (p-PI3K)[367-370]. Ginsenoside Rd, also increased the expressions of p-PI3K in the study of anti-apoptotic[371]. In addition, ginsenoside Rb1 rapidly increased the expression of p-Akt in human aortic endothelial cells[372], and ginsengoside Rg3 elevated the enzyme activities of PI3-kinase in ECV 304 human endothelial cells[373]. However, similar with the regulation on NF-κB activation, in the inflammatory condition, ginsenoside Rb1 significantly inhibited the phosphorylation of

AKT induced by high glucose[357]; CK, the metabolite of Rb1, also prevented the phosphorylation of Akt in basic fibroblast growth factor (bFGF) treated human umbilical vein endothelial cells (HUVECs)[358].

1.2.4.7 Actions of Ginseng/Ginsenosides on TNF-α Production

Most of the studies showed the down-regulation of ginseng and ginsenosides on the expression and /or lease of TNF-α (Table5). Only one paper reported that ginseng extracted by saline increase the protein level of TNF-α in anti-CD3 and the outer membrane protein of P. aeruginosa (OMP)/ Concanavalin A (ConA) treated the primary lung cells from CBA/J mice[374]. In this paper, Song et al. established a T helper type 2 (Th2)-like response cell model through being treated by monoclonal hamster anti-mouse CD3 without sodium azide (anti-CD3), and then stimulated by OMP/ConA to release TNF-α. The extract of ginseng collaborating with OMP and ConA promoted a Th1-like response to up-regulate the release of TNF-α and IFN-γ. However, in Th1-like response to cell model, ginseng demonstrated the significant down-regulation on the release of TNF-α. As we all know, that activated macrophages induced a strong polarized Th1-like T cell response[375]. The extract of red RG significantly reduced the expression of TNF-α in LPS induced monocyte-derived macrophage THP-1 cells[376]. Thus, there should be a bidirectional regulation of ginseng on the release of TNF-α in different type of cell model. In addition, different extraction methods may be another possible factor which influences the effect of ginseng extracts on the release of TNF-α.

Ginseng extracted by saline up-regulated the production of TNF-α, but RG extracted by water down-regulated it. Previous study provided a similar result that the aqueous and alcoholic extracts of ginseng have opposite regulation on the release of TNF-α. In this

paper, aqueous extract of ginseng , but not alcoholic extract of ginseng up-regulated the release of TNF-α[377]. We couldn't confirm whether different extract methods were the reason leading to opposite regulation of the ginseng extracts on TNF-α, as there are no -results of HPLC in these studies. It is just an assumption and need more experimental data to prove it.

All of the studies involved in ginsenosides showed the significant inhibition on the production of TNF-α (Table5). There seemed to be no difference of the regulation on the production of TNF-α between PDGs and PTGs[184]. Wu showed both PDGs (Rb2& Rd) and PTGs (Rg1& Re) inhibited the protein level of TNF-α in LPS induced N9 microglial cells[184]. Although Rg1 showed stronger inhibition compared with other ginsenosides, another PTG (Re) showed the similar inhibition with PDGs.

There are two aspects affect the widespread use of ginseng, low absorption rate and complex actions (sometime opposing or contradictory). Many studies focused on finding novel ginseng extracts to increase absorption rate and/or be beneficial to biological activity. Different ginseng extracts, such as fermented ginseng (FG)[359]and transgenic ginseng(TG)[378] have been used in the studies, and they showed significant inhibition on TNF-α

.

Table 1-7 Actions of ginseng and ginsenosides on the TNF-α expression

Products	**cell/tissue**	**Treatment**	**Effects**
FG	RINm5F insulinoma cells	streptozotocin	↓[*359]
RG	pancrease tissue, *in vivo*	diethyldithiocarbamate	↓[Δ379]
	THP-1 cells	LPS	↓[*376]
TG	HMC-1 cells	PMA&PMACI*	↓[#378]
Ginseng	lung cells of CBA/J mice	anti-CD3,OPM&ConA	↑[#374]

Rb1	PBMC&FLS cells	IFN-γ, LPS or IL-1	↓[#380]
	Raw264.7& U937 cells	LPS	↓[#381]
	HaCaT cells	Capsaicin	↓[#382]
Rb2	N9 microglial cells	LPS	↓[#184]
	Raw264.7& U937 cells	LPS	↓[#381]
Rc	Raw264.7& U937 cells	LPS	↓[#381]
Rd	N9 microglial cells	LPS	↓[#184]
Rg1	N9 microglial cells	LPS	↓[#184]
Rg3	murine BV2 microglial cells	β-amyloid	↓[Δ351]
	ears tissue, *in vivo*	Oxazolone	↓[*383]
Re	N9 microglial cells	LPS	↓[#184]
Rf	ears tissue, *in vivo*	Oxazolone	↓[*383]
Rh2	ears tissue, *in vivo*	Oxazolone	↓[*383]
	murine BV2 microglial cells	LPS/IFN-γ	↓[Δ384]

*: gene expression

Δ: protein expression

#: protein level

1.2.5 Yin and Yang Actions of Ginseng and Ginsenosides

"Yin" and "Yang" are two important ancient philosophical concepts in China. "Yin" covers a variety of inhibition and down-regulating response, and "Yang" covers a variety of promotion and up-regulating response. It has been reported previously that ginseng has a complex action on modulation of cell functions with both "Yin" and "Yang" aspects. For example, it has been shown that Rg1 stimulates angiogenesis, whereas Rb1 exerts an opposing effect, and mechanistic studies revealed that such responses were mediated through the PI3K/Akt pathway[385] . In this review, ginseng and ginsenosides also displayed the "Yin" and "Yang" actions on the activity of NF-κb pathway and the production of TNF-α. We found different conditions may be the main reason resulting in these opposite actions. In non-inflammatory condition, such as cancer cell models, immune cell models and normal cells, ginseng and ginsenosides display up-regulation (Yang action) on the activity of NF-κB pathway and the production of TNF-α, but in inflammatory model

ginseng and ginsenosides demonstrate significant down-regulation(Yin action). In addition, opposite regulation of individual ginsenosides maybe also play a role in it, as different extract method also result in the opposite action of ginseng in the same inflammatory cell model[377].

In conclusion, ginseng and ginsenosides have multiple actions on NF-κB signalling pathway and TNF-α production, including modulation of NF-κB activation and related DNA binding and transcriptional activities, upstream regulating pathways such as TAK1/ERK1/2 and PI3K/ Akt, as well as other potential mechanisms through P38 MARK and cAMP pathways. The difference in actions of individual ginsenosides may be involved in diverse pharmacological activities of ginseng. Given TNF-α and certain NF-κB signalling components are defined or potential drug targets, further studies on the actions of ginsenosides on TNF-α and NF-κB pathways may facilitate the development of novel agents to target the relevant mechanisms for potential therapeutic application for treating chronic inflammatory diseases.

1.3 Objectives of my Ph.D Thesis

In this project, we hypothesized that ginsenosides, G115 and GHMFs may target TNF-α and NF-κB signalling pathway. To test our hypothesis, we have:

a) Finished the review: Regulations of cellular NF-κB pathway by ginseng and ginsenosides

b) Finished the review: Inflammatory cells, mediators and singling pathways in COPD

c) Set up inflammatory models *in vitro.*

d) Investigated the effect of ginseng and ginsenosides on cytokines production induced by LPS.

e) Investigate the effect of ginseng and ginsenosides on NF-κB pathway induced by LPS

f) Investigate the effect of ginseng and ginsenosides on cAMP pathway induced by LPS

In addition, we also put forward hypothesis that TNF-α inhibitors used in the conjunction in the MTX provide a better efficacy against the progression of JD in patients with active RA comparing with MTX used alone; the degree of the disease activity of RA is important factor for the efficacy of TNF-α inhibitors on the progression of DJ in active RA patients. To test our hypothesis, we have used Meta analysis to -

g) Compare the effect of TNF-α inhibitors used alone and combined with MTX on the RP of active RA patient.

h) Investigate the correlation between the degree of activity of RA and the efficacy of TNF-α inhibitors on RP in active RA patients

i) compare the effect of different type of TNF-α inhibitors on RP in active RA patients

Chapter 2. Establishment of Inflammatory Cell Models Related to COPD

2.1 Introduction

The main pathology of COPD is persistent inflammation in the airway and lungs (see Section1.1.1). In order to understand the actions of ginseng and its cellular mechanisms on COPD, it is important to study the effects of ginseng on inflammatory mechanisms using an appropriate cell model.

LPS and CSE are commonly used in studies on inflammatory mechanism of COPD and macrophages are the common cell type to study various inflammatory mediators[386,387]. As there are no commercially available human macrophage cell lines, researchers use PMA induced U937 cells differentiation to macrophage-like U937 cells in related studies (see Section1.1.5.2.4). Previous studies have shown that LPS and CSE can stimulate macrophages to release TNF-α, IL-1β, IL-6 and other cytokines through the activation of the NF-κB pathway[241,388,5,6]. LPS can also elevate the production of cAMP to activate the cAMP pathway[389]. Thus, aim of this part of my project was to establish the inflammatory cell models using LPS and CSE induced macrophage-like U937 cells. The focus was on the cytokine, in particular TNF-α production and the changes of NF-κB and cAMP pathways.

2.2 Materials and Method

2.2.1 Materials

Phorbol 12-myristate 13-acetate (PMA), Lipopolysaccharide (LPS), Bay11-7082, Forskolin, H89, Cilostamide (PDE3 inhibitor), Rolipram (PDE4 inhibitor), Sildenafil

citrate salt (PDE5 inhibitor) and Penicillin-Streptomycin were purchased from Sigma (Aldrich PTY LTD, Australia).

RPMI1640, heat-inactivated fatal bovine serum, Opti-MEM® Reduced Serum Medium and MEM Non-Essential Amino Acids Solution were purchased from Gibco (Invitrogen Australia Pty Limited, Australia). The ELISA Kits of TNF-α, IL-1β and IL-6 were from GE (GE Healthcare Australia Pty. Ltd, Australia). Cyclic AMP EIA Kit was purchased from Cayman (Sapphire Bioscience Pty. Ltd, Australia). Phospho-IKKα/β (Ser176/180) antibody, IKKβ antibody, Phospho-NF-κBp65 (Ser536) antibody, NF-κB p65 antibody, Phospho-CREB (Ser133) antibody, CREB (48M2) antibody, Phospho-IκBα antibody and IκBα antibody were purchased from Cell Signalling (Genesearch PTY. Ltd.). NE-PER nuclear & cytoplasmic extraction kit was obtained from Thermo Fisher (Thermo Fisher Scientific Australia Pty Ltd). Dual-Glo® Luciferase Assay System and with reporter lysis buffer was purchased from Promega (Promega Australia). Attractene Transfection Reagent and Signal NF-κB Reporter (luc) Kit was purchased from QIAGEN (QIAGEN Pty. Ltd.).

2.2.2 Method

2.2.2.1 Cell Culture

U937 cells were obtained from the lab of Bernard O'Brien (Bernard O'Brien Institute of Microsurgery, The University of Melbourne, Victoria, Australia) and cultured in RPMI1640 medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum at 37°C and 5% CO_2 in humidified air and harvested at the log phase of growth.

To differentiate suspension U937 cells into adherent macrophages, cells (10^6cells/ml) were incubated in petri dish ($24x10^6$cells/dish) for 24 hours in the presence of 10 ng/ml PMA. The differentiated cells were made quiescent in fresh completed RPMI without PMA for 48 hours and during which the culture medium was replaced daily. U937 macrophages were obtained as slightly modified method described previously[390-392].

2.2.2.2 Preparation of Cigarette Smoke-treated Cell Culture Medium

CSE was obtained from four cigarettes (Marlboro Red, 12 mg tar, 0.9 mg nicotine each). Briefly, smoke from combustion cigarettes was bubbled through 50 ml of culture medium and the burning time of each whole cigarette is 3 minutes. In order to avoid the difference among different cigarettes, the absorbance of this CSE was adjusted to 1.0 (optical density, OD) at 320 nm, which was used as the stock CSE (defined as 100 %)[393]. For cell experiments, the stock CSE was diluted with the cell culture medium at 5-fold dilution (20%).

2.2.2.3 Cytokines Assay

Macrophage-like U937 cells were detached with trypsin, washed, resuspended in medium at 10^6cells/ml and incubated in 96-well plates (10^5cells/well). After 24 hours, treated with LPS (1μg/ml) for 6 hour or added 5–fold dilution of raw CSEM for 18 hours; or treated with LPS (1μg/ml) in absence or presence of Bay11-7082, Forskolin, H89, Cilostamide, Rolipram or Silderafil for 6 hours. The supernatant of the test samples was collected and stored at -80°C to determine TNF-a, IL-1β and IL-6.The concentrations of TNF-a, IL-1β and IL-6 in the culture supernatants were determined by human enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's protocol.

2.2.2.4 cAMP Assay

Macrophage-like U937 cells were cultured in 24-well cell culture plates at a concentration of $1x10^6$ cells/well. After 24 hours, cells were treated with 1μg/ml LPS in the absence or presence. After 6 hours, the cell culture medium was aspirated. The cells were placed on the ice and washed by cold PBS for three times. Adding 0.25ml lysis buffer (0.1 M HCl) in each well. After 10 minutes, the total cell lysates were centrifuged at 6000g for 10 minutes. The supernatants were assayed directly using the cAMP EIA kit from Cayman Chemical according to the manufacturer's instruction (Sapphire Bioscience Pty. Ltd, Australia).

2.2.2.5 Western Blot

Macrophage-like U937 cells were detached with trypsin, washed, resuspended in the medium at 10^6cells/ml and incubated in 6-well plates ($2.5x10^6$ cells/ well) for 24 hours. Cells were treated with LPS (1μg/ml) for 5, 15, 30 or 60 minutes. Samples were washed by cold PBS and put on ice for protein collection. The cells were lysed using RIPA buffer and plus protease and phosphatase inhibitors on the day of use to obtain total protein (Appendix1). The lysate was immediately scraped, collected into microcentrifuge tubes and vortexed for 15 seconds. The samples were extracted by high-speed centrifugation at 14000Xg for 15 minutes to precipitate the insoluble materials. The nuclear and cytoplasmic protein were lysed and separated by NE-PER nuclear & cytoplasmic extraction kit (Thermo Fisher Scientific Asutralia Pty Ltd) according to manufacturer's instructions. Protein was quantified using Bradford Protein concentration assay (Appendix 2). The total protein was adjusted at 1.5μg/μl. The nuclear and cytoplasmic protein was adjusted at 1μg/μl.

Protein levels were measured by Western blot analysis. Protein samples were loaded in wells of SDS-polyacrylamide gel (10%acrylamide, Appendix 3) for electrophoresis at 90V for 30 minutes and then increased the voltage to 150v for another 60 minutes in running buffer (Appendix 4). The separated gel was transferred to PVDF by electrophoresis at 100 V for 120 minutes in transferring buffer (Appendix 4). The membranes were washed once by TBS-T (Appendix 4) and then blocked for 1 hour in TBS-T containing 1% (wt/vol) bovine serum albumin (BSA). After washing the membranes with TBS-T 6 times, the membranes were incubated with primary antibody of target protein overnight at 4°C. Polyclonal antibodies were diluted in different concentrations in TBS-T containing 3% (wt/vol) BSA before incubation. The concentrations of these antibodies were 1:2000 (Phospho-IKKα/β), 1:600 (Phospho-Iκα, NF-κB and Phospho-NF-κB) and 1:1000 (CREB and Phospho-CREB). After incubation with primary antibody in TBS-T containing 5% (wt/vol) skim milk or 3% BSA, membranes were washed with TBS-T for 6 times again and treated with anti-rabbit antibody (1:3000) for 2 hours in room temperature. After six washes with TBS-T, the blots were incubated with chemiluminescence reagent and exposed to X-ray film. After exposure, membranes were stripped by stripping buffer (Appendix 4) at 50°C for 15 minutes, washed for 3 times, incubated with blocking buffer for 1 hour and washed for another6 times. The stripped membranes were prepared for the second- time incubation of primary antibodies, including IKKβ (1:2000), IκBα(1:600) and β-Tublin used as internal control(1:3000). These membranes were also incubated with the second antibody, including anti-rabbit antibody (1:2000) or anti-mouse antibody (1:3000) for 2 hours.

2.2.2.6 NF-κB Reporter Assay

Macrophage-like U937 cells were seeded in 24-well plate (2.5x 10^5/well). After 24h, refresh medium with 500µl Opti-MEM® containing 1.5µl attractene transfection reagent (QIAGEN Pty. Ltd.) and 0.6µg NF-κB promoter-firefly/Renilla luciferase (40/1)/negative control/positive control (QIAGEN Pty. Ltd.) each well. After 18 hours incubation, refreshed the cell with normal culture medium for another 6 hours, and then refreshed the medium with 1µg/ml LPS. After 6 hours, the cells were rinsed with cold PBS and lysed with 100µl reporter lysis buffer (Promega) in each well. The lysate was centrifuged at 15,000 × g for 5 minutes at 4°C, and the supernatant was harvested. Both firefly and Renilla luciferase levels were measured by a luminometer using the Dual-Glo® Luciferase Reporter Assay System (Promega Australia).

2.2.2.7 PDE Assay

Macrophage-like U937 cells were seeded in 6-well plates (2x 10^6/well). After 24h, cells were treated with or without 10µM Rolipram[394], following incubation with 1µg/ml LPS for 4 hours. The medium were aspirated and the cells washed with ice-cold PBS, which was then aspirated before lysis buffer was added. The lysis buffer used to extract PDE for PDE assay was made from 50 mM Tris (pH 7.5), 1.5 mM EDTA, 1 mM benzamidine, 0.1 mM sodium orthovanadate, 1 mM DTT and supplemented with a protease inhibitor cocktail for use with mammalian cells. Lysis was allowed to progress on ice for 1 hour before gentle agitation. The homogenate was subjected to centrifugation 10 minutes at 12,000xg (4°C) and the supernatant was stored at –80°C for further analysis.

PDE activity was determined using the method described by Thompson and Appleman two-step procedures with modification[394-396]. Briefly, cells were incubated with the incubation mixture and treated with various test agents (0.1 ml/sample) at 30°C for 10

minutes. The incubation mixture (0.4 ml) contained 1 μM cAMP, [3H]cAMP in 40 mM Tris-Cl buffer (pH 8.0) containing 3.75 mM β-mercaptoethanol and 15 mM $MgCl_2$. The reaction was terminated by incubation for 1 minute in a boiling water bath, and the crotalus atrox snake venom was added for 5-min incubation at 30°C. The hydrolyzed nucleotide was separated using an A-25 anion exchange resin column. The reaction combined with scintillation cocktail, which was quantified by scintillation counting. Protein concentration was determined using the Bradford method.

2.3 Statistical Analysis

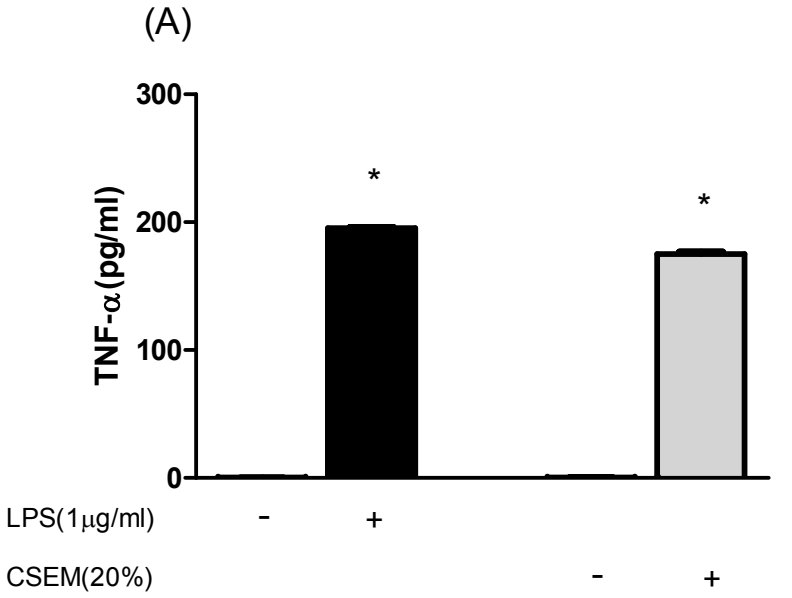
All values presented are mean ± SEM of the given number (n) of experiments. The two-group comparison was conducted by Student's t-test. The multi-group comparisons were conducted by one-way analysis of variance (one-way ANOVA) followed by Bonferroni's test. P values of 0.05 or less were considered to be statistically significant and tests were performed using GraphPad Prism version 5.0 Software (GraphPad Software, Inc La Jolla, CA).

2.4 Results

2.4.1 Release of Cytokines in LPS and CSEM Induced Cell Models

In unstimulated macrophage-like U937 cells, the basal level of TNF-α was barely detectable. Cells treated with LPS (1μg/ml) or CSE (20%) significantly increased the release of TNF-α. There is a significant increase in TNF-α release (600 and 500-fold) in cells after the treatment with LPS(6 hours) or CSE(18 hours) respectively, compared with the blank control ($p<0.001$; Fig.2-1-A). There was no statistically difference between LPS

and CSEM on TNF-α release ($p>0.05$; Fig.2-1-A). In addition, LPS also significantly stimulated macrophage-like U937 cells to release IL-1β and IL-6 ($p<0.001$; Fig.2-1-B).



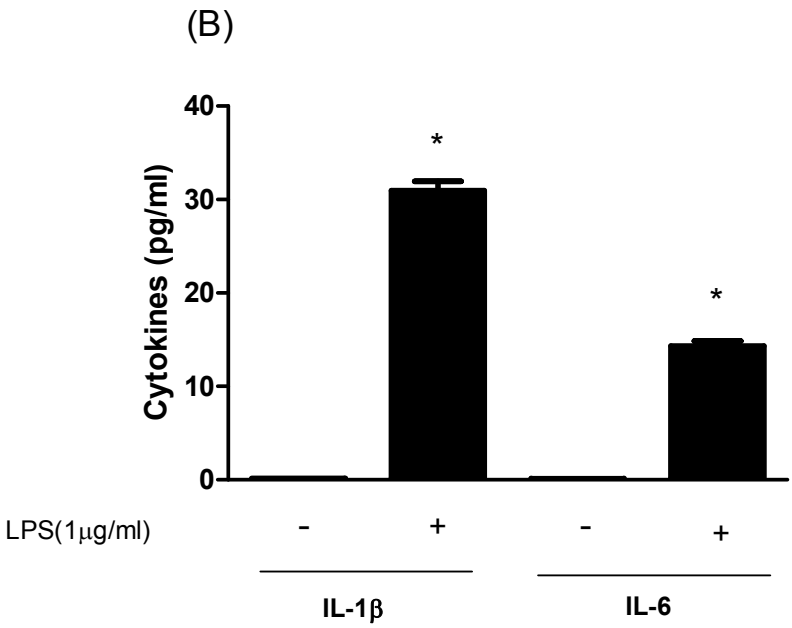


Figure 2-1 Release of TNF-α induced by LPS&CSE and the release of IL-1β & IL-6 induced by LPS in macrophage-like U937 cells.

Macrophage-like U937 cells were treated with or without 1ug/ml LPS for 6 hours; or treated with or without CSE (20%) for 18 hours. The concentration of TNF-α (A), IL-1β and IL-6(B) were tested by ELISA. Data represent the mean ±SEM (n=3) performed in duplicate. *p < 0.001 Vs blank vehicle by Student's t-test. Student's t-test also was used to compare the difference between LPS and CSE group.

2.4.2 Production of cAMP in LPS Induced Macrophage-Like U937 Cells

Macrophage-like U937 cells treated with LPS (1μg/ml) for 6 hours had a significant higher level of cAMP, determined by EIA method, compared with the blank control.($p<0.05$;Fig.2-2).

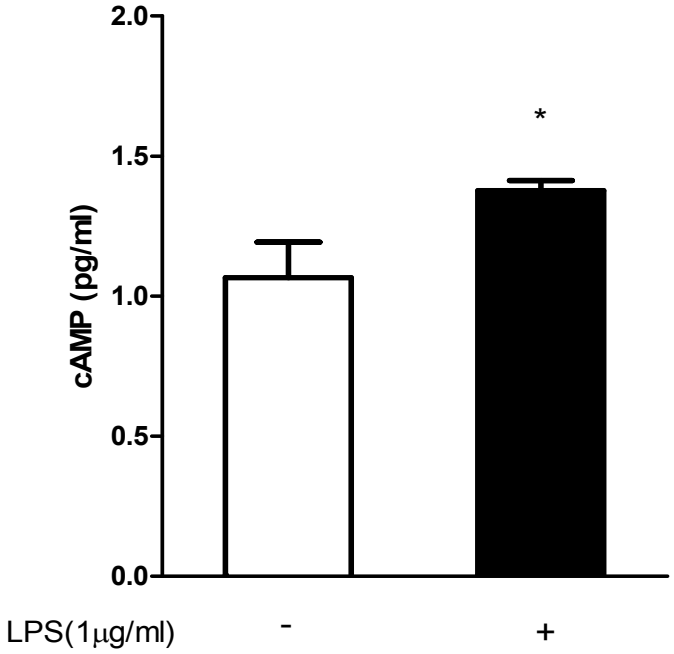


Figure 2-2 Production of cAMP in LPS Induced Macrophage-Like U937 Cells.

Macrophage-like U937 cells were treated with or without 1μg/ml LPS for 6 hours. The level of cAMP was tested by EIA method. Data represents the mean±SEM (n=3) performed in duplicate. *p < 0.05 Vs blank vehicle by Student's t-test.

2.4.3 Expression of Phosphorylated Proteins in NF-κB and cAMP Pathways in LPS Induced Macrophage-Like U937 Cells

To confirm the involvement of the activation of IKK, IκBα, p65 and CREB in LPS induced macrophage-like U937 cells, the time course of expressions of p-IKK, p-IκBα, p-p65 and p-CREB were analysed by Western blot. Stimulation with LPS (1μg/ml) induced a rapid phosphorylation of IKK as early as 5 minutes, with the maximal activity was observed at 15 minutes. Similarly, the activated IκBα was appeared at 5 minutes, and the maximal activity of at 30 minutes. LPS increased the expression of p-p65 in a time-dependent manner; with the maximal effect at 30 minutes and then declined after 30 minutes. The expression of p-CREB was not significantly changed at 5 and 15 minutes, but it was increased at 30 minutes, and this increase was sustained at 60 minutes. (Fig.2-3)

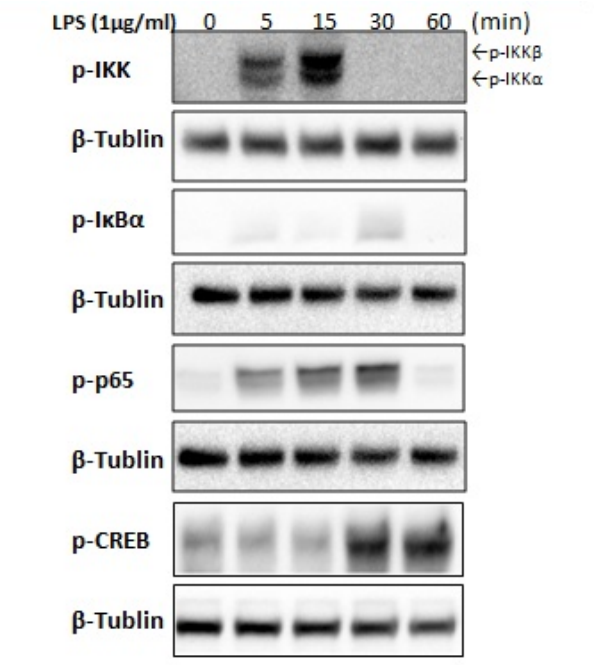


Figure 2-3 Expression of Phosphorylated Proteins in NF-κB and cAMP Pathways in LPS Induced Macrophage-Like U937 Cells.

Macrophage-like U937 cells were treated with or without LPS (1μg/ml) at 5, 15, 30, 60 minutes. The expression of p-IKK, p-IκBα, p-p65 and p-CREB were analysed by western blot using p-IKK, p-IκBα, p-p65 and p-CREB antibodies as described in the methods. B-Tublin was used as a loading control.

2.4.4 Effects of Bay11-7028 and Foskolin on the Expression of Proteins in NF-κB and cAMP Pathways in LPS Induced Macrophage-Like U937 Cells

Bay11-7082, but not Foskolin, significantly inhibited the expression of p-IKK, p-IκBα, p-p65 and IKK, IκBα, p65 induced by LPS ($p<0.001$, $p>0.05$; Fig. 2-4-A&B). Foskolin statistically increased the expression of p-CREB ($p<0.001$), but not CREB ($p>0.05$) induced by LPS (Fig.2-4-A&B).

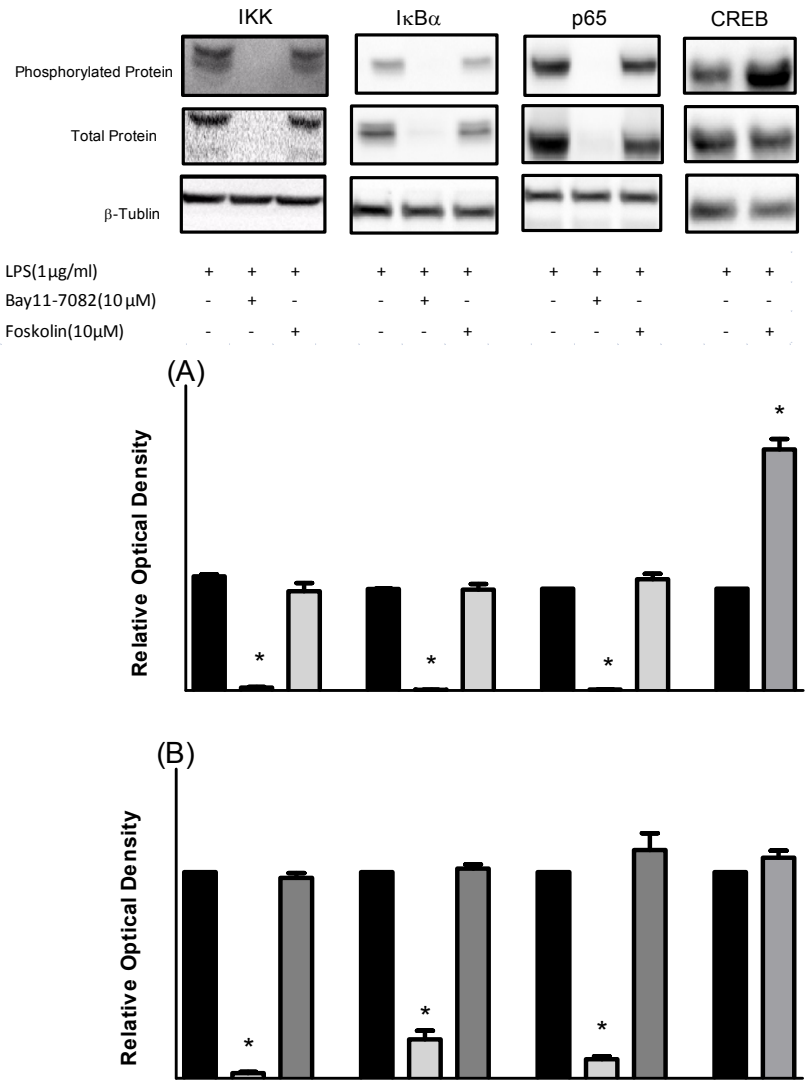


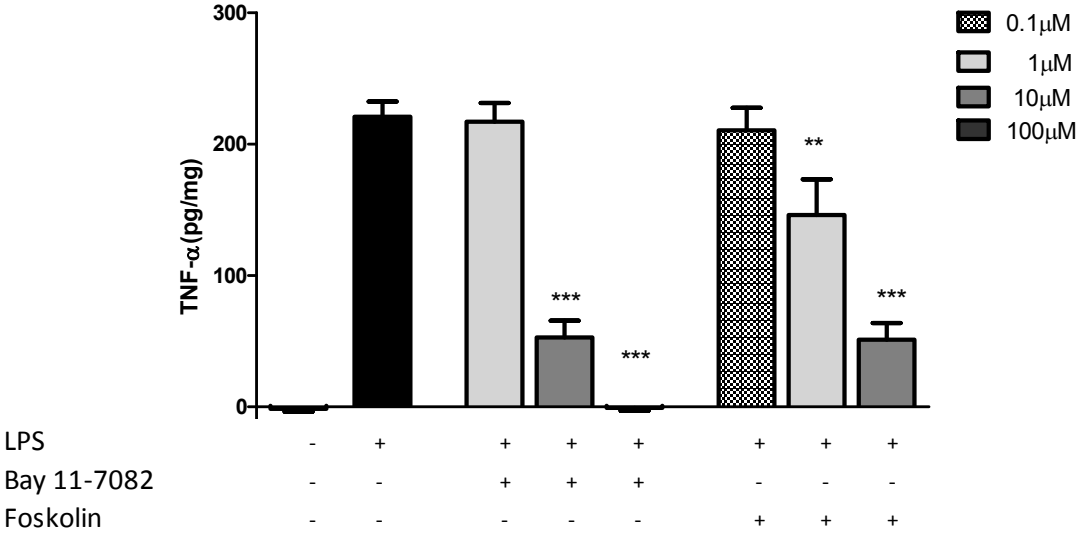
Figure 2-4 Effects of Bay11-7028 and Foskolin on the Expression of Proteins in NF-κB and cAMP Pathways in LPS Induced Macrophage-Like U937 Cells.

Macrophage-like U937 cells were treated with or without LPS(1μg/ml) in absence or presence of Bay11-7082(10μM) or Foskolin(10μM) for 15mins (p-IKK&IKK), for 30 mins (p-IκBα & IκBα and p-p65&p65) or for 60mins(p-CREB&CREB).The expressions of p-IKK, p-IκBα, p-p65,p-CREB(A) and IKK, IκBα, p65,CREB(B) were measured by western blot using corresponding antibodies as described in the methods. B-Tublin was used as a loading control. Data represents the mean ± SEM (n=3). *p<0.001, Vs control vehicle using Student's t-test.

2.4.5 Effect of NF-κB and cAMP Pathways on the Release of TNF-α by LPS Treated Macrophage-Like U937 Cells

The activated NF-κB and cAMP pathways were associated with increased TNF-α production by LPS, the effects of Bay11-7082, the inhibitor of NF-κB pathway (1, 10 and 100μM) and Foskolin, the elevator of cAMP pathway (0.1, 1 and 10μM) on the release of TNF-α were tested with or without LPS (1μg/ml) treatment. BAY11-7082 significantly inhibited the release of TNF-α at 10μM and 100μM ($p<0.001$; Fig.2-5-A). Foskolin also statistically inhibited TNF-α release at 1μM ($p<0.01$) and 10μM ($p<0.001$; Fig.2-5-A). At 100μM, BAY11-7082 almost completely inhibited the LPS-induced release of TNF-α (Fig. 2-5-A). At 10 μM, both Bay11-7082 and Foskolin reduced 70% the release of TNF-α induced by LPS (Fig. 2-5-A). The presence of Bay11-708(10μM) and Foskolin (10μM) caused a further inhibition of TNF-α release ($p<0.001$), and the inhibition of Foskolin (10μM) was significantly reduced by H89 (2μM; Fig. 2-5-B), an antagonist of cAMP pathway. In addition, in order to investigate the role of PDEs on LPS-induced TNF-α release, the effects of specific PDE inhibitors (PDE3 inhibitor, Cilostamid; PDE4 inhibitor, Rolipram and PDE5 inhibitor, Sildenafil) on the release of TNF-α were studied. Macrophage-like U937 cells were treated with LPS (1μg/ml) in the absence or presence of Cilostamid, Rolipram and Sildenafil (0.1μM, 1μM and 10μM) for 6 hours. Rolipram, a cAMP-specific PDE inhibitor, significantly inhibited the release of TNF-α at all concentrations tested ($p<0.001$). Cilostamid, an inhibitor of cAMP & cGMP PDE, only reduced the release of TNF-α at 0.1μM ($p<0.001$). In contrast, Sildenafil, a cGMP PDE inhibitor, had no significant effect at 0.1 and 1 μM, but increased the release of TNF-α induced by LPS at 10μM. (Fig. 2-5-C)

(A)


300
200
100
0
TNF-α(pg/mg)
0.1μM
1μM
10μM
100μM
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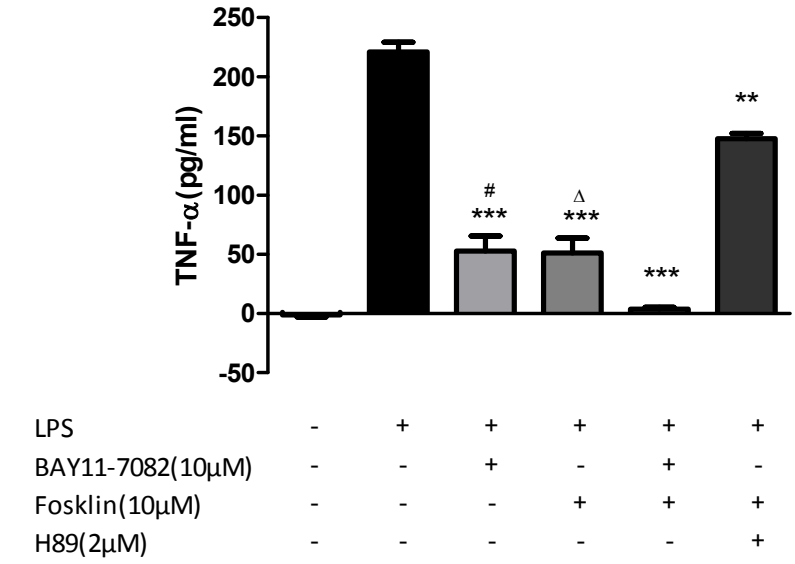
LPS - + + + + + + +
Bay 11-7082 - - + + + - - -
Foskolin - - - - - + + +

(B)


250
200
150
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50
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-50
TNF-α(pg/ml)
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Δ
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LPS - + + + + +
BAY11-7082(10μM) - - + - + -
Fosklin(10μM) - - - + + +
H89(2μM) - - - - - +

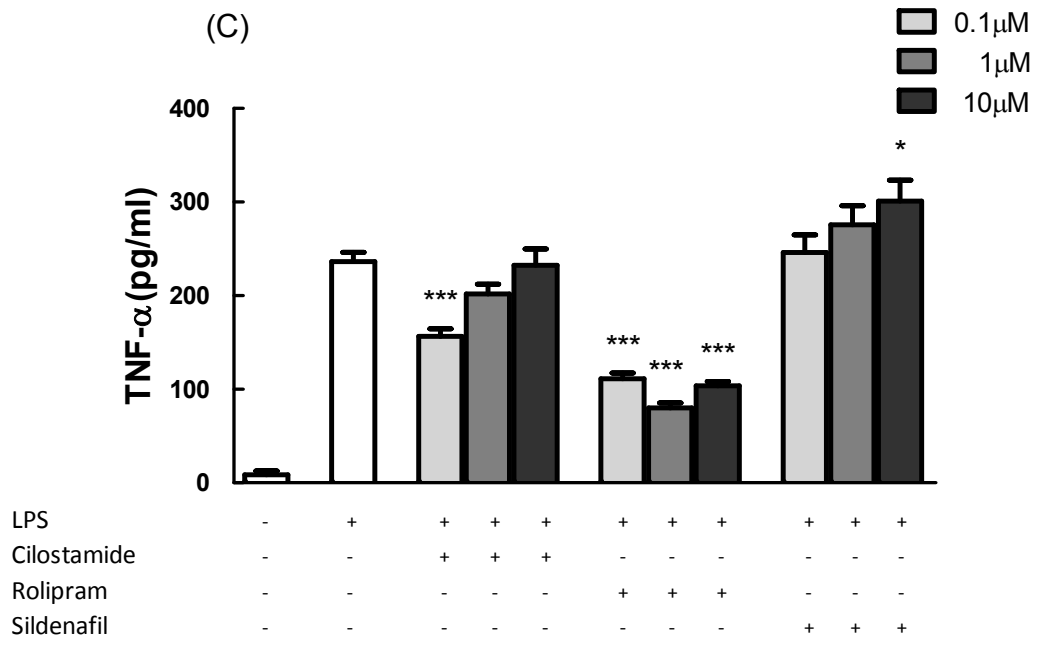


Figure 2-5 Effect of NF-κB and cAMP pathways on the release of TNF-α by LPS treated macrophage-like U937 cells.

Macrophage-like U937 cells were treated with or without LPS(1μg/ml) in absence or presence of Bay11-7082(1μM, 10μM, 100μM), Foskolin (0.1μM, 1μM, 10μM)(A), Bay11-7082(10μM)&Foskolin (10μM), Foskolin (10μM) &H89(2μM)(B), Cilostamide (0.1μM, 1μM, 10μM), Rolipram (0.1μM, 1μM, 10μM) or Sildenafil (0.1μM, 1μM, 10μM) (C) for 6 hours. The concentration of TNF-α was tested by ELISA. Data represents the mean±SEM (n=3) in duplicate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, Vs control vehicle, by one-way ANOVA followed by Bonferroni's test. # $p<0.001$, Vs Bay11-7081&Foskolin treatment vehicle by one-way ANOVA followed by Bonferroni's test. Δ $p<0.001$, Vs Foskolin&H89 treatment vehicle by ne-way ANOVA followed by Bonferroni's test.

2.4.6 Effect of NF-κB and cAMP Pathways on the Transcriptional Activity of NF-κB in LPS Induced Macrophage-Like U937 Cells

In order to investigate the involvement of NF-κB and cAMP pathways in the transcriptional activity of NF-κB induced by LPS, the effects of Bay11-7028, Foskolin and H89 on the transcriptional activity of NF-κB induced by LPS were determined.

Macrophage-likeU937 cells were pre-treated with or without LPS (1μg/ml) in absence or presence with Bay11-7082(10μM), Foskolin (10μM), H89(2μM), Bay11-7082(10μM)&Foskolin (10μM) or Foskolin (10μM)&H89(2μM) for 6 hours. LPS increased 90% transcriptional activity of NF-κB, and this was significantly inhibited by Bay11-7082($p<0.001$) and Foskolin ($p<0.001$) respectively. There was no significant difference of the effect between Bay11-7082 and Foskolin ($p>0.05$). Bay11-7082 plus Foskolin further inhibited the transcriptional activity of NF-κB induced by LPS ($p<0.05$,$p<0.001$). H89, an inhibitor of cAMP pathway, didn't affect the transcriptional activity of NF-κB induced by LPS ($p>0.05$). However, it significantly weakened the effect of Foskolin on the transcriptional activity of NF-κB induced by LPS ($p<0.001$; Fig.2-6)

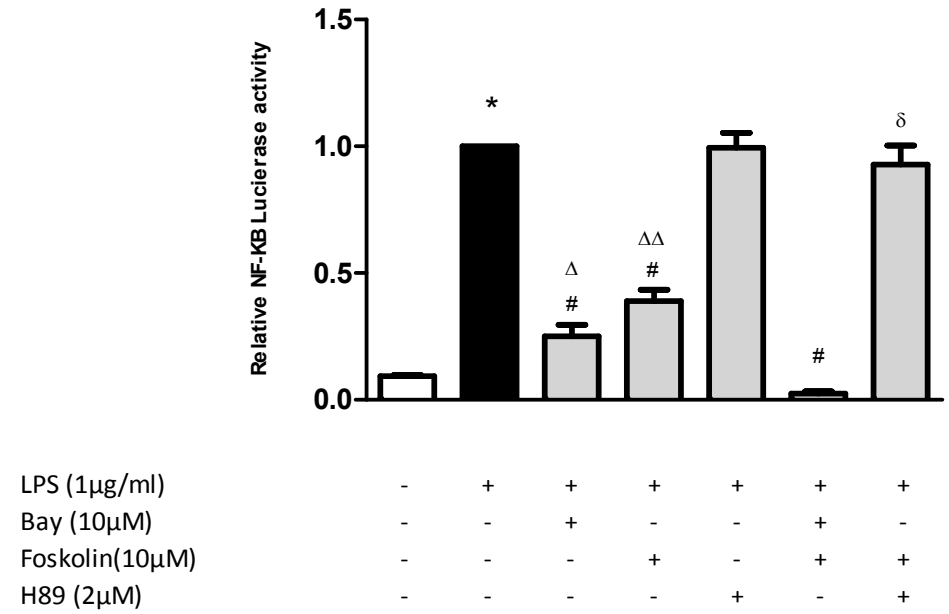


Figure 2-6 Effect of NF-κB and cAMP Pathways on the Transcriptional Activity of NF-κB in LPS Induced Macrophage-Like U937 Cells.

Macrophage-like U937 cells were treated with or without LPS(1ug/ml) in absence and presence with Bay11-7082(10μM), Foskolin (10μM), H89(2μM), Bay11-

7082(10μM)+Foskolin(10μM) or Foskolin(10μM)+ H89(2μM) for 6 hours. The transcriptional activity of NF-κB was measured by the Dual-Glo® Luciferase Reporter Assay System. Data represent the mean ± SEM (n=3) in duplicate. *<0.001, Vs blank vehicle Student t-test. #<0.001, Vs control vehicle by one-way ANOVA followed by Bonferroni's test. Δ<0.05; ΔΔp< 0.001, Vs Bay11-7082+Foskolin vehicle by one-way ANOVA followed by Bonferroni's test. δ< 0.001, Vs Foskolin vehicle by one-way ANOVA followed by Bonferroni's test.

2.4.7 PDE4 Activity in LPS Induced Macrophage-Like U937 Cells

PDE4 activity in LPS-induced U937 cells were studied using a specific PDE4 inhibitor Rolipram. Cells pre-treated with Rolipram (30μM) for 2 hours, then treated with LPS(1μg/ml) for 4 hours, showed a significant increase in PDE4 activity (p<0.001; Fig. 2-7).

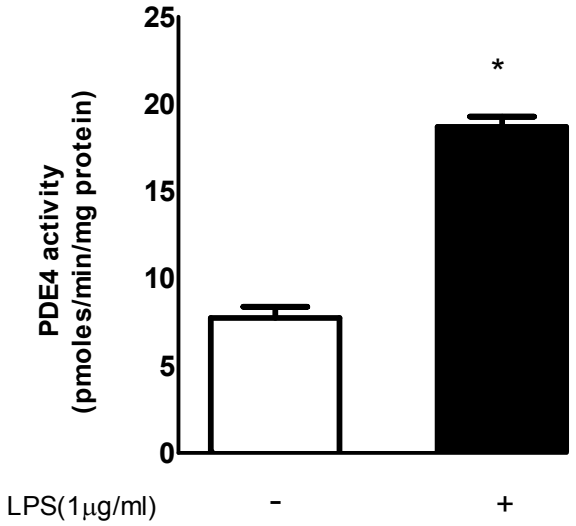


Figure 2-7 PDE4 Activity in LPS Induced Macrophage-Like U937 Cells.
Cells were pre-treated with or without Rolipram(30μM) for 2 hours, and then treated with or without LPS(1μg/ml) for 4 hours. PDE4 activity was quantified by scintillation counting. Data represents the mean ± SEM (n=3) performed in duplicate. *p<0.001, Vs blank vehicle by Student t-test.

2.5 Discussion

LPS and CSE induced macrophage-like U937 cells have been frequently used as the inflammatory cell model to study the pathogenesis of COPD[46,300]. Findings from our study showed that both LPS and CSE caused a massive release of TNF-α in macrophage-like U937 cells (600-fold and 500-fold increase), which is consistent with previous studies[46,393,397]. In addition, LPS also significantly increased the release of IL-1β and IL-6. The findings on the activation of IKK, IκBα, p65, and the increased transcriptional activity of NF-κB by LPS indicate that NF-κB pathway is significantly activated in LPS induced macrophage-like U937 cells. Overall, these results suggest that LPS and CSE inducing macrophage-like U937 cell models are suitable for studying the inflammatory mechanisms related to TNF-α and NF-κB pathways. In addition, the present study also observed that LPS elevated cAMP levels and increased the expression of activated p-CREB and PDE4 activity, indicating that cAMP pathway is also activated by LPS, which is also consistent with previous studies[394,398,399].

The effects of activated NF-κB and cAMP pathways on the release of TNF-α induced by LPS is further supported by the findings of using NF-κB pathway inhibitor (Bay11-7082), cAMP pathway elevator (Foskolin) and cAMP pathway inhibitor(H89). The activated cAMP pathway seems to involve in the regulation of TNF-α release induced by LPS, as Foskolin reduced the release of TNF-α and this effect was blocked by H89. There seems to be a synergy effect between the inhibited NF-κB pathway and the activated the cAMP pathway on the release of TNF-α induced by LPS, as Foskolin further enhanced the action of Bay11-7082 on TNF-α release and both of them almost abolished the release of TNF-α induced by LPS .

The effect of activated cAMP pathway on the transcriptional activity of NF-κB induced by LPS was also demonstrated in this part of my project. Foskolin showed no effect on the expression of IKK, IκBα and p65, but it significantly inhibited the transcriptional activity of NF-κB induced by LPS, and this inhibition of activity was almost completely eliminated by H89. It indicated that the activated cAMP pathway inhibited the transcriptional activity of NF-κB induced by LPS, but not through down-regulating the expression of the key proteins in NF-κB pathway, which was consistent with previous studies[279,280]. In addition, there seems to be a synergy effect between the inhibited up-stream activity of NF-κB pathway and the activated the cAMP pathway on the transcriptional activity of NF-κB induced by LPS, as Foskolin further elevated the inhibition of Bay11-7082 on the transcriptional activity of NF-κB and both of them almost completely inhibited the transcriptional activity of NF-κB.

In addition, we also showed that Bay11-7082 not only inhibited the expression of total IKK, but also non-specifically inhibited the expression of p-IκBα, p-IKK, p-p65 IκBα and p65. Interestingly, IκBα as a stable factor binding with NF-κB to inhibit the activation of NF-κB (see Section1.1.4.4), was also inhibited by Bay11-7082. Previous study proved that down-regulating expression of IκBα can elevate the activity of NF-κB pathway. Our results indicate that there is a bidirectional regulation of Bay11-7082 on the activity of NF-κB pathway.

There is evidence that PDE4 plays an important role in the production of TNF-α in macrophages induced by LPS. It has been reported that there was a 90% decrease in TNF-α mRNA accumulation in the LPS inducing macrophages from PDE4B-deficient mice[394]. Rolipram (PDE4 inhibitor) also significantly inhibited the release of TNF-α in macrophages induced by LPS and *in vivo*[400,401]. The effect of Cliostamide (PDE3 inhibitor)

on LPS-inducing release of TNF-α in macrophages has not been reported, although some studies showed that Cliostamide significantly inhibited the production of TNF-α in HUVECs induced by LPS, this result also was proved *in vivo* [402]. On the other hand, sildenafil was shown to have no effect on the release of TNF-α in ovalbumin (OVA) challenged mice[403]. In the present study, it was found that LPS significantly increased the PDE4 activity in macrophage-like U937 cells, Rolipram and Cliostamide but not Sildenafil significantly inhibited the release of TNF-α induced by LPS. These findings are consistent with previous observations, confirming the involvement of mainly PDE4, also PDE3 but not PDE5 in LPS-induced cytokine release in macrophage-like U937 cells. The reason for a slight increase of TNF-α production by high concentration of sildenafil is not clear. One possibility is that the down-steam product of PDE5, cGMP, may have an opposite effect of cAMP to regulate TNF-α release[404]. Further study is necessary to elucidate the exact mechanism involved.

In summary, LPS inducing macrophage-like U937 cells exhibited an elevated cytokine levels and activated NF-κB pathway which is the main source of TNF-α production. LPS also activates cAMP and PDE4 pathways to regulate cytokine release to avoid the excess production of inflammatory mediators. These findings indicate that this cell model is ideal for studying the mechanism involved in the regulation of cytokines and related NF-κB and cAMP pathways, as well as the effects of ginseng and ginsenosides on inflammatory mediators relevant to COPD.

Chapter 3. Regulation of Ginseng and Ginseng-Related Products on Cytokines

3.1 Introduction

Ginseng is a well-known medicinal herb which has been widely used in the treatment of COPD and other conditions (see Section1.2.3). Ginseng and ginseng related products, such as G115 and some ginseng formulas have been shown in clinical trials with significant improvement on lung function and quality of life of COPD patients[182,336]. However, the pharmacological mechanism of G115 and ginseng formula in COPD is still not clear. Given the importance of inflammation in the pathogenesis mechanisms of COPD, it is possible that the anti-inflammatory effect of ginseng may play an important role in its therapeutic efficacy on COPD. The support evidence is that ginsenosides, the major active components of ginseng and G115, have been demonstrated with anti-inflammatory actions (see Section1.2.4.7).

One of the key inflammatory mediators involved in COPD is cytokine, such as TNF-α, IL-1β and IL-6, which plays an important role in inducing, maintaining and amplifying the inflammatory response in COPD[13]. Previous studies have demonstrated that inhibiting the release and activity of cytokines improved the symptom of COPD patients (see Section1.1.3). However, there is certain deficiency in the study on the effects of ginseng and ginseng related products on the release of cytokine. Firstly, most previous studies used individual ginsenosides, often ignored their metabolites. It has been known that the absorption of some ginsenosides through the gastrointestinal tract is very low *in vivo*[326,405]. For some ginsenosides, such as Rb1, the metabolites are more important in mediating the biological effects of ginseng *in vivo*[405]. Secondly, there is deficiency in the study on the correlation between the regulation of the ginseng and ginseng formula on the release of

cytokines. Therefore, we are difficult to determine that the regulation of ginseng formula on cytokines is related to that of ginseng.

Thus, the objective of the study described in this chapter was to investigate the effects of ginseng, ginseng formulas and ginsenosides on the release of cytokines in the established inflammatory cell model as described in Chapter2. The specific research aims were to investigate the effect of different kinds of GHMFs, G115 and ginsenosides on the release of TNF-α and other cytokines (IL-1β and IL-6) in LPS-induced macrophage-like U937 cells, and the study possible correlations of their actions.

3.2 Materials and Method

3.2.1 Materials

All herbal compounds tested were the highest grade (purity > 99%) available from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). GHMFI & II were obtained from Guangdong Provincial Hospital and GHMFIII &IV were prepared by Mr Xifeng Zhai from the Chinese medicine research laboratory at RMIT University. G115 was supplied by Pharmaton SA (Switzerland). The details of other materials are described in section 2.2.1

3.2.2 Method

3.2.2.1 Cell culture

See Section 2.2.2.1

3.2.2.2 Preparation of Cigarette Smoke-Treated Medium

See Section 2.2.2.2

3.2.2.3 Cytokines Assay

PMA induced Macrophage-like U937 cells were treated with Rb1, Rg1, Rg3, Rh1, Rh2, CK (1, 10 and 100μM), GHMF-I, GHMF-II(0.1, 1 and 10mg/ml), GHMF-III and GHMF-IV, G115(0.01, 0.1 and 1mg/ml) or vehicles for 2 hours, and with LPS (1μg/ml) for 6 hours. The supernatant of the test samples was collected to determine the concentrations of TNF-α, IL-1β and IL-6 using ELISA kits, according to the manufacturer's protocol. Other details see section 2.2.2.3.

3.3 Statistical Analysis

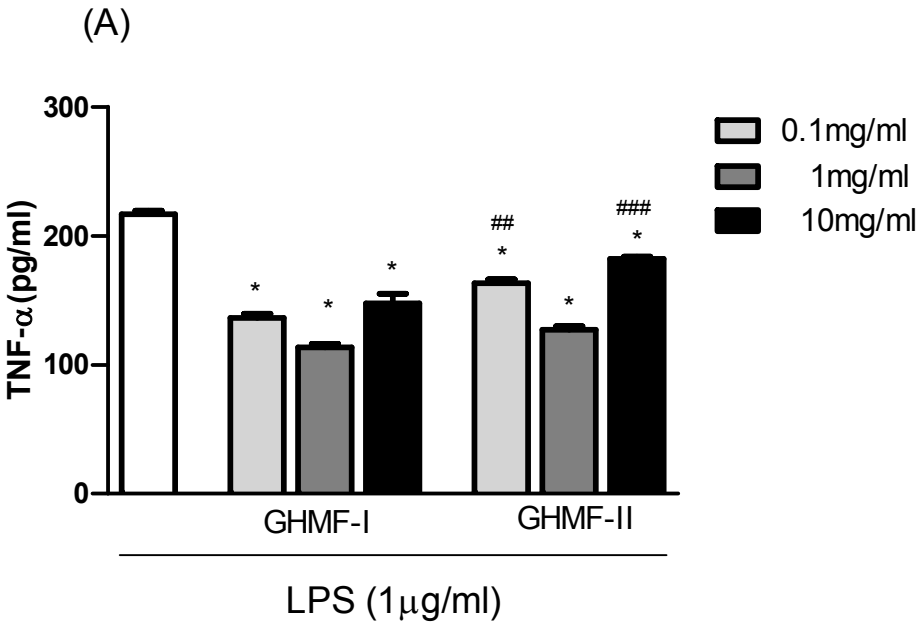
All values are presented as mean ± SEM of the given number (n) of experiments. The two-group comparison was conducted by Student's t-test. The multi-group comparisons were conducted by one-way ANOVA followed by Bonferroni's test or by Tukey's post hoc according different aims. Correlation analysis was performed to determine the relationship between G115 and GHMF-III on the release of TNF-α in different cell models. P values of 0.05 or less were considered to be statistically significant and tests were performed using GraphPad Prism version 5.0 software (GraphPad Software, Inc La Jolla, CA).

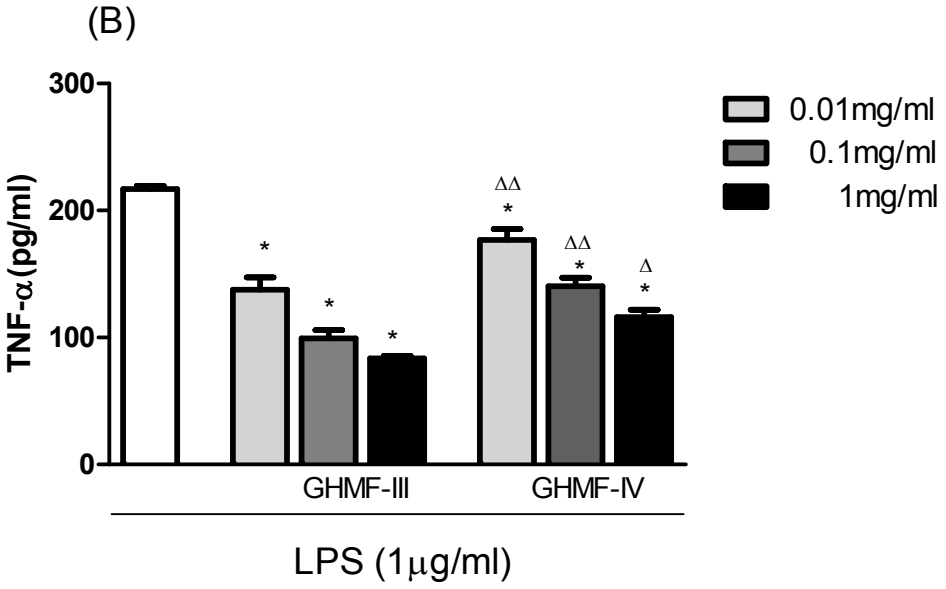
3.4 Result

3.4.1 Effects of GHMFs on the Release of TNF-α in LPS induced macrophage-like U937 cells

The release of TNF-α from macrophage-like U937 cells was significantly inhibited by all GHMFs tested (0.1, 1 and 10mg/ml for GHMF-I and GHMF-II, and 0.01, 0.1 and 1mg/ml

for GHMF-III and GHMF-IV) ($p<0.001$; Fig 3-1). The most effective concentration of GHMF-I and GHMF-II was at 1mg/ml. GHMF-I demonstrated more potent inhibition on TNF-α release than GHMF-II at 10mg/ml, but not at 1mg/ml ($p<0.001$; Fig.3-1-A). The most effective concentration of GHMF-III and GHMF-IV was at 1mg/ml, and GHMF-III showed more potent inhibition on TNF-α release than GHMF-IV at any concentration ($P<0.01$), especially at 0.1mg/ml and 1mg/ml ($p<0.001$, Fig.3-1-B). At 1mg/ml, GHMF-III produced a more potent inhibition on the release of TNF-α than GHMF-I ($P<0.001$; Fig. 3-1-C).





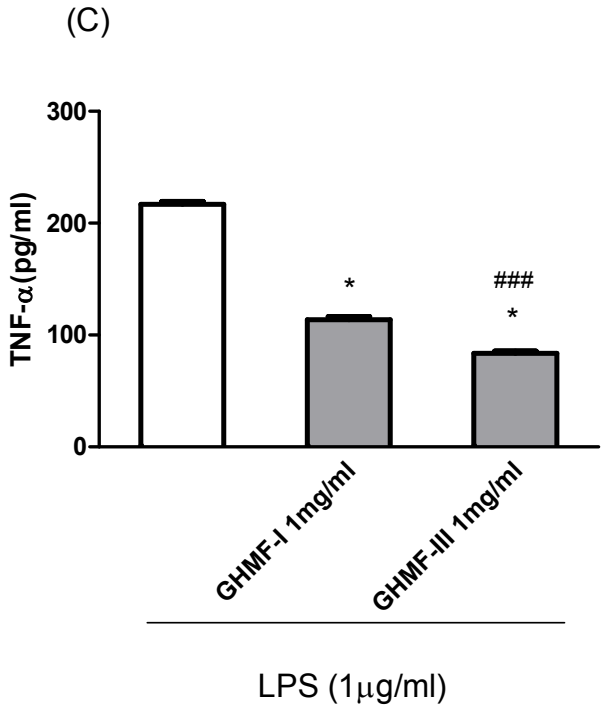


Figure 3-1 Effects of GHMFs on the Release of TNF-α in LPS induced macrophage-like U937 cells.

Macrophage-like U937 cells were treated with different concentrations (0.01-10 mg/ml) of GHMFs or vehicles. GHMF were composed by the commercial herbal extracts (A), or raw-herbs (B). Cells were treated with test agents for 2 hours, and then induced by

LPS(1μg/ml) for 6 hours. The effect of GHMF-I and GHMF-III on the release of TNF-α was also compared at 1mg/ml (C). The concentration of TNF-α was tested by ELISA. Data represent the mean±SEM (n=3) in duplicate. *p < 0.001, Vs control vehicle by one-way ANOVA followed by Bonferroni's test. #<0.01; ##p < 0.001, Vs GHMF-I and Δ<0.01; ΔΔ<0.001,Vs GHMF-III at the same concentration by one-way ANOVA followed by Tukey's post hoc test.

3.4.2 Effects of GHMF-III and G115 on the Release of TNF-α in macrophage-like U937 cells Induced by LPS or CSE

After the pre-treatment of cells with G115 (0.01, 0.1 and 1mg/ml) for 2 hours and then stimulation with LPS for another 6 hours, the release of TNF-α was significantly inhibited by G115 at 0.01mg/ml ($p<0.05$,$p<0.01$), 0.1mg/ml ($p<0.001$) and 1mg/ml($p<0.001$). The most effective concentration of G115 is at 1mg/ml, which was more potent than that at 0.01mg/ml ($p<0.001$). No significant differences between the inhibitions of G115 and GHMFIII on the LPS-induced TNF-α release at the same concentration were observed ($p>0.05$; Figure3-2-A). In CSE induced cell model, G115 and GHMF-III significantly decreased the level of TNF-α at 0.1mg/ml and 1mg/ml ($p<0.001$). At 0.01mg/ml, GHMF-III but not G115 statistically inhibited the release of TNF-α ($P<0.001$). GHMF-III demonstrated more powerful inhibition than G115 at 0.01mg/ml ($p<0.001$) and at 0.1mg/ml ($p<0.01$; Fig.3-2-B). The correlation between the effects of GHMF-III and G115 on the release of TNF-α in LPS or CSE inducing cell models was conducted. There was a significant correlation between the effects of GHMF-III and G115 on the release of TNF-α in both cell models ($P<0.0001$, $R=0.9658$; $p<0.0001$, $R=0.8685$).

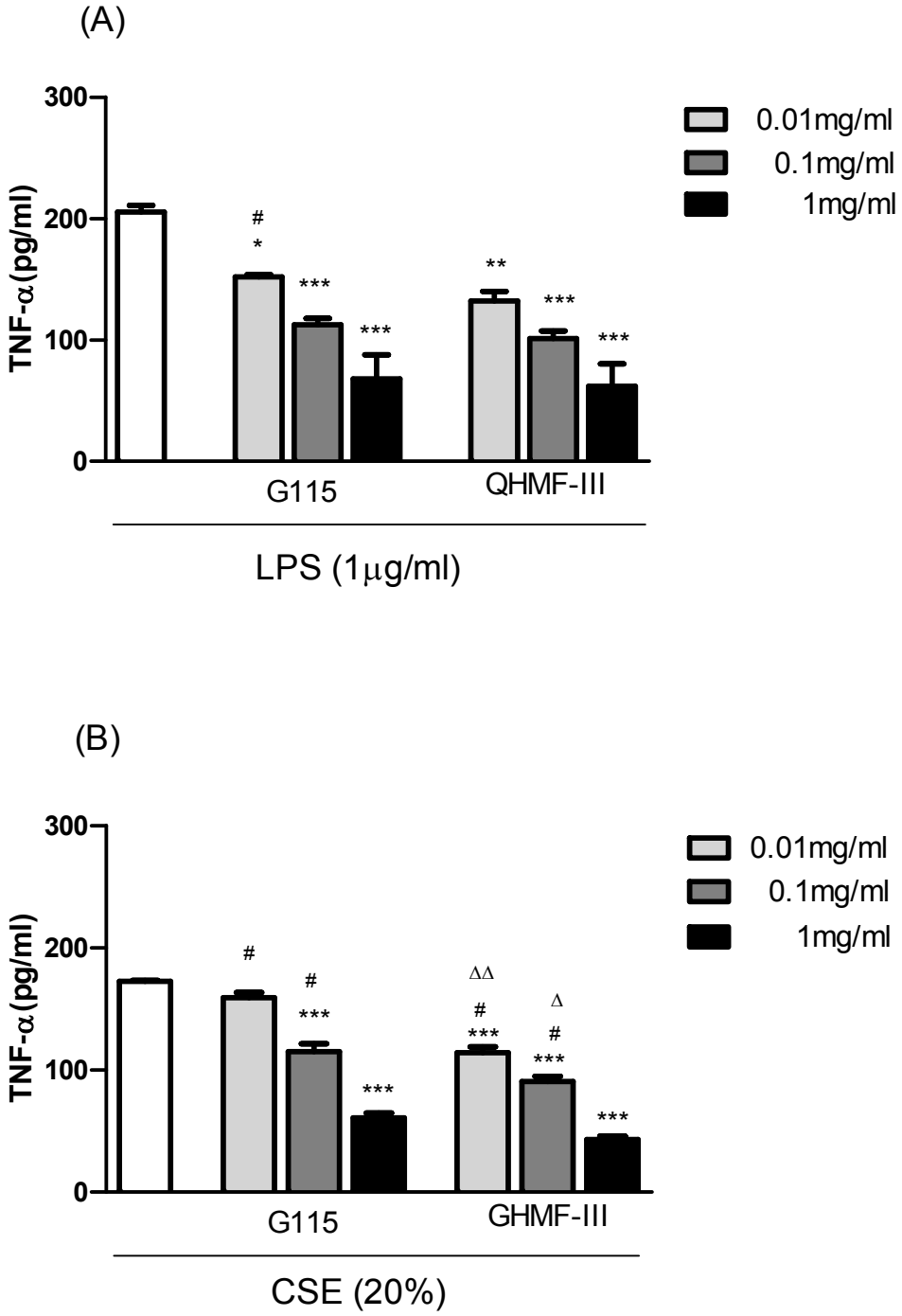


Figure 3-2 Effects of GHMF-III and G115 on the Release of TNF-α in macrophage-like U937 cells Induced by LPS or CSE.

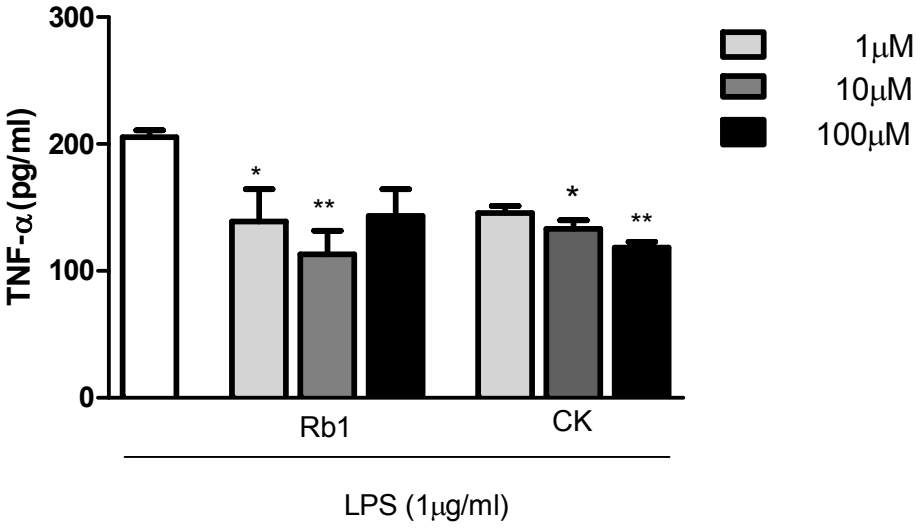
After treatment with or without G115 or GHMF-III (0.01, 0.1 and 1mg/ml) for 2 hour, macrophage-like U937 cells were induced by LPS(1μg/ml)(A) or CSE (20%)(B) in absence or presence for 6 or 18 hours respectively, The concentration of TNF-α was tested by ELISA. Data represent the mean ± SEM (n=3) in duplicate. *$p < 0.05$; **$p < 0.01$;

***p < 0.001, Vs control (LPS or CSE) vehicle by one-way ANOVA followed by Bonferroni's test. # p<0.001, Vs G115 or GHMF-III at 1mg/ml by one-way ANOVA followed by Bonferroni's test. Δ p < 0.001; ΔΔp< 0.001, Vs G115 at the same concentration by one-way ANOVA followed by Tukey's post hoc test. Correlation analysis was performed to determine the relationship between G115 and GHMF-III on the release of TNF-α in different cell models.

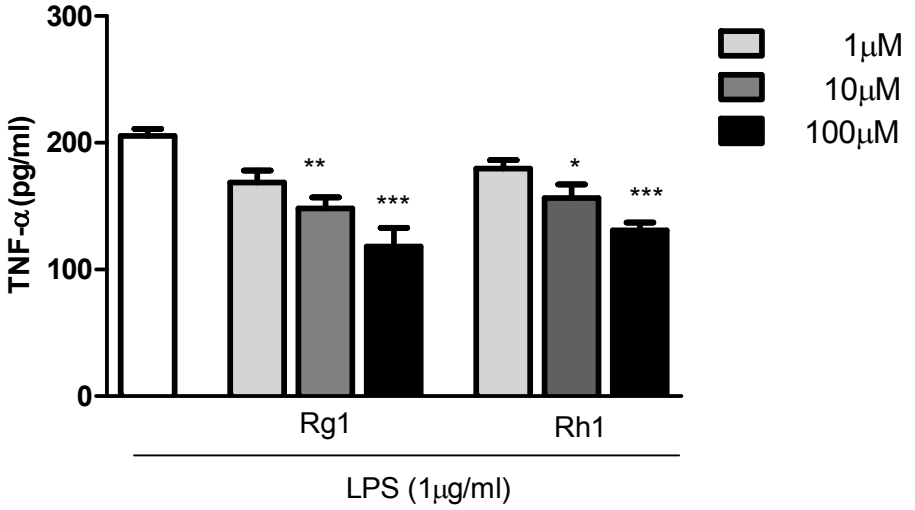
3.4.3 Effects of Ginsenosides and Their Metabolites on the Release of TNF-α in Macrophage-Like U937cells Induced by LPS.

In order to investigate the effects of main active components of G115 on the release of TNF-α, cells were treated with ginsenoside Rb1, Rg1, Rg3 and their metabolites(CK, Rh1 and Rh2) at 0.1, 1 and 1μM for 2 hours and then stimulated with LPS for another 6 hours. Ginsenoside Rb1 significantly inhibited the release of TNF-α at 1μM ($p<0.05$) and 10μM ($p<0.01$), but not at 100μM ($p>0.05$). Its metabolites, CK, also significantly inhibited the TNF-α release at 10μM ($p<0.05$) and 100μM ($p<0.01$), but not at 1μM ($p>0.05$) (Fig.3-3-A). Both Rg1 and its metabolite (Rh1) significantly inhibited the release of TNF-α at 10μM ($p<0.01$; $p<0.05$) and at 100 μM ($P<0.001$), but not at 0.1μM ($p>0.05$, Fig.3-3-B). There was no significant difference in the effects of Rb1/Rg1 and its metabolite(CK/Rh1) at any concentrations ($p>0.05$, Fig.3-3-A&B). Similarly, Rg3 significantly inhibited TNF-α release at 1μM ($p<0.01$) and 10μM ($p<0.001$), but not at 100μM ($p>0.05$). The metabolite (Rh2) had no effect on the release of TNF-α at 1μM and 10μM ($p>0.05$). At 100μM, Rh2 enhanced the release of TNF-α ($p<0.01$, Fig.3-3-C). Ginsenoside Rb1 and Rg3 display more powerful inhibitions on the release of TNF-α at 10μM than at 100μM, however, there was no significant difference between them ($p>0.05$; Fig.3-3-A&C).

(A)


300
200
100
0
TNF-α(pg/ml)
*
**
*
**
1μM
10μM
100μM
Rb1
CK
LPS (1μg/ml)

(B)


300
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TNF-α(pg/ml)
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1μM
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100μM
Rg1
Rh1
LPS (1μg/ml)

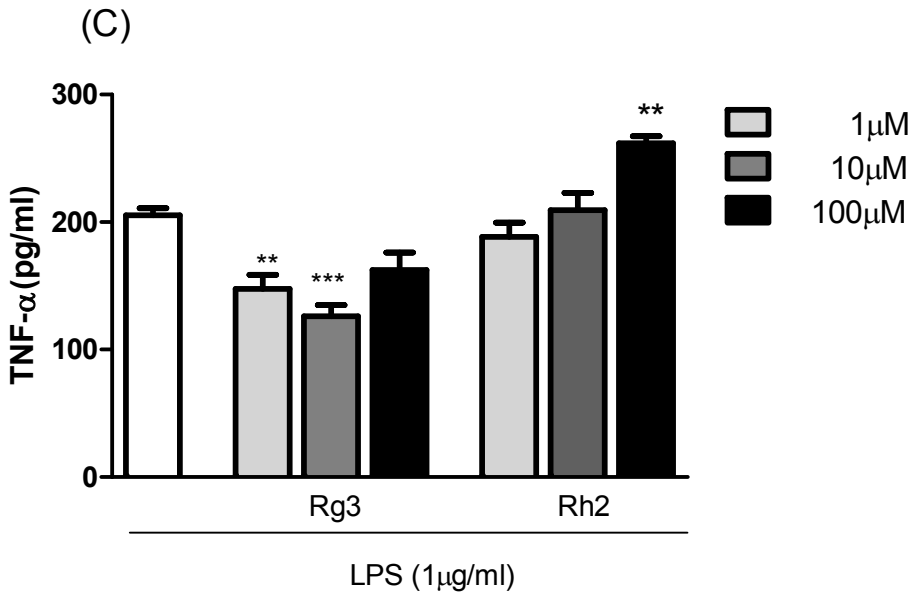


Figure 3-3 Effects of Ginsenosides and their metabolites on the release of TNF-α in LPS induced macrophage-like U937 cells.

Macrophage-like U937 cells were treated with different concentrations (1μM, 10μM, 100μM) Rb1 / CK(A), Rg3 / Rh2(B) or Rg1 / Rh1(C) or vehicle for 2 hours, and then induced by LPS (1ug/ml)for 6 hours. The concentration of TNF-α was tested by ELISA. Data represent the mean±SEM (n=3) in duplicate. *p < 0.05; **p < 0.01; ***p < 0.001, Vs control vehicle by one-way ANOVA followed by Bonferroni's test. One-way ANOVA followed by Tukey's post hoc test was used to test the difference between the effects of ginsenosides and its metabliltesat any concentrations.

3.4.4 Effects of GMHM-III, G115, Rb1 and Rg1 on the Release of IL-1β and IL-6 in Macrophage-Like U937 Cells Induced by LPS.

In order to investigate the effects of G115, GHMF-III, Rb1 and Rg1 on the release of other cytokines, cells were treated with GHMF-III(1mg/ml), G115(1mg/ml), Rb1(10μM) or Rg1(10μM) for 2 hours, and then stimulated with LPS(1μg/ml) for another 6 hours. The secretion of IL-1β was significantly inhibited by GHMF-III ($p<0.001$), G115 ($p<0.001$), Rb1 ($p<0.05$) and Rg1 ($P<0.01$) compared with the control group. There was no significant

difference in the effects of these agents ($p>0.05$; Fig. 3-4-A). The secretion of IL-6 induced by LPS was also significantly inhibited by GHMF-III ($p<0.001$), G115 ($p<0.01$), Rb1 ($p<0.001$) and Rg1 ($P<0.001$). GHMF-III and Rg1 displayed the more powerful inhibition on the release of IL-6 than G115 ($p<0.05$; Fig. 3-4-B).

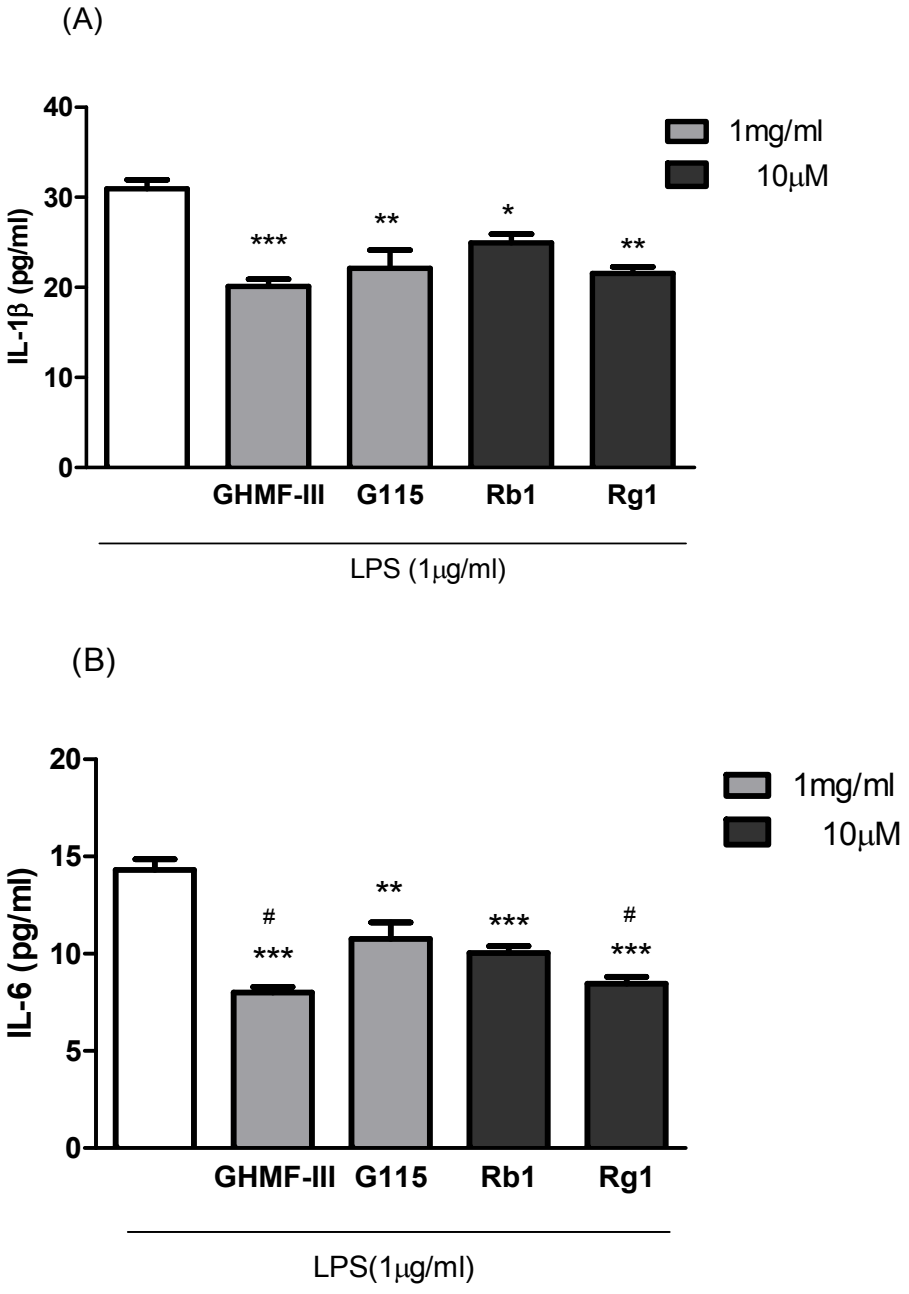


Figure 3-4 Effects of GHMF-III, G115, Rb1 and Rg1 on the release of IL-1β and IL-6 in LPS induced macrophage-like U937 cells.

The concentrations of IL-1β(A) and IL-6(B) were determined by ELISA. Data represent the mean ± SEM (n=3) in duplicated. *<0.05; **p < 0.01; ***p < 0.001, Vs control vehicle by Bonferroni's test. One-way ANOVA followed by Tukey's post hoc test was used to test the different regulation among GHMF-III, G115,Rb1 and Rg1 on the release of TNF-α, # p<0.05.

3.5 Discussion

As mentioned in Chapter 1, the key pathological mechanism of COPD is consistent inflammation of the lung and airway, and cytokines play an important role in this process (see Section 1.1.3). It has been shown that TNF-α leads to the amplification of inflammation, emphysema and the weight loss of COPD[50,171,172]. TNF-α inhibitors, as powerful drugs against inflammatory response, have been used in the treatment of COPD[176]. However, the side effect, medical cost and uncertain efficacy have affected their clinical application in COPD[180,181]. Therefore, identifying other agents with actions of TNF-α inhibition may help to improve the current therapies for COPD. In this regard, the present study has found for the first time that ginseng and several ginseng formulas inhibited TNF-α release from macrophage-like U937 cells induced by LPS. The inhibition of LPS induced cytokine release by ginseng and ginseng formula is further supported by the finding that ginseng and ginseng formula as well as ginsneosides also inhibited IL-1β and IL-6 release induced by LPS, indicating they may act through inhibition of NF-κB pathway (Further evidence on that will be discussed in Chapter 4).These findings provide supporting evidence at cell level for the use of ginseng and ginseng related products for treating COPD.

Among several ginseng formulas tested, GHMF-III showed more effective inhibitory effect on the release of TNF-α than other formulas ($p<0.01$). GHMF-III is a GHMF excluding polysaccharide. It is possible that polysaccharide in the ginseng extracts may be responsible for such difference. Previous studies have demonstrated that polysaccharides can up-regulated the product of many inflammatory factors such as TNF-α, IL-1β, IL-6, IL-12, GM-CSF and IFN-γ to aggravate the inflammatory response[406-408], so the polysaccharides in GHMF-I, II and IV may actually weaken the anti-inflammatory action of these formulas. In addition, GHMF-I demonstrated more significant inhibition on the release of TNF-α than GHMF-II. The difference between GHMF-I and GHMF-II may be caused by the different content of active components in each GHMF. Further study is necessary to determine the contents of active components in these formulas.

G115, a standardized extract of ginseng, and GHMF-III showed a significant inhibition on the release of TNF-α in LPS and CSE induced macrophage-like U937 cells. Previous study demonstrated that G115 significantly increased the release of TNF-α in LPS induced animal model[329]. There may be an opposite regulation of G115 on the release of TNF-α *in vivo* and *in vitro*. The present study also found a correlation between the effect of GHMF-III on the release of TNF-α and that of G115 in both LPS and CSE cell models, indicating the main active component of GHMF-III is likely to be similar to that of G115. In addition, GHMF-III showed a more powerful inhibition on the secretion of TNF-α than G115 in CSE but not LPS- cell model, indicating it may more suitable for the treatment of cigarette smoking induced inflammation.

Given the ginsenosides are the main active compounds for ginseng (G115) and GHMF-III, the present study also investigated the effects of different ginsenosides on LPS-induced cytokine release. Ginsenosides Rb1 and Rg3 significantly inhibited the release of TNF-α at

a non-concentration-dependent manner. Ginsenosides Rg1 significantly inhibited the release of TNF-α at a concentration-dependent manner. These results were similar to previous studies[184,351,380,382,383]. The difference of inhibition among Rb1, Rg3 and Rg1 may be caused by the difference of absorption rate of them, as previous study showed that the gastrointestinal-tract absorption rate of Rg1 was higher than that of Rb1 and Rg3[405]. As a proof of this guess, the metabolite of Rb1, CK displayed the similar concentration-dependent inhibition on the release of TNF-α with Rg1. The metabolite of Rg1 (Rh1) also inhibited the release of TNF-α induced by LPS at a concentration-dependent manner. In contrast, the metabolite of Rg3 (Rh2) didn't affect TNF-α release at low concentrations, but enhanced the release of TNF-α at high concentration. However, this result is not consistent with previous studies, as Rh2 was found to inhibit the release of TNF-α in LPS/ INF-γ inducing murine BV2 microglial cells, and in the ears tissue from the rat induced by oxazolone[383,384,409]. It is not clear whether such difference is due to different cell type involved. Comparing the inhibition of ginsenosides and their metabolits, we found there was a similar inhibition on TNF-α between Rg1 and Rh1; the difference of inhibition between Rb1 and CK seemed to be related with the low absorption rate of Rb1. However, it is still uncertain that the up-regulating mechanism of Rh2 on TNF-α induced by LPS.

In summary, the present study demonstrated that ginseng and several ginseng formulas significantly inhibited LPS and CSE induced the release of TNF-α and/or other cytokines from macrophage-like U937 cells. Among these, GHMF-III and G115 are the most effective. The actions of ginseng and ginseng formula are likely to be mediated by the active compounds ginsenosides. Among the ginsenosides studied, Rb1, Rg3 and Rg1 may be the main contributors to the actions of ginseng. Further study is warranted to investigate if the effects of GHMF-III, G115, Rb1 and Rg1 on cytokine release are through inhibition of NF-κB pathway.

Chapter 4. Regulation of G115, GHMF-III and Ginsenosides on NF-κB Pathway

4.1 Introduction

NF-κB is the key transcriptional factor which regulates the transcription of many inflammatory factors/proteins involved in COPD[235], such as TNF-α, IL-IL-1β and IL-6[410,411]. Findings as described in the Chapter 3 have demonstrated that G115, GHMF-III, Rg1, Rb1, Rg3, CK, Rh1 significantly inhibited the production of TNF-α induced by LPS. Moreover, the inhibition of G115, GHMF-III, Rb1 and Rg1 on TNF-α release was accompanied by a significant inhibition of IL-1β and IL-6 release induced by LPS. These findings indicate that ginseng and ginsenosides may inhibit the TNF-α release through down-regulating the activation of NF-κB. Thus, we hypothesise that ginseng and ginsenosides inhibit the release of TNF-α through decreasing the activity of NF-κB pathway in LPS induced macrophage-like U937 cells.

LPS activates NF-κB through a series of phosphorylation reactions (PRs), including the phosphorylation of IKK, IκBα and NF-κB[412]. Phosphorylated IKK can activate IκBα, and then activate NF-κB. In the study described in the last Chapter3, the inhibitor of IKK (Bay11-7082) almost completely inhibited the release of TNF-α induced by LPS, indicating that PRs are likely to be involved in the cellular generation of TNF-α. Evidence from previous studies suggests that ginseng/ginsenosides may regulate NF-κB pathway. For example fermented ginseng significantly inhibited the expression of p-IκBα[345]; ginsenosidesRb1, Rb2, Rd, Rg1 and the metabolits of ginsenosides(CK and Rh2)also significantly inhibited the expression p-IκBα[340,184,341]; in addition, the expression of p-IKK was also down-regulated by CK and Rh2[341]. However, the regulating mechanism of G115, GHMF-III, Rg1, Rb1, Rg3, CK, Rh1 and Rh2 on PRs in NF-κB pathway was still

uncertain. As in these PRs, the activation of any upstream protein (IKK, IκBα), finally resulting in the activation of NF-κB, if we want to confirm initiator-protein which is triggered by the ginseng and ginseng-related products, we should screen the effect of them on the expression of all total proteins and their corresponding phosphorylated products in these PRs. However, there is no one paper that comprehensively analysed the regulation of G115, GHMF-III, Rg1, Rb1, Rg3, CK, Rh1 or Rh2 on the protein expression in these PRs.

In the NF-κBpathway, activated NF-κB needs to be transmitted into the nucleus to achieve its transcriptional activity. Transcriptional activity of NF-κB is another important regulating target in NF-κB pathway. It displays the terminal action of NF-κB pathway, and can be regulated by various factors including cAMP dependent pathway[279,280]. Previous studies showed that activated CREB, the downstream factor of cAMP-dependent pathway, competitively inhibited transcriptional activity of NF-κB[9,10]. Similarly, as described in Chapter 3, Foskolin(the elevator of cAMP) reduced the LPS-induced TNF-α production which was eliminated by the PKA inhibitor H89, indicating an interaction between cAMP and NF-κB pathway. Although there is no previous studies on the involvement of such mechanism in the anti-inflammatory actions of ginseng, there is evidence that ginsenoside Rb1 and Rg1 increased intracellular cAMP and the activity of PKA[353]. Interestingly, the metabolite of Rg1(Rh1) significantly inhibited the transcriptional activity of NF-κB , but not the DNA binding activity of NF-κB in LPS induced BV-2 cells, and this inhibited transcriptional activity of NF-κB by Rh1 accompanying with the up-regulating expression of p-CREB in nucleus[352]. Therefore, G115, GHMF-III, Rg1, Rb1, Rg3, CK, Rh1 or Rh2 may up-regulated the activity of cAMP pathway to decrease the transcriptional activity of NF-κB.

The level of cellular cAMP is regulated by PDE (see Section 1.1.4). Since cAMP-specific PDE inhibitors, especially PDE4 inhibitor (Rolipram) significantly inhibited the LPS-induced TNF-αproduction in U937 cells (See Chapter 3), it is possible that ginseng and certain ginsenosides may act as PDE inhibitors to regulate cellular cAMP . Previous studies showed that ginsenoside Rg1 and Rg3 were used as cAMP-specific PDE inhibitors for decreasing the activity of cAMP-specific PDE to up-regulate the level of cAMP[356]. We inferred that G115, GHMF-III, Rg1, Rb1, Rg3, CK, Rh1 or Rh2 could regulate the activity of cAMP pathway induced by LPS, and they perhaps played these actions through regulating the activity of PDE4. Thus the aim of studies described in this chapter is to investigate the effects of GHMF-III, G115 and ginsenosides on regulation of activity of NF-κB and cellular cAMP pathways and their relationships to inhibition of the transcriptional activity of NF-κB. Specifically, to investigate ⑴the regulations of GHMF-III, G115, ginsenoside Rb1, Rg1, Rg3 and their metabolites on expression of total & p-IKK, IκBα and p65; ⑵the regulations of GHMF-III, G115, ginsenoside Rb1, Rg1, Rg3 and their metabolites on the level of cAMP and the expression of CREB & p-CREB; (3) the effects of G115, GHMF-III and ginsenoside Rg1 on the transcriptional activity of NF-κB ; (4) the effects of G115, GHMF-III and ginsenoside Rg1 on the activity of PDE4, using LPS induced macrophage-like U937 cells.

4.2 Materials and Method

4.2.1 Materials

See Section 2.2.1&3.2.1

4.2.2 Method

4.2.2.1 Cell Culture

See Section 2.2.2.1

4.2.2.2 cAMP Assay

Cells were incubated with G115 (1mg/ml), GHMF-III (1mg/ml) or Rb1, Rg1, Rg3, CK, Rh1, Rh2 (10μM) for 2 hour, and then treated with 1μg/ml LPS in the absence or presence for 6 hours. The supernatants of cell lysates were assayed directly using EIA method according to the manufacturer's instruction (Sapphire Bioscience Pty. Ltd, Australia). The details see Section 2.2.2.4

4.2.2.3 Western Blot

Cells were incubated with or without G115 (1mg/ml), GHMF-III (1mg/ml) or Rb1, Rg1, Rg3, CK, Rh1, Rh2 (10μM) for 2 hour, and then treated with 1μg/ml LPS in the absence or presence for 15, 30 or 60 mins respectively. The protein from the lysed cells was measured by Western blot analysis. The details see Section 2.2.2.5

4.2.2.4 NF-κB Reporter Assay

The transfected macrophage-like U937 cells were pre-incubation with or without G115 (1mg/ml), GHMF-III (1mg/ml) or Rb1, Rg1, Rg3, CK, Rh1, Rh2(10μM) for 2 hour, and then treated with 1μg/ml LPS in the absence or presence for 6 hours. The lysate of cells were measured by Dual-Glo® Luciferase Reporter Assay System (Promega Australia). The details see Section 2.2.2.6

4.2.2.5 PDE Assay

Macrophage-like U937 cells were treated with or without 10μM Rolipram[394], 1mg/ml G115, 1mg/ml GHMF-III or 10μM Rg1 for 2 hour, following incubation with or without 1μg/ml LPS for 4 hour. The lysate were assayed for PDE activity using the method of Thompson and Appleman two-step procedures with modification[394-396]. The details see Section 2.2.2.7.

4.3 Statistical Analysis

See Section 3.3

4.4 Result

4.4.1 Effects of G115, GHMF-III, Ginsenosides and their Metabolites on the Expression of IKK, IκBα, P65 and their phosphorylated products in NF-κB Pathway in Macrophage-like U937 Cells Induced by LPS.

In order to observe the effect of G115(1mg/ml), GHMF-III(1mg/ml), Rb1(10μM), CK(10μM), Rh2(10μM), Rg1(10μM) and Rh1(10μM) on the expression of p-IKK, p-IκBα, p-p65 and total IKK, IκBα, p65, the macrophage-like U937 cells were pre-treated with the test agents for 2 hours, and then induced by LPS for 15 minutes (for p-IKK and IKK expressions) or 30 minutes (forp- IκBα&IκBαand p-p65&p65 expressions). Bay11-7082, the inhibitor of IKK, was used as a positive control. Bay11-7082(10μM) displayed a significant inhibition on the expression of p-& total IKK, p-& total IκBα and p-& total p65($p<0.001$) (Table-4-1-A,B&C). Foskolin, an elevator of cAMP pathway, had no significant effect on the expression of these proteins ($p>0.05$; Table4-1-A, B&C). Rh2, the

metabolite of Rg3, also couldn't inhibit the expression of these proteins ($p>0.05$; Table4-1-A, B&C).

As the start point in PRs of NF-κB pathway, the expression of p-IKK induced by LPS was significantly inhibited by G115 ($p<0.001$), GHMF-III($p<0.001$), Rb1($p<0.001$), CK($p<0.05$), Rg3($p<0.05$), Rg1($p<0.001$) and Rh1($p<0.05$; Fig.4-1-A). Only G115($p<0.01$), GHMF-III($p<0.001$), Rb1($p<0.001$) and Rg3($p<0.05$)inhibited the expression of IKK induced by LPS at the same time(Fig.4-1-B).

The expression of p-IκBα was significantly inhibited by G115 ($P<0.01$), GHMF-III($P<0.01$), Rb1($P<0.001$), CK($P<0.01$), Rg3($P<0.05$), Rg1($p<0.001$)and Rh1($p<0.05$) (Fig.4-2-A). Although Rg1 and Rb1 tended to have more powerful inhibition on the expression of p- IκBα, no statistically significant difference with other test agents($p>0.05$)was observed. G115 and GHMF-III significantly up-regulated the expression of IκBα ($p<0.05$, $p<0.01$). Gisenoside Rb1 also increased the expression of IκBα induced by LPS ($p<0.05$; Figure4-2-B).

The expression of p-p65 was significantly inhibited by G115 ($p<0.001$), GHMF-III($p<0.01$),Rb1($p<0.001$), CK($p<0.01$), Rg3($p<0.001$), Rg1($p<0.01$) and Rh1($p<0.05$;Fig.4-3-A). There was no significant difference among the test agents on p-p65 expressions ($p>0.05$). Only ginsenoside Rg3 displayed the down-regulation on the expression of p65 ($p<0.05$; Figure4-3-B).

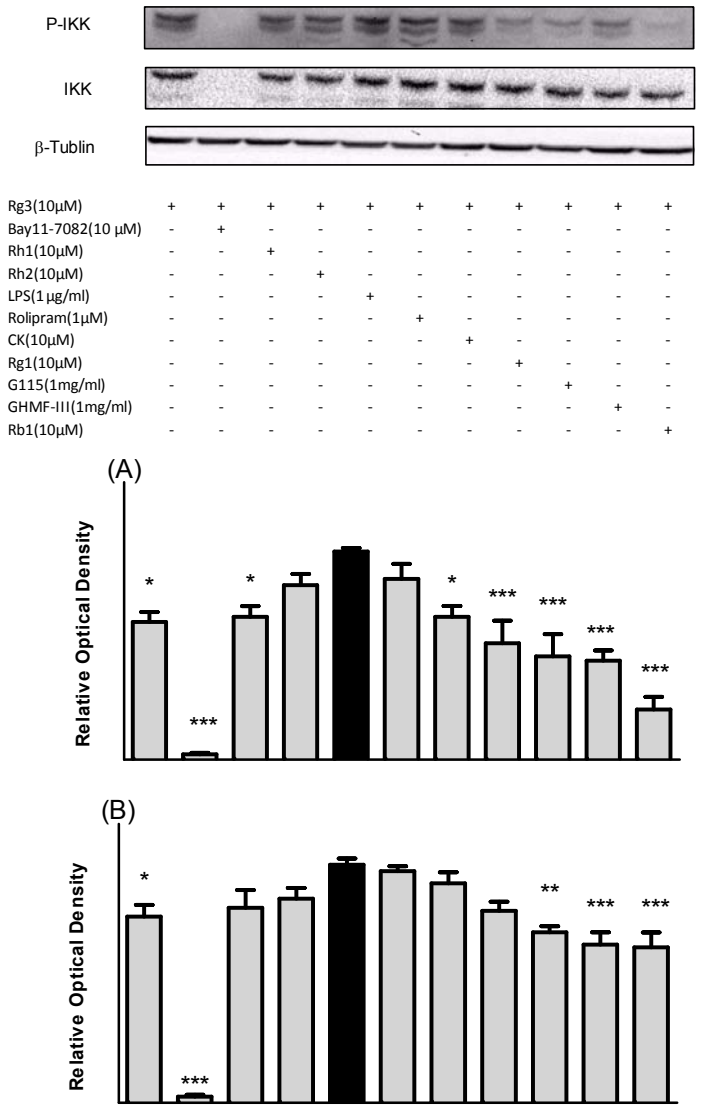


Figure 4-1 Effects of G115, GHMF-III, Ginsenosides and their Metabolites on the Expression of p-IKK and IKK in NF-κB Pathway in Macrophage-like U937 Cells Induced by LPS.

Macrphage-like U937 cells were treated with or without Bay11-7082(10μM), Foskolin(10μM), G115(1mg/ml), GHMF-III(1mg/ml) and Rb1, Rg1, Rg3, CK, Rh1, Rh2(10μM) for 2 hour, and then stimulated with or without LPS(1μg/ml) for 15 minutes. The expression of p-IKK and IKK was measured by western blot using p-IKK antibody and IKK antibody as described in the methods (A&B). B-Tublin was used as a loading control. Data represent the mean ± SEM (n=3). *<0.05; **p < 0.01; ***p < 0.001, Vs control vehicle by one-way ANOVA followed by Bonferroni's test.

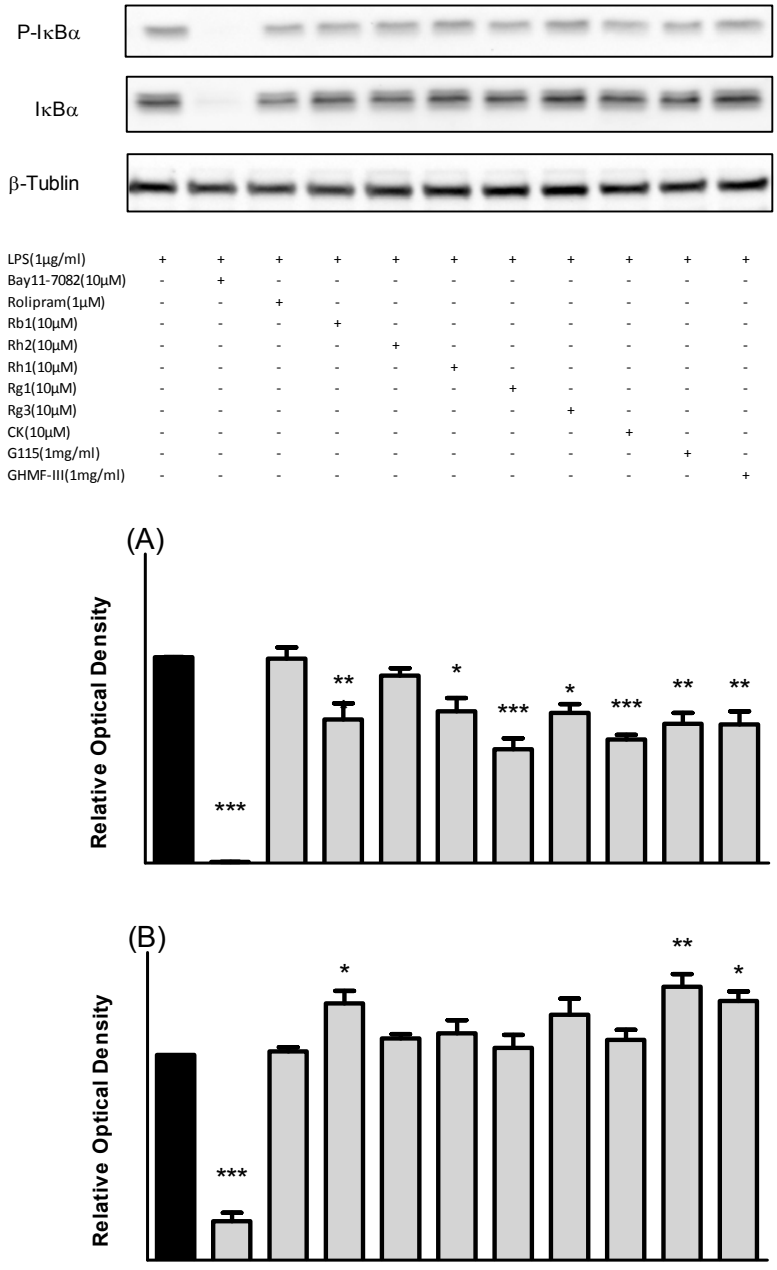


Figure 4-2 Effects of ginseng and ginseng related products on the expression of p-IκBα and IκBα in macrophage-like U937 cells induced by LPS.

Macrphage-like U937 cells were treated with or without Bay11-7082(10μM), Foskolin (10μM), G115 (1mg/ml), GHMF-III(1mg/ml) and Rb1, Rg1, Rg3, CK, Rh1, Rh2(10μM) for 2 hour, and then stimulated with or without LPS(1μg/ml) for 30 minutes. The expression of p-IκBα (A) and IκBα(B) was measured by western blot using p- IκBα antibody and IκBα antibody as described in the methods. B-Tublin was used as a loading control. Data represent the mean ± SEM (n=3)*<0.05; **p < 0.01; ***p < 0.001, Vs control vehicle by one-way ANOVA followed by Bonferroni's test.

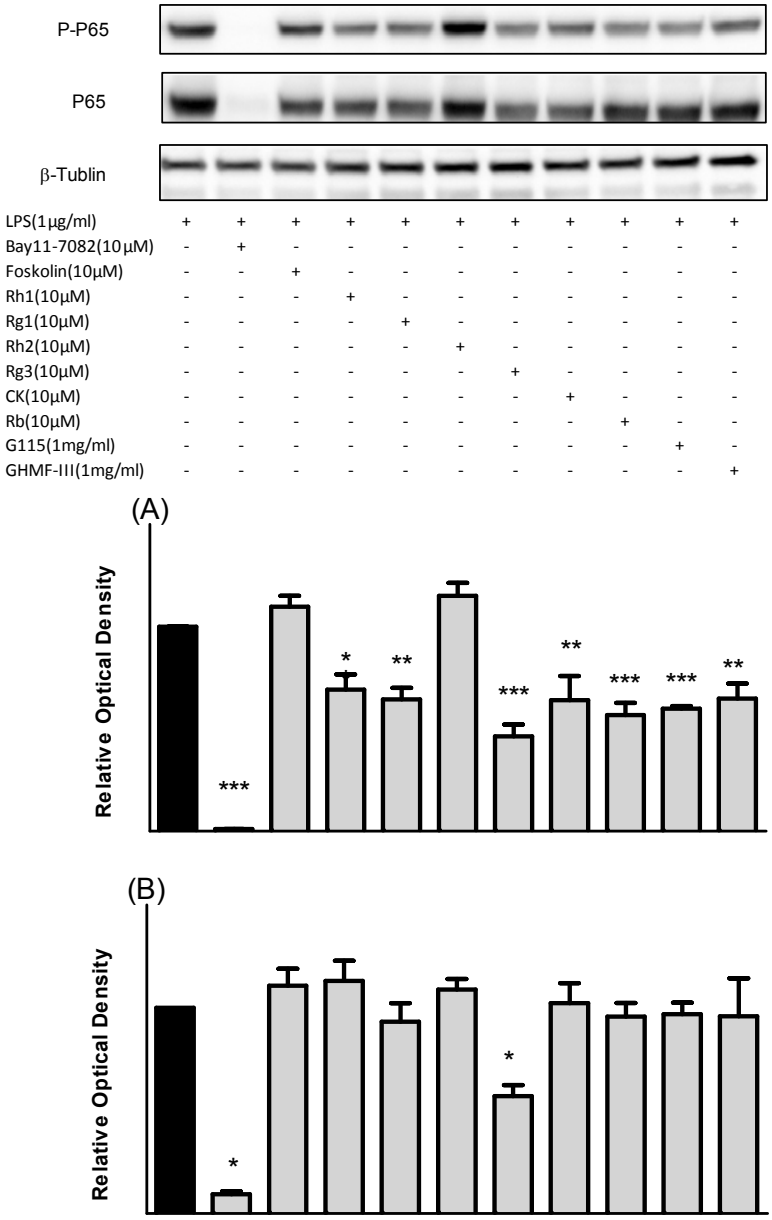
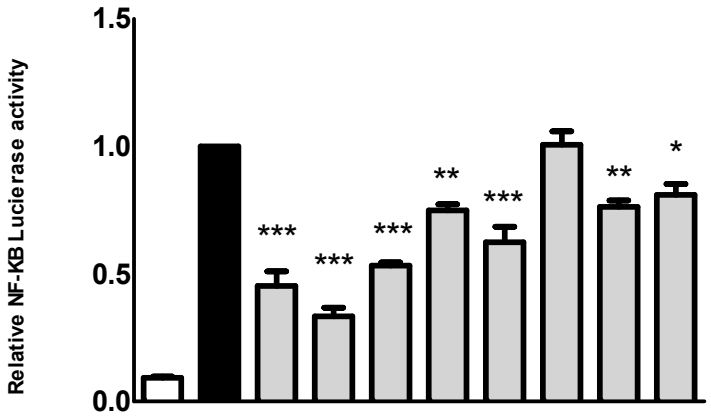


Figure 4-3 Effects of ginseng and ginseng related products on the expression of p-p65 and p65 in macrophage-like U937 cells induced by LPS.

Macrphage-like U937 cells were treated with or without Bay11-7082(10μM), Foskolin (10μM), G115(1mg/ml), GHMF-III(1mg/ml) and Rb1, Rg1, Rg3, CK, Rh1, Rh2(10μM) for 2 hour, and then stimulated with or without LPS(1μg/ml) for 30 minutes. The expression of p-p65 (A) and p65(B) was measured by western blot using p- p65 antibody and p65 antibody as described in the methods. B-Tublin was used as a loading control. Data represent the mean ± SEM (n=3). *<0.05; **$p < 0.01$; ***$p < 0.001$, Vs control vehicle by one-way ANOVA followed by Bonferroni's test.

4.4.2 Effects of G115, GHMF-III, Ginsenosides and their Metabolites on the Transcriptional Activity of NF-κB Induced by LPS.

To investigate the effects of G115, GHMF-III, Rb1, Rg1, Rg3, CK, Rh1 and Rh2 on the transcriptional activity of NF-κB induced by LPS, U937 cells were pre-treated with or without G115(1mg/ml), GHMF-III(1mg/ml) and Rb1, Rg1, Rg3, CK, Rh1, Rh2(10μM) for 2 hours, and then were induced by LPS(1μg/ml) for 6 hours. The transcriptional activity of NF-κB induced by LPS was significantly inhibited by G115($p<0.001$), GHMF-III($p<0.001$), Rb1($p<0.001$),Rg1($p<0.01$), Rg3($p<0.001$), CK($p<0.001$) and Rh1($p<0.01$), but not Rh2($p>0.05$; Figure4-4)



LPS (1μg/ml)	-	+	+	+	+	+	+	+	+	+
GHMF-III(1mg/ml)	-	-	+	-	-	-	-	-	-	-
G115(1mg/ml)	-	-	-	+	-	-	-	-	-	-
Rb1(10μM)	-	-	-	-	+	-	-	-	-	-
CK(10μM)	-	-	-	-	-	+	-	-	-	-
Rg3(10μM)	-	-	-	-	-	-	+	-	-	-
Rh2(10μM)	-	-	-	-	-	-	-	+	-	-
Rg1(10μM)	-	-	-	-	-	-	-	-	+	-
Rb1(10μM)	-	-	-	-	-	-	-	-	-	+

Figure 4-4 Effects of GHMF-III, G115, Ginsenosides and their Metabolites on the transcriptional activity of NF-κB in macrophage-like U937 cells induced by LPS.
Macrphage-like U937 cells were treated with or without GHMF-III (1mg/ml), G115 (1mg/ml) and Rb1, CK, Rg3, Rh2, Rg1, Rh1 (10μM) for 2 hours, and then stimulated with or without LPS(1μg/ml) for 6hours. The transcriptional activity of NF-κB was measured by the Dual-Glo® Luciferase Reporter Assay System. Data represent the mean ± SEM (n=3) in duplicate. *p<0.05;**p<0.01; ***p<0.001 Vs LPS vehicle by one-way ANOVA followed by Bonferroni's test.

4.4.3 Effects of G115, GHMF-III, Ginsenosides and their Metabolites on the Level of cAMP in LPS Induced macrophage-like U937 cells

In order to test the effects of G115,GHMF-III,Rb1, Rg1, Rg3, CK, Rh1 and Rh2 on cAMP pathway, U937 cells were treated with or without Rolipram (1μM), G115 (1mg/ml), GHMF-III(1mg/ml), Rb1(10 μM), CK(10 μM), Rg3(10 μM), Rh2 (10 μM), Rg1(10 μM) and Rh1(10 μM) for 2 hours, and then stimulated by LPS (1μg/ml) for 6 hours. LPS slightly increased the level of cAMP, however, there was no statistically difference on the level of cAMP between LPS and blank group ($p>0.05$). As positive control, Rolipram almost increased 40% of production of cAMP induced by LPS. The level of cAMP induced by LPS was also significantly up-regulated by G115 ($p<0.05$), GHMF-III ($p<0.0g$) and ginsenoside Rg1 ($p<0.01$), but not Rb1, Rg3, CK, Rh1 or Rh2 ($p>0.05$). Rolipram displayed a significant increase in cAMP level than G115 ($p<0.001$), GHMF-III ($p<0.001$) and Rg1($p<0.01$; Fig.4-5).

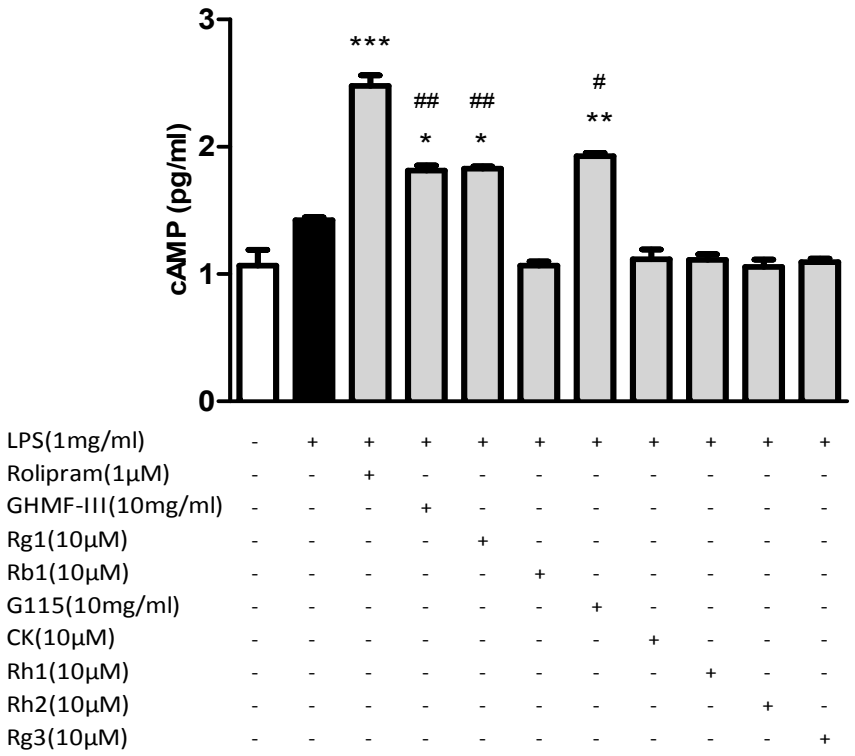


Figure 4-5 Effects of G115, GHMF-III, Ginsenosides and their Metabolites on the Level of cAMP in LPS Induced macrophage-like U937 cells.

Macrophage-like U937 cells were treated with or without Bay11-7082(10μM), Rolipram(1μM), G115(1mg/ml), GHMF-III(1mg/ml) and Rb1, Rg1, Rg3, CK, Rh1, Rh2(10μM) for 2 hour, and then stimulated with or without LPS(1μg/ml) for 6 hours. The level of cAMP was measured by EIA. Data represent the mean ± SEM (n=3) in duplicate.*p<0.05; **p < 0.01,***p<0.001 Vs control vehicle by one-way ANOVA followed by Bonferroni's test.#p<0.01; ##p<0.001, Vs Rolipram vehicle by one-way ANOVA followed by Bonferroni's test.

4.4.4 Effects of G115, GHMF-III and Ginsenosides Rg1 on the Expression of CREB and p-CREB in LPS Induced Macrophage-like U937 Cells.

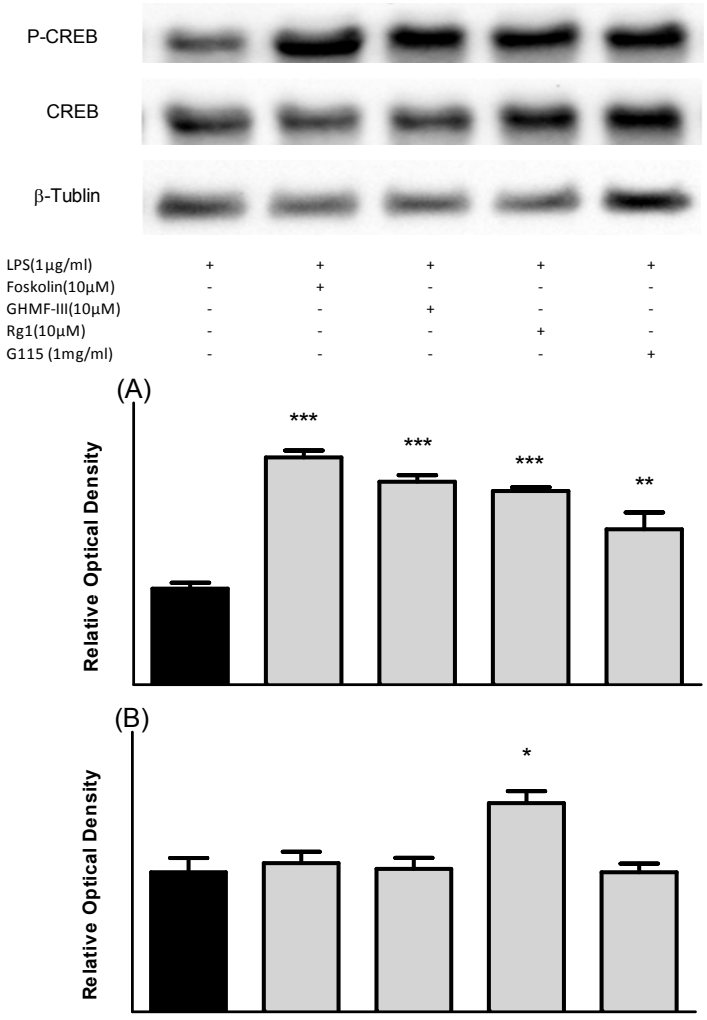


Figure 4-6 Effects of G115, GHMF-III and Ginsenosides Rg1 on the Expression of CREB and p-CREB in LPS Induced Macrophage-like U937 Cells.

Macrphage-like U937 cells were treated with or without Rolipram (1μM), G115(1mg/ml), GHMF-III(1mg/ml) and Rg1(10μM) for 2 hours, and then stimulated with or without LPS(1μg/ml) for 30 minutes. The expression of p-CREB(A) and CREB(B) was measured by western blot using p-CREB antibody and CREB antibody as described in the methods. B-Tublin was used as a loading control. Data represent the mean ± SEM (n=3). *p<0.05; **p < 0.01; ***p < 0.001, Vs control vehicle by one-way ANOVA followed by Bonferroni's test.

As the positive control, Rolipram significantly increased the expression of c-CREB induced by LPS, compared with LPS group ($p<0.001$; Fig.4-6-A). However, it had no significant effect on the expression of total CREB ($p>0.05$; Fig.4-6-B). The expression of p-CREB induced by LPS was also significantly increased by ginsenoside Rg1 ($p<0.001$), GHMF-III ($p<0.001$) and G115 ($p<0.01$). Ginsenoside Rg1 displayed the more significant up-regulation on the expression of c-CREB than G115 ($P<0.05$; Fig.4-6-A). Among Rg1, GHMF-III and G115, only Rg1 up-regulated the expression of total CREB compared with LPS group ($p<0,05$;Fig.4-6-B)

4.4.5 Effects of G115, GHMF-III and Ginsenoside Rg1 on the Activity of PDE4 in LPS Induced Macrophage-like U937 Cells

U937 cells with 1μg/ml LPS elicited a significant increase in PDE4 activity. The LPS increased PDE4 activity was significantly inhibited by 10μM Rg1 ($p<0.001$), 1mg/ml GHMF-III ($P<0.001$) and 1mg/ml G115 ($P<0.05$). No significant difference was observed among the effects of Rg1, GHMF-III and G115on Rolipram-sensitive PDE activity induced by LPS ($P>0.05$; Fig. 4-7).

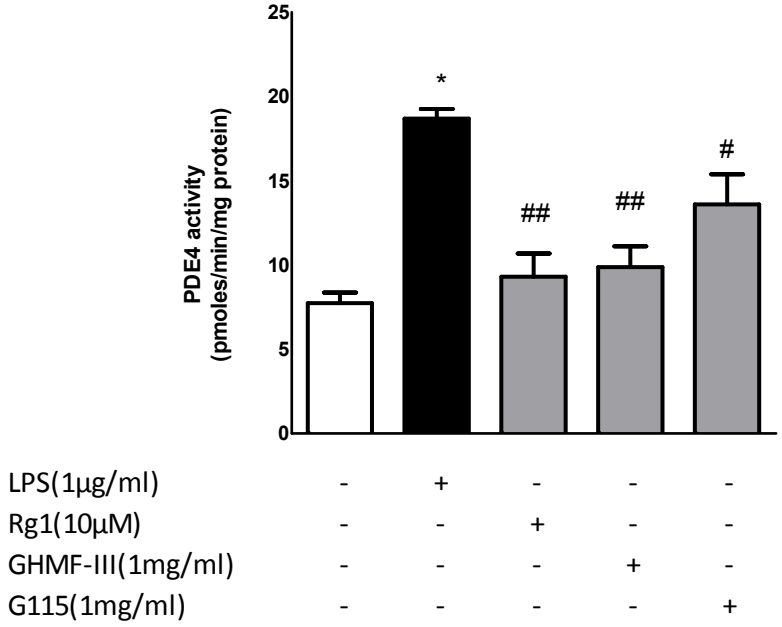


Figure 4-7 Effects of G115, GHMF-III and Ginsenoside Rg1 on the Activity of PDE4 in LPS Induced Macrophage-like U937 Cells.

Macrophage-like U937 cells were pre-treated with or without Rolipram (30μM), Rg1(1mg/ml), GHMF-III(1mg/ml) and G115(1mg/ml) for 2 hour, and then induced by LPS(1μg/ml) for 4 hours. PDE4 activity was quantified by scintillation counting. Data represent the mean ± SEM (n=3) performed in duplicate. *<0.001, Vs blank vehicle by T'student test.#<0.05; ##<0.001, Vs control vehicle by one-way ANOVA followed by Bonferroni's test.

4.5 Discussion

NF-κB pathway is the important inflammation-related pathway, which involves the pathogenesis of abnormal inflammation of COPD (see Section 1.1.4.4). In this part of my project, ginseng and its related products displayed the different regulation on the activity of NF-κB pathway induced by LPS.

GHMF-III, as a ginseng formula, displayed a similar and more potent regulation on the activation of PRs in NF-κB pathway induced by LPS with G115 (the standardised extract

of ginseng), as both of them not only inhibited the expression of activated products of PRs in NF-κB induced by LPS, including p-IKK, p-IκBα and p-p65, but also inhibited the expression of total IKK and IκBα induced by LPS. Only PDG type ginsenoside Rb1 displayed the similar regulation on the activation of PRs induced by LPS in NF-κB pathway with G115 and GHMF-III, so it seemed that G115 and GHF-III displayed the pharmacological actions of Rb1 on the activation of PRs induced by LPS in NF-κB pathway. Another PDG ginsenoside Rg3 displayed the analogous regulation with Rb1 on the activation of PRs induced by LPS in NF-κB pathway, but couldn't increase the expression of IκBα. Previous study showed that Rg3 inhibited the expression of IκBα in RAW264.7 cells without any stimulation[339]. We inferred that Rg3 perhaps displayed the dissimilar regulation on the expression of IκBα at different condition. The PTG type ginsenoside Rg1 also inhibited the expression of p-IKK, p-IκBα and p-p65, but couldn't inhibit the expression of total protein.

There were some differences of the regulation on the activity of RPs between the metabolite and the PDG type ginsenoside. CK, the metabolite of Rb1 showed the similar effect as Rg1 and Rh1. It inhibited the expression of phosphorylated proteins, but not the expression of total protein of PRs. Rh2, the metabolite of Rg3 had no obvious effect on the activation of PRs in NF-κB, which however, was different with a previous study showing that Rh2 inhibited the expression of p-IκBα in TNF-α induced human astroglial cells[341]. The reason for these discrepancies is not clear, but it may be related to different cell lines used. Further study is necessary to elucidate the exact mechanisms involved.

Previous studies only involved in the regulation of Rb1, Rg1 and Rh1 on the transcriptional activity of NF-κB, and all of them display significant inhibition on it (see Section 1.2.4.3). In this part of our project, we also investigated the regulation of other

ginseng and its related products on the transcriptional activity of NF-κB induced by LPS as supplementary. We found that G115, GHMF-III, Rg3 and CK also inhibited the transcriptional activity of NF-κB induced by LPS and the inhibition of Rb1, Rg1 and Rh1 on it also was proved.

Interestingly, both GHMF-III and G115 up-regulated the activity cAMP pathway, the effect may link directly to their inhibitory actions on the transcriptional activity of NF-κB. GHMF-III and G115 mainly displayed the pharmacological action of PTG type ginsenoside Rg1 on the regulation of cAMP pathway, as only GHMF-III, G115 and ginsenoside Rg1, but not Rb1, Rg3, CK, Rh1 or Rh2, up-regulated the level of cAMP induced by LPS, which was accompanied with the increase of the expression of p-CREB. Thus, it is possible that the cAMP-component actions of G115 and GHMF-III are mediated by Rg1 in these ginseng extracts. Given these agents also inhibited the transcriptional activity of NF-κB, it is likely that a synergistic action may be involved in the regulatory actions of ginseng on NF-κB pathway. .In addition, the up-regulation of cAMP by GHMF-III, G115 and Rg1 is associated with the decreased in activity of PDE4, which indicates that GHMF-III, G115 and Rg1 may act as PDE4 inhibitor to reduce the PDE4 activity, resulting in the increase of cellular cAMP levels. Liang reported earlier that Rb1 also increased the level of intracellular cAMP in rat liver compared with that in controls[353] , but this was not confirmed in our study.

G115 is a ginsenoside-extract from ginseng and PDGs (Rb1, Rb2, Rc and Rd) and PTGs(Re, Rg1 and Rg2) are the main active components of it[324]. Thus, G115 maybe display both pharmacological actions of PDGs and PTGs. This hypothesis has been proved in my project that G115 exhibited more potent inhibition on the activity of NF-κB pathway and up-regulation on the activity of cAMP pathway than Rb1(PDGs) and Rg1(PTGs) used

alone. It indicates that crude extracts of ginseng may target more broadly than purified individual ginsenosides. GHMF-III showed the similar regulation on the activity of NF-κB and cAMP pathways, however as the chemical information for GHMF-III is not fully characterized, further study about the chemical construction of GHMF-III is necessary.

In conclusion, the study has clearly demonstrated that ginseng and ginsenosides can regulate NF-κB pathway in LPS induced macrophage-like U937 cells. They act via at least two separate mechanisms: inhibition of the activity of PRs in NF-κB to decrease the activity of NF-κB, and activation of cAMP pathway, via inhibition of PDE, which in turn to inhibit the transcriptional activity of NF-κB. The study also discovered a non-specific inhibition of Bay11-7082 on the expression of p-IKK, p-IκBα, p-p65, IκBα and p65. In addition, there are differences among ginseng and ginseng related products on the regulations of cAMP pathway, as only G115, GHMF-III and ginsenoside Rg1 share similar actions on activation of cAMP pathway. Some of the findings in the study have not been reported previously. These new findings may help to understand the mechanism of anti-inflammatorty actions of ginseng and ginseng related products.

Chapter 5. Efficacy of TNF-α Inhibitors on Radiological Progression in Active Rheumatoid Arthritis- A systematic review

5.1 Introduction

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disorder characterized by inflammation of the articular synovium, resulting in bony erosions, deformity and ultimately, joint destruction (JD). RA is one of the most common and costly diseases, affecting about 0.3-1.2% of the population and 20%-35% of the patients stop work during first 2-3 years in disease[3,413-416]. The prevalence of work disability can be decreased through slowing and arresting the progression of JD. Therefore preventing the progression of JD is the key treatment objective of RA[417].

5.2 JD and Active RA

5.2.1 JD of RA Patients

JD is an important part of illness development in RA[418] and occur throughout the whole course of disease, the functional loss and irreversible disability caused by JD are the most important manifestations leading to the medical cost of RA[419-421]. Preventing the progression of JD not only can improve life quality of patients, but also can lead to substantial savings in medical costs[422-424]. Hence, the control of the progression of JD is delineated as the key treatment goal outlined in the Guidelines of American College of Rheumatology (ACR)[417].

JD in RA is the result of a series of inflammatory responses. First, a large number of inflammation-related cells is attracted to the joint cavity to cause secretion of various inflammatory mediators which results in the occurrence of synovitis secondary to swelling

of synovial cells, excess synovial fluid, and the development of fibrous tissue (pannus) in the synovium[425]. These inflammatory response lead to the destruction of articular cartilage and the fusion of bone under cartilage. JD is the most important pathological manifestations of RA and the progression of JD can be evaluated by x-ray[426].

5.2.1.1 Scoring of the Radiological Progression of JD in RA

It has been known that joint external manifestations do not correspond to the inner JD in patients with RA. Therefore, radiographic outcome, the gold standard for JD progression assessment, is widely used in the clinical setting[426]. There are many kinds of scoring systems for calculating the radiographic outcome, including Steinbrocke, Kellgren, Sharp and Lanser scoring systems[420,421,427,428]. Among these, Sharp system is commonly used to monitor the progression of JD through continuous improvement [429-431].

The improved Sharp method defines erosions as: 0 = Normal, 1 = Discrete erosions, 2 to 3 = Larger erosions according to surface area involved, 4 = Erosions extending over middle of the bone, and 5 = Complete collapse. Generally, 15 areas from the hands to wrists and six areas from the feet are used to calculate the joint space narrowing score. The maximum joint space narrowing score is 120; the maximum erosion score is 160 for hands and wrists, and 120 for feet. Thus, the maximum Sharp radiographic score is 448.17[431]. The progression of JD in RA patients and the efficacy of therapeutic drugs can be evaluated using the Sharp scoring system.

5.2.2 Active RA

American Rheumatism Association (ARA) first proposed that RA should be divided into active RA and inactive RA according to the classification criteria for RA [432]. Later the

Rome criteria was introduced since some histological features as proposed in ARA criteria could not be used in the population setting[433].

5.2.2.1 Criteria of Active RA

Currently, there is still no real "gold standard" to judge RA disease activity. Nevertheless, many clinical trials have used the Disease Activity Score (DAS) as an assessment method to value RA activity. DAS is a combined index to assess important impact factors affecting RA activity, such as the number of swollen and tender joints and measures the Erythrocyte sedimentation rate (ESR)/C-reactive protein (CRP). DAS is usually scored between 0 and 10 to indicate RA activity[434]. The DAS28 is analogous to the DAS but includes simplified 28-joint counts[435].

5.2.3 JD in Patients with Active RA

Many rheumatologists believe that patients with active RA have most JD. Kirwan et al collected related clinical data and illustrated that continued disease activity is obvious in the whole course of RA, accompanied with temporal fluctuations reflected in a gradual increase in JD[436]. Further studies support that active RA is the main developing period of JD[437,438]. Interestingly, although the patients in early stage of RA displayed slow and slight disease progression and only some of these patients will develop into RA[439], the patients in early stage of active RA also showed the significant JD[438,440,441].

In addition, one study showed that the fluctuation in RA disease activity is directly related to changes in radiologic progression in patients with active RA[442]. This indicates that the change of RA disease activity may affect the progression of JD, and also the efficacy of therapeutically drugs of RA for treating JD.

5.3 Treatment of JD in patients with active RA

5.3.1 Treatment of RA

In 1970s, some rheumatologists proposed a "pyramid" treatment protocol for RA treatment based on past experience[443]. They believe that the treatment of RA should follow a step-by-step three line administration protocol. RA treatment drugs can be divided into Non-steroidal anti-inflammatory drugs (NSAIDS) as the first-line drugs, disease modifying anti-rheumatic drugs(DMARDs), such as Methotrexate (MTX), as the second-line drugs, and the glucocorticoid as the third-line drug. Patients from early, middle and late stages of RA are corresponded to the use of the first-line, the second-line and the third-line drugs respectively. However, subsequent accumulating clinical evidence indicates that the use of a single drug was not enough to control the RA progression, and then many rheumatologists proposed an "inverted pyramid"(step down) treatment protocol in 1989[444]. This protocol suggests to treat RA patients using all three line drugs in conjunction, and gradually reduces the types and doses of drugs, and finally using one or two kinds of drugs with small doses for long-term maintenance of steady state of RA. Although the "inverted pyramid" protocol caused some controversy, it has been used as the reasonable and preferred treatment protocol for RA treatment by global rheumatologists. However, the therapeutic treatment of RA has not been significantly improved until the clinical application of TNF-α inhibitors. TNF-α inhibitors significantly improved the American College of Rheumatology core criteria 20 (ACR20), DAS score and Health Assessment Questionnaire(HAQ) scores, as well as swollen joint count, tender joint count, and CRP level[445-447]. Most importantly, TNF-α inhibitors significantly inhibited the progression of JD in active RA [437,438,446,448-452].

MTX and TNF-α inhibitors are the most important and popular anti-JD progression drugs in patients with RA. However, according to the efficacy, side effect and medical costs, these drugs are often used in different conditions. For example, low-dose MTX was used for treating the patients with early RA[443,453,454], but for the patients with active RA TNF-α inhibitors displayed showed more efficacy on the progression of JD than MTX[437,438,446,448-452].

5.3.2 Efficacy of MTX on the Progression of JD in Patients with Early RA

MTX, formerly known as amethopterin, is an anti-metabolite and anti-folate drug. It has been widely used in the treatment of autoimmune diseases, including RA, psoriatic arthritis and Crohn's disease[455-457]. MTX in low doses is generally safe and well tolerated as the preferred therapy for the treatment of RA[458]. Previous studies demonstrated that low-dose MTX used for up to one year significantly slowed the radiographic progression of JD in patients with early RA, though not anyone was responsive to the treatment with MTX[453,454]. As patients with early RA always displayed slight JD, MTX is suitable for retarding mild JD, but not moderate or severe JD. Many studies showed that MTX used in conjunction with other DMARD type drugs, such as Sulfasalazine, Ciclosporin and Azathioprine, to increase the efficacy of these drugs[459-461]. Thus, there is a synergy effect between MTX and other drugs to inhibit the progression of JD.

5.3.3 Efficacy of TNF-α Inhibitors on the JD Progression in Active RA Patients

TNF-α is an important inflammatory mediator and play an important role in initiating and perpetuating inflammatory and tissue damage in the rheumatic joint[462,463]. Thus, down-regulating the activity of inflammatory mediators to inhibit the progression of JD in patients with active RA became the core therapeutic objective in RA treatment.

TNF-α inhibitors have been used in the treatment of RA since 2000. Three TNF-α inhibitors are currently approved by the FDA and the European commission(EC) for the treatment of RA, which are infliximab (Remicade), etanercept (Enbrel) and adalimumab (Humira). These drugs are also listed in the Pharmaceutical Benefits Scheme (PBS) in Australia. ACR recommended using TNF-α inhibitors as biological DMARD to treat RA, especial to treat active RA[464]. There are strong evidence that TNF-α inhibitors displayed more powerful inhibition on the progression of JD in active RA comparing with MTX used alone[437,438,446,448-452].

TNF-α inhibitors for the treatment of active RA can be used alone and in combination with MTX. Many studies have shown that TNF-α inhibitors, Infliximab (INF), Adalimumab (ADA), and Etanercept (ETA), can retard the progression of JD when used alone or in combination with MTX in active RA patients[437,446,448-452,422]. In the guideline of biological drug in RA, ACR believed there was no distinction between these two different administration of TNF-α inhibitor on the efficacy of active RA[417]. However, many rheumatologists were more inclined to use TNF-α inhibitors in conjunction with MTX to inhibit the progression of JD in active RA patients[437,446,448-452]. The rational for this is not well defined as all drugs for RA have obvious side effects[465,466]. It is important for the patients and rheumatologists to select drugs based on evidence of efficacy and safety. In addition, there are still certain unanswered questions about the treatment of TNF-α inhibitors in the JD of active RA patients, including factors affecting the efficacy of TNF-α inhibitors, and which type of TNF-α inhibitor provide a better efficacy. The aim of this systemic review is to quantitatively assess all the randomised controlled trials that assessed the effect of TNF-α inhibitors on radiological progression (RP) of JD in active RA to answer these questions.

Based on the available evidence, we hypothesized that TNF-α inhibitors used in conjunction with MTX provide a better efficacy against the progression of JD in patients with active RA comparing with TNF-α used alone, as well as MTX used alone. In addition, the degree of the disease activity of RA is an important factor for the efficacy of TNF-α inhibitors on the progression of JD in active RA patients. To test our hypothesis, we have used meta-analysis to -

- Compare the effect of TNF-α inhibitors used alone and in combination with MTX on the RP of active RA patient.
- Investigate the correlation between the degree of activity of RA and the efficacy of TNF-α inhibitors on RP in active RA patients
- Compare the effect of different types of TNF-α inhibitors on RP in active RA patients

5.4 Methods

5.4.1 Search Strategy

A search was conducted which was not restricted by language for all publications on TNF-α inhibitors and RA between January 1966 and July 2012. The electronic database included PubMed, Embase, Cumulative Index to Nursing and Allied Health Literature (CINAHL), Database of Abstracts of Reviews of Effects (DARE), Cochrane Central Register of Controlled Trials (CENTRAL), and the Cochrane Database of Systematic Reviews. We also searched for the articles published in the conference, unpublished trials and those trials in progress. We supplemented search by checking references cited in published systematic reviews and by references to the bibliographies of the articles extracted from the literature reviews.

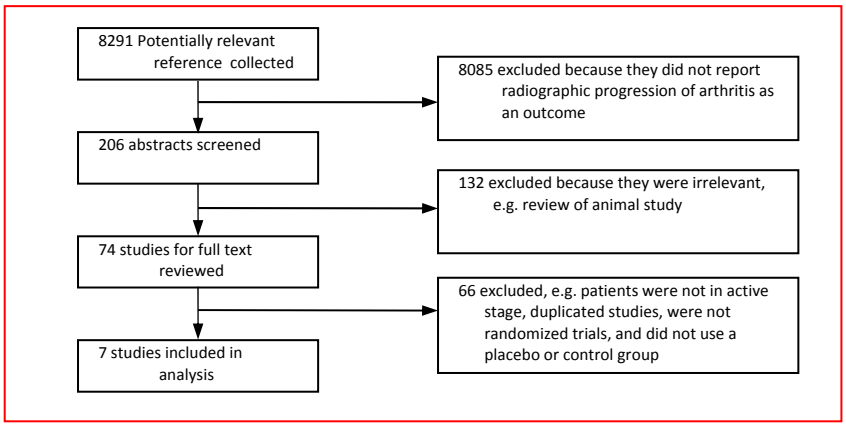
The search used the following medical subject headings (MeSH) and relevant keywords: (1) “Arthritis, Rheumatoid, or “Rheumatoid Arthritis”; (2) “TNFR-fc”, “Infliximab”, “Adalimumab”, or “Etanercept”; (3) “methotrexate” or “MTX” ; (4) “joint destruction” or “joint damage”. (5) “Radiological progression”.

5.4.2 Inclusion Criteria

All randomised trials that used TNF-α inhibitors alone, or in combination with MTX, reported the radiological progression of arthritis as an outcome, and patients with active RA, were included.

Articles were excluded based on abstracts if they did not pertain to RA and TNF-α inhibitors. Selected abstracts were excluded if they did not report radiological progression of arthritis as an outcome, and they were reviews or animal studies. Full texts of selected articles were reviewed and excluded if they were duplicated studies, were not randomized trials, and subjects were not at active stage (Fig.5-1).

Figure 5-1: Selection of Articles



5.4.3 Outcome Measurement

Radiological outcome is deemed as the gold standard for assessing the progression of JD in RA[426]. Sharp and Lanser scoring systems were recommended as the main method to assess the progression of JD in all radiological assessment method[429]. According to these methods, researcher can calculate the mean change (MC) of radiological scores between the baseline and after treatment of patients with RA and the percentage progression of JD above a certain cut-off level, which is equivalent to the risk ratio (RR) of every study. Recommended cut-off levels for progression were 0 or 0.5, as well as the smallest detectable difference (SDD) in order to account for measurement errors. In this project, all papers used the Sharp scoring system as the measure method at the end point. Using this method, MC and RR, as two outcomes of the end point, can be calculated in patients with active RA.

5.4.4 Data Extraction

Data were extracted from the papers which were collected according to the inclusion criteria by two independent reviewers. Disagreements were resolved by consensus involving in a third investigator. If the results which we needed were not expressed in the article, we approached the investigators by letter for more information. Four investigators were approached; two of them responded.

Descriptive information was also collected, including the name of the study, year of publication, total number of participants, intervention, the list of study providing available data about RR & MC and trial quality.

5.4.5 Assessment of Study Quality

Two independent reviewers blindly evaluated methodological quality of each study using Jadad scale, and disagreements were resolved by a third assessor. It contains three questions: the type of the study (randomized and double-blinded), the description of the withdrawals and dropouts; one point is awarded for each affirmative answer. One additional point is given or deducted if the study satisfy or dissatisfy the demand of the quality of the randomization and blindness. The Jadad scale goes from 0 to 5, and scores of 3 or higher indicate a good methodological quality[467].

5.5 Statistical Analysis

In all selected study, the RR was calculated as the primary outcome. MC in radiological scores between baseline and after therapy, along with the 95 % confidence intervals (95% CL) was calculated as the secondary outcome. Data were dealt with Comprehensive meta-analysis to pool results of comparable studies (RevMan5.1).

As data exclusion was finished by two reviewers independently, to quantify the level of agreement between reviewers, Cohen's kappa coefficient was calculated. The κ statistic is a statistical measure of inter-rater agreement, and values were ranged from +1 (perfect agreement) to -1(complete disagreement).

Heterogeneity was evaluated with Cochran's Q test statistic for collection of studies with large sample sizes, and quantified using the I^2 statistic. Values of I^2 equal to 25%, 50%, and 75% representing low, moderate, and high heterogeneity respectively. When there was a significant heterogeneity (I^2>50%) among the independent studies, we used random-

effects meta-analysis; when the heterogeneity was insignificant (I^2<50%) among the related studies, the fix-effects meta-analysis was done.

To assess whether the efficacy of TNF-α inhibitors on the progression of JD in active RA patients was modified by clinical and demographic variables, we pre-specified a list of 13 variables for subgroup analysis. Variables were chosen on the basis of either biological plausibility (e.g., age, the percent of baseline administration medicine, or different kinds of TNF-α inhibitors) or known risk factors of JD in RA (e.g. DAS28, ESR and CRP). Each variable was used as the standard to divide available studies into different subgroup, and the subgroup analysis was done for them to confirm whether there was a significant distinction between subgroups.

To analyse the heterogeneity, studies were divided into different subgroups according to the results of subgroup analysis. “Complemented with MTX” and “different type of TNF-α inhibitors” were confirmed as two major factors to modify the efficacy of TNF-α inhibitors on RR of the progression of JD in patients with active RA, so we divided our studies into 5 subgroups (INF combination with MTX, ETA combination with MTX, ADA combination with MTX, ETA alone and ADA alone) according to these two factors. Meta-analysis was done for pooling results of independent studies in each subgroup.

To assess the correlation between the treatment efficacy and the duration of RA, age, DAS28, or CRP, meta-regression was used. Duration of RA, age, DAS28, CRP and ESR were used as independent variables, and log risk ratio was used as dependent variable. There are statistically significant correlation between two variables, if $p<0.05$.

We assessed publication bias using the Egger’s regression. Publication bias was assessed via a funnel plot—in which the log RR was plotted against the standard error for all studies

included. There is not publication bias if $p>0.05$. If publication bias was detected, the effect of such bias was assessed with the fail-safe number method. The fail-safe number was the number of unpublished studies that would be needed to nullify the observed result to statistical non-significance at $\alpha=0.05$ level.

5.6 Results

Of the 8291 references screened, 7 clinical trials were included in the final analysis (Fig.5-1). There were three types of TNF-α inhibitors involved in these 7 trials, including INF, ADA, and ETA. All of these TNF-α inhibitors were recommended by ACR[417]. 7 trials included 11 types of treatment groups (different drug administration methods), such as MTX+INF 3mg/kg every 8wk, MTX+INF 3mg/kg every 4wk, MTX+INF 10mg/kg every 8wk, MTX+INF 10mg/kg every 4wk, MTX+INF 6mg/kg every 8wk, MTX+ETA 50mg weekly, MTX+ ETA 25mg twice week, MTX+ADA 40mg every other week, ETA 25mg twice week, ADA 40mg every other week and ADA 20mg every week treatment groups (Tab.5-1). As all treatment groups in these trails were analysed separately, we regarded these treatment groups as the independent studies for further meta-analysis. As a result, the placebo group was counted repeatedly. Every independent study which provided available data about RR and MC was marked in the "table 5-1". In total 14 independent studies, there were 12 independent studies providing available results of RR of the progression of JD in active RA patients, and 11 independent studies providing available results of MC of the progression of JD in active RA patients (Tab.5-1). In all of 7 clinical trials, there were 2 types of comparisons involving in the effect of TNF-α and MTX inhibitors on RR of the progression of JD in active RA patients, including TNF-α inhibitors used alone comparing with MTX and TNF-α inhibitors in combination with MTX comparing with MTX. In the first comparison, there were 9 independent studies providing available results of RR of the

progression of JD in active RA patients. In the second comparison, there were 3 independent studies providing available results of RR of the progression of JD in active RA patients.

In total, 5704 individuals were analysed, whose mean age were between 49.99 and 56.62 years old. The mean span of the disease duration was very large. It was between 8.73 and 131.58 months. All the studies performed measure of radiographic progression after one year（48, 52 or 54 weeks）, and so one year was chosen as observation time of end point (Tab.5-1).

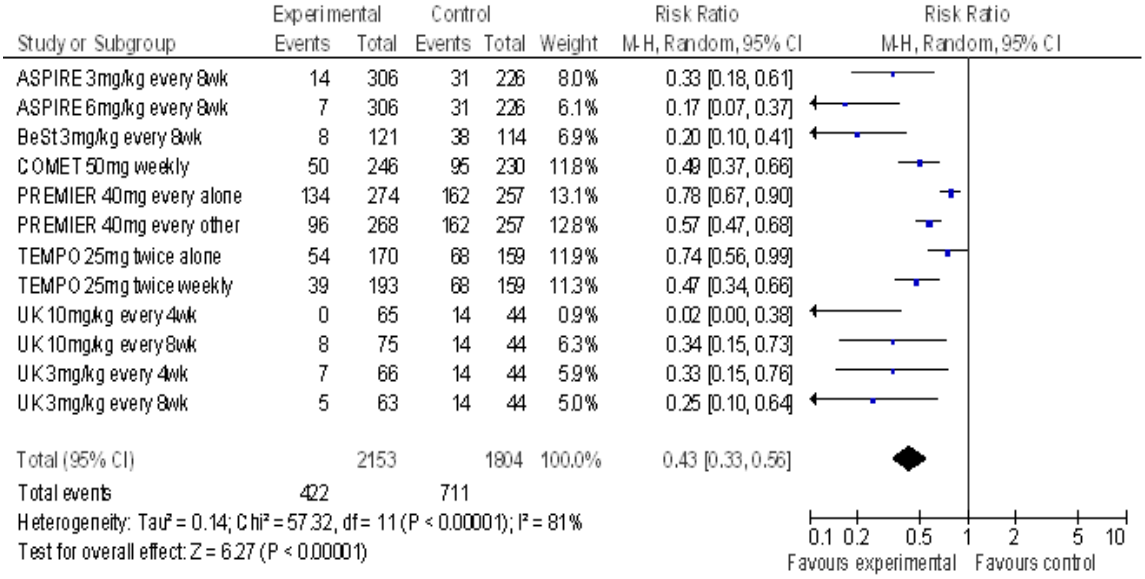
The quality of all trials was good. The Jadad scales of all trials were 3 or higher, which meant a good methodological quality (Tab.5-1). Only two studies didn't use double-blind method[446,451]. Only one study didn't record the result of withdrawal and dropout[451]. Cohen's kappa coefficient was calculated, and result was 0.79. It meant that data exclusion finished by two reviewers independently were substantial agreement.

Table 5-1 Characteristics of Studies

Study (year)	No. of patients	duration of radiographs (weeks)	Intervention	RR*	MC	Trial Quality
UK group (1999)[446]	428	Base-line /54wks /102wks	MTX+INF(3mg/kg every 8wk) Vs MTX MTX+INF(3mg/kg every 4wk) Vs MTX MTX+INF(10mg/kg every 8wk) Vs MTX MTX+INF(10mg/kg every 4wk) Vs MTX	√ √ √ √	√ √ √ √	5
ASPIRE group (2004)[449]	1490	Base-line /54wks	MTX+INF(3mg/kg every 8wk) Vs MTX + placebo MTX+INF(6mg/kg every 8wk) Vs MTX + placebo	√ √	√ √	5
BeSt study (2006)[451]	508	Base-line /48wks	MTX+INF(3-10mg/kg every 8wk) Vs MTX	√	√	3
COMET study (2008)[452]	542	Base-line /52wks	MTX+ETA(50mg weekly) Vs MTX + placebo	√	-	5
TEMPO study (2005)[450]	686	Base-line /48wks /96wks /144wks	ETA(25mg twice weekly)+placebo Vs MTX+placebo MTX+ETA(25mg twice weekly) Vs MTX + placebo	√ √	- -	5
PREMIER study (2006)[437]	799	Base-line /48wks /96wks	ADA(40mg every other week)+placebo Vs MTX+placebo MTX+ ADA(40mg every other week) Vs MTX + placebo	√ √	- -	4
BBOTT study (2004)[468]	619	Base-line /52wks	ADA(40mg every other week)+ placebo Vs MTX+ placebo ADA(20mg every week)+ placebo vs MTX+ placebo	- -	√ √	5

RR= the risk ratio; MC= Mean change in radiographic scores between baseline and after treatment.
* Number of RR and MC may not add up to 100% of total because data provided by some studies were empty or not available.

Figure 5-2 Comparison between the Effect Size of TNF-α Inhibitors and MTX on RR of JD in Patients with Active RA



In 12 independent studies which compared the effect of MTX and TNF-α inhibitor(including TNF-α inhibitor used alone and in combination with MTX) on RR of joint damage in active RA patients, we used random effects model for heterogeneity (P=0.00001, I^2=81%). The effect size of TNF-α inhibitors group was significantly better than MTX group (Z=6.27, P<0.00001). The use of TNF-α inhibitors was associated with a 57% reduction in progression of JD comparing with MTX used alone (RR 0·43, 95% CI 0.33–0.56; Fig.5-2). All the studies displayed the consistent beneficial efficacy of TNF-α inhibitors comparing with the efficacy of MTX on RR of the progression of JD in active RA patients (Fig. 5-2). Of 11 independent studies which compared the effect of MTX and TNF-α inhibitors(including TNF-α inhibitor used alone and in combination with MTX) on MC in active RA patients, we used random effect model for heterogeneity (P=0, I^2=84%). The effect size of TNF-α inhibitors groups was also significantly better than MTX groups (Z=-6.921, P=0). The use of TNF-α inhibitors was associated with a JD scores improvement of -2.313(-2.663 - -1.963; Table:5-2).

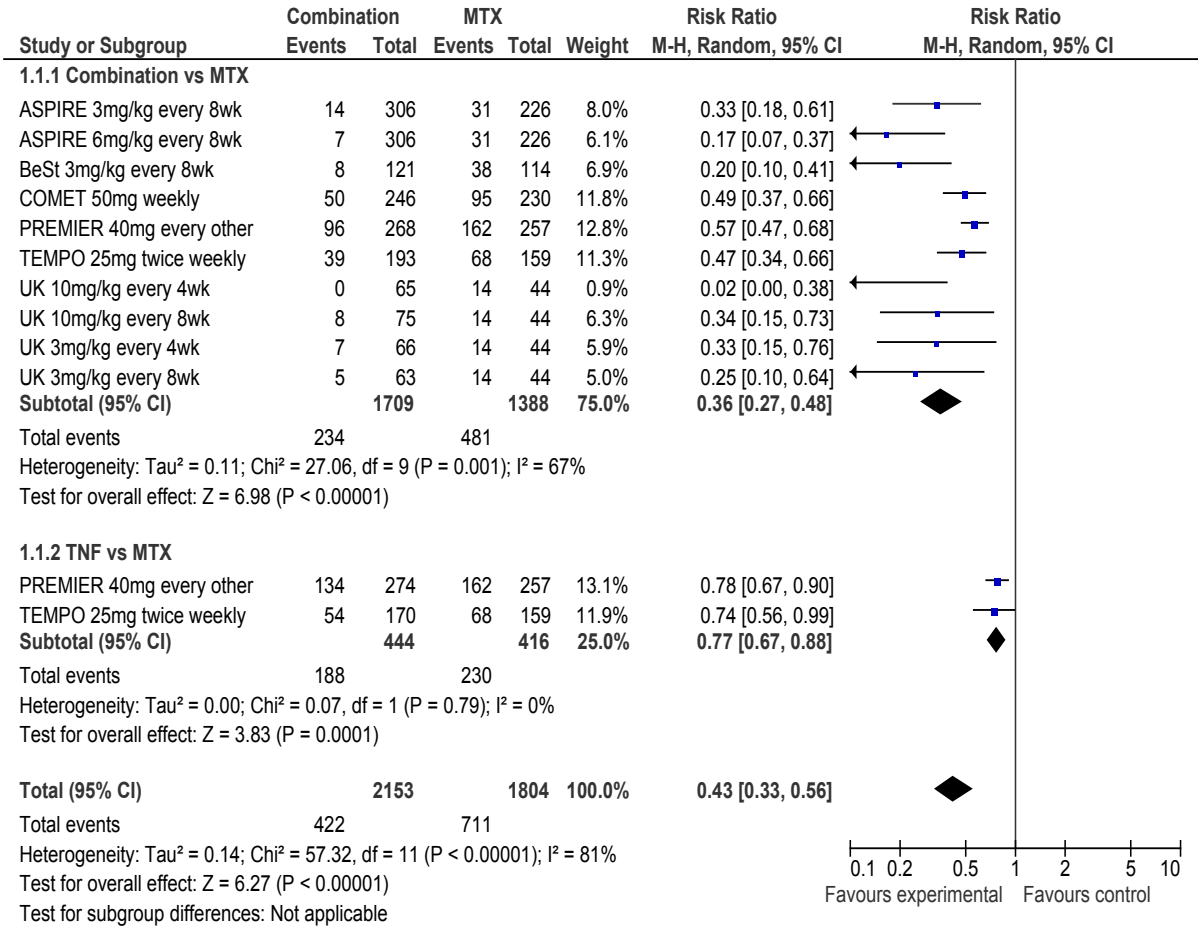
Table 5-2 Comparison between the effect size of TNF-α inhibitors and MTX on MC of the progression of JD in patients with active RA.

Study name	Difference in means	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
UK MTX+INF3mg/kg every 8wk	-5.7	1.55	2.401	-8.737	-2.663	-3.678	0
UK MTX+INF3mg/kg every 4wk	-5.4	1.776	3.155	-8.881	-1.191	-3.04	0.002
UK MTX+INF10mg/kg every 8wk	-6.8	1.273	1.621	-9.295	-4.305	-5.341	0
UK MTX+INF10mg/kg every 4wk	-7.7	1.364	1.861	-10.374	-5.026	-5.645	0
ASPIRE MTX+INF3mg/kg every 8wk	-3.3	0.65	0.423	-4.574	-2.026	-5.077	0
ASPIRE MTX+INF6mg/kg every 8wk	-3.2	0.641	0.411	-4.457	-1.943	-4.989	0
BeSt MTX+INF3-10mg/kg every 8wk	-5.8	1.449	2.1	-8.64	-2.960	-4.003	0
TEMPO ETA25mg twice weekly	-1.17	0.288	0.083	-1.734	-0.606	-4.067	0
TEMPO MTX+ETA25mg twice weekly	-2.3	0.363	0.132	-3.012	-1.588	-6.336	0
BBOTT 40mg every other week	-2.6	0.675	0.455	-3.923	-1.277	-3.853	0
BBOTT MTX+20mg every other week	-1.9	0.667	0.455	-3.207	-0.593	-2.848	0.004
Effect size	-2.313	0.179	0.032	-2.663	-1.963	-12.935	0

We also compared the effect between TNF-α inhibitor combined with MTX/TNF-α inhibitor used alone and MTX on RR of the progression of JD in active RA patients. In 10 independent studies which compared the effect of MTX and TNF-α inhibitor in combination with MTX on RR of the progression of JD in active RA patients, we used

random effects model for heterogeneity (P=0.001, I^2=67%). We found the effect size of TNF-α inhibitor in combination with MTX on RR of the progression of JD was significantly inhibited than that of MTX (Z=6.98; P=0.00001). In 2 independent studies which compared the effect of MTX and TNF-α inhibitor used alone on RR of the progression of JD in active RA, we used fixed effects model for heterogeneity (P=0.79, I^2=0). We found TNF-α used alone also displayed a more distinctive advantage than MTX on RR of the progression of JD in active RA patients (Z=3.83, P=0.0001; Fig.5-3). Comparing the effect of these two subgroups, we found there was a significant difference between TNF-α inhibitors used alone and combined with MTX on RR of the progression of JD in active RA patients(P=0.0001;Table5-3). In addition, TNF-α inhibitors combined with MTX was associated with a 31% reduction on RR of the progression of JD comparing with TNF-α inhibitors used alone (Fig.5-3).

Figure 5-3 Comparison between the size effects of TNF-α inhibitors/combined with MTX and MTX on RR of the progression of JD in patients with active RA



There was a significant heterogeneity among the data from studies selected in our project, when we tried to put the related data together to do meta-analysis (I^2>50%). In order to find which reason caused these heterogeneities, the subgroup analysis was done. We found that there was a significant distinction among the effect size of subgroups on RR of the progression of JD in activated RA patients, when we divided the group according to whether using MTX as a complementary or different type of TNF-α inhibitors(p<0.0001, p=0.00007). In addition, dividing the group according to the degree of HAQ also resulted in the significant distinction between the effect size of subgroups on RR of the progression

of JD in activated RA patients (p=0.02). However, there was no statistic difference between the effect sizes of subgroups on RR of the progression of JD in activated RA patients, when we divided the group according to whether took DMARDs before, whether took Corticosteroids/ Glucocorticoid before, whether took NSAIDs before, whether RF appeared a positive response, the level of CRP(mg/L), the level of ESR(mm/h), the degree of DAS28, the number of swelling joints, the number of tender joints, the compliance of RA patients, the age of patients or the duration of RA(months)(p>0.05;Table5-3)

Table 5-3 Subgroup analysis for confirming the influencing factors of heterogeneities.

	Subtotal(n)	RR(95%CL)	P value
Complementarity			
TNF-α inhibitors Combinated with MTX	860 3957	0.77(0.67,0.88) 0.43(0.33,0.56)	0.0001
Different type of TNF-α inhibitor			
Inflixmab	1744	0.23(0.17,0.32)	0.00007
Etanercept	767	0.56(0.47,0.66)	
Adalimab	1056	0.67(0.6,0.76)	
Previous took DMARDs,No.%			
20-40	2596	0.48(0.34,0.68)	0.84
0-19	916	0.45(0.25,0.81)	
Previous took Corticosteroids or Glucocorticoid ,No.%			
40-65	1602	0.45(0.32,0.62)	0.8
15-39	2120	0.47(0.31,0.72)	
Previous took NSAID,No.%			
≥80	1745	0.41(0.24,0.70)	0.62
60-79	921	0.34(0.21,0.56)	
RF positive,No.%			
≥75	445	0.28(0.15,0.56)	0.56
55-74	1980	0.36(0.21,0.61)	
CRP(mg/L)			
250-350	681	0.60(0.38,0.93)	0.2
20-50	3041	0.41(0.29,0.56)	
ESR(mm/h)			
46-55	921	0.34(0.21,0.56)	0.48
35-55	1064	0.25(0.13,0.49)	
DAS28			

6.0-8	532	0.61(0.47,0.80)	0.14
4-5.9	916	0.41(0.26,0.65)	
HAQ			
≥1.6	1532	0.48(0.35,0.66)	0.02
＜1.6	235	0.20(0.10,0.41)	
Number of swelling joints(0-68 possible joints)			
20-29	3246	0.45(0.33,0.60)	0.62
10.0-19	476	0.49(0.37,0.66)	
Number of tender joints(0-71 possible joints)			
27-36	3246	0.45(0.33,0.60)	0.62
17-26	476	0.49(0.37,0.66)	
Compliance			
>95	235	0.20(0.10,0.41)	0.11
60-80	2908	0.38(0.27,0.54)	
Age(years)			
≤52	1736	0.40(0.26,0.60)	0.5
>52	681	0.33(0.31,0.64)	
Duration of RA(months)			
125-132	1501	0.47(0.32,0.69)	0.83
60-80	2221	0.44(0.31,0.64)	

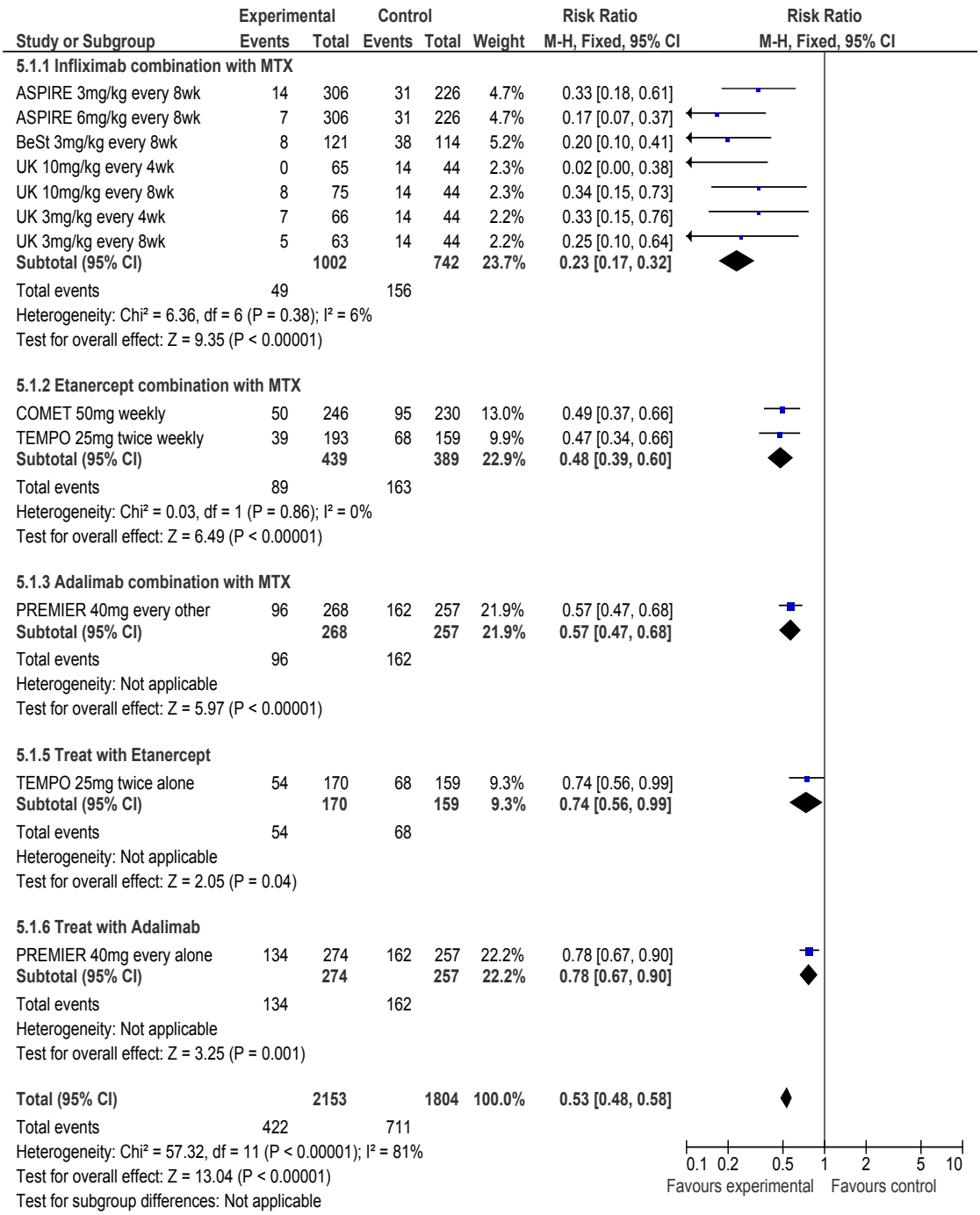
NSAID =non-steroidal anti-inflammatory drugs; RF= Rheumatoid factor; CRP=C-reactive protein; ESR= erythrocyte sedimentation rate; DAS28=28-joint Disease Activity Score; HAQ=Health Assessment Questionnaire.

* Number may not add up to 100% of total because of missing data in some variables.

Through subgroup analysis, we found “whether using MTX as a complementary” and “different types of TNF-α inhibitors” were two major factors which strongly modify the efficacy of TNF-α inhibitors on RR of the progression of JD in activated RA patients. Thus, we divided 12 independent studies into 5 subgroups (including INF combination with MTX, ETA combination with MTX, ADA combination with MTX, treated with ETA and treated with ADA groups) according these two major factors. In five subgroups, only first two subgroups (INF combination with MTX and ETA combination with MTX) were composed by multi-studies, and other subgroups just comprised one trial. There was low heterogeneity in first two subgroups (p=0.38 I^2=6%; p=0.86 I^2=0). Both effect sizes of INF combination with MTX and ETA combination with MTX on RR of the progression of JD

in activated RA were better than that of MTX used alone ($Z=9.35, p<0.00001$; $Z=6.49, p<0.00001$). INF used with MTX displayed a 77% (RR 0·23, 95% CI 0.17–0.32) and ETA displayed a 52% (RR 0·48, 95% CI 0.39–0.60) reduction RR of the progression of JD in patients with activated RA comparing with MTX used alone. (Figure 5-4)

Figure 5-4 Comparing between the effect size of TNF-α inhibitors combined with MTX and MTX on RR of the progression of JD in active RA patients after grouping according the result of subgroup analysis



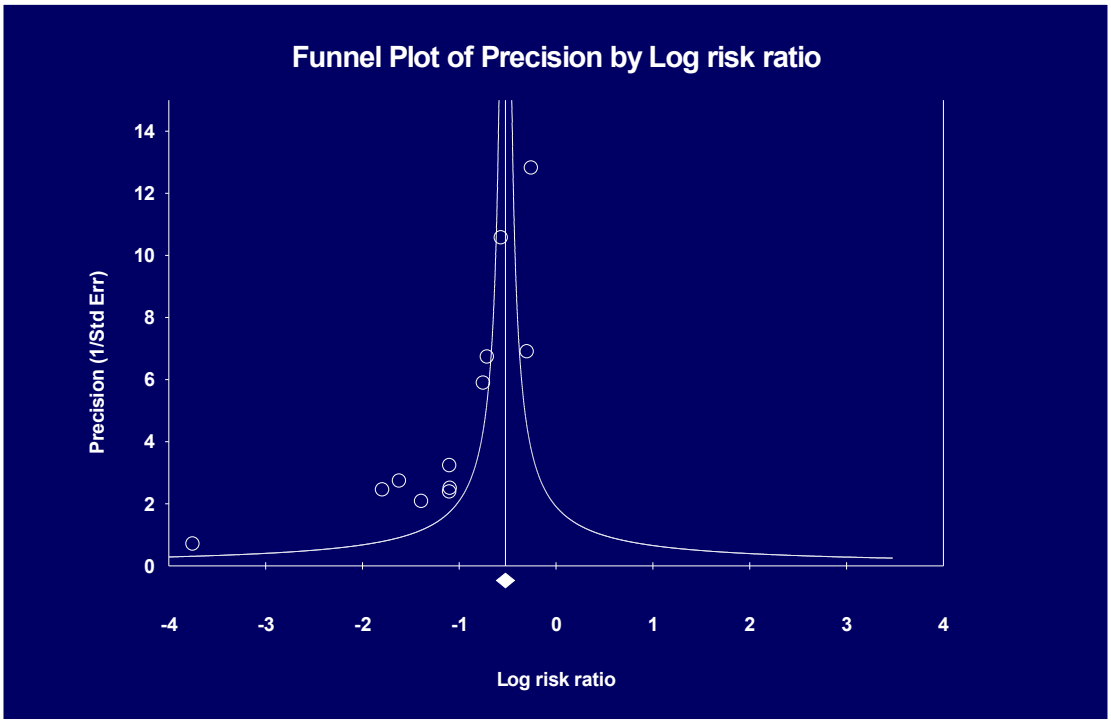
We used meta-regression analysis to examine the correlation between the common risk leading to the progression of JD and the effect size of TNF-α inhibitors on the RR of the progression of JD in active RA patients. These risk factors included the duration of RA, age, DAS28, CRP and ESR. We selected the available independent studies for these risk factors, 10 studies for the duration of RA, 11 studies for the age, 6 studies for DAS28, 9 studies for CRP and 7 studies for ESR (Table5-4).We noted there was no correlation between log RR and the increased disease duration/age (p=0.23, p=0.25; Table5-4). There was a significant negative correlation between log RR and the increased the degree of DAS28, CRP and ESR (p=0.026, p=0.0073, p=0.014; Table5-4)

Table 5-4 Summary of meta-regression analysis

	Slope (CL95%)	**P value**	**Available studies**
Duration of RA	0.00189(0.00026, 0.00352)	0.23	10
Age	0.075 (0.036, 0.114)	0.25	11
DAS28	0.23(-0.009,0.47)	0.026	6
CRP	0.00017 (-0.0008,0.0012)	0.0073	9
ESR	0.085 (-0.028, 0.198)	0.014	7

Egger's regression analysis showed that publication bias was present (p=0·0002; Figure5-5). We therefore used the fail-safe methods to estimate the number of potential missing studies needed to significantly change the conclusion of our findings. This analysis showed that, to nullify our estimated effect size, 484 studies with non-significant findings would be needed. In view of the fact that there have been no more than 10 studies published over the last 8 years, it is highly improbable that such a large number of similar studies would have gone unpublished or have been missed out by our extensive search strategy. Furthermore, the missing studies were likely to be small, the effect of which is probably very negligible.

Figure 5-5 Funnel plot to assess publication bias



5.7 Discussion

The main finding of this systemic review is that TNF-α inhibitors (used alone and in combination with MTX) significantly improved the progression of JD compared with MTX alone in active RA patients. Through further analysis, we found that both two administrations of TNF-α inhibitors significantly retarded the progression of JD compared with MTX in active RA patients respectively, and TNF-α inhibitors in combination with MTX displayed the more powerful inhibition on the progression of JD than TNF-α inhibitors used alone in active RA patients. These findings indicate a better efficacy can be achieved when using TNF-α in conjunction with MTX to prevent the progression of JD in active RA patients.

It should be pointed out that heterogeneity was observed in the present study with the pooled data from the selected studies, but not with dividing these studies into different

subgroups, according to identified impact factor for meta-analysis. Many factors may affect the heterogeneity in meta-analysis. We have summarized 13 common factors specific to RA, including complementary with MTX, different types of TNF-α inhibitors, HAQ, took DMARDs before, took / Glucocorticoid before, took NSAIDs before, RF appeared a positive response, CRP, ESR, the number of swelling joints, the number of tender joints, the compliance of RA patients, the age of patients and the duration of RA(months)[437,450,469-473]. Among these factors, complementary with MTX and different types of TNF-α inhibitors seems the main reason for the heterogeneity. After we divided the all involved studies into subgroups according these two main factors, the heterogeneities in subgroups disappeared. TNF-α inhibitors in combination with MTX displayed the most powerful inhibition on the progression of JD in active RA patients comparing with TNF-α inhibitors/ MTX used alone.

Among the three types of TNF-α inhibitors approved by the FDA, ADA is constructed from a fully human monoclonal antibody, while INF is a mouse-human chimeric antibody and ETA is a TNF receptor-IgG fusion protein. Comparing the effect of different types of TNF-α inhibitors on the progression of JD in active RA patients, we found that INF in combination with MTX displayed more powerful inhibition on the progression of JD than EDA or ADA in combination with MTX. This result indicates that to control the progression of JD of active RA patients, INF in combination with MTX seems a good choice. There is no one paper about INF used alone to improve the progression of JD in active RA. However, comparing the effect of EDA and ADA used alone on the progression of JD in active RA patients, we found that there is no distinction between the inhibitions of them on the progression of JD.

The degree of activity of RA was directly related to changes in radiologic progression in patients with active RA[442]. In this study, we have also found that the degree of activity of RA affected the efficacy of TNF-α inhibitors on the progression of JD in active RA. Three important markers of RA activity are CRP, ESR and DAS28. We found there was a negative correlation between the therapeutic effect by TNF-α inhibitors and the increased level of CRP, ESR or DAS28. This suggests that a better outcome for the patients with higher level CRP, ESR or DAS28 than those patients with lower level CRP, ESR or DAS28.

In summary, the present study has demonstrated that TNF-α inhibitors in combination with MTX has a better efficacy on the progression of JD in active RA than TNF-α inhibitors/MTX used alone, and INF in combination with MTX exhibits more powerful effect than other types of TNF-α inhibitors. In addition, CRP, ESR and DAS28 are factors affecting the efficacy of TNF-α inhibitors on progression of JD in active RA patients. The evidence obtained may provide a guide /support for rheumatologists and patients to select and best use of anti-RA drugs.

Chapter 6. General Discussion

6.1 Major Findings of this Project

As mentioned in Chapter 1, COPD is characterised by a persistent inflammation in the airway and lungs which results in airway obstruction. Current treatments for COPD are still not satisfactory and there is a need to develop new therapies targeting inflammatory mechanisms. Ginseng is a well-known medicinal herb and has been used in the treatment of COPD. Understanding the mechanism of anti-inflammatory actions of ginseng and ginseng based formulas will facilitate the development of novel agents for treating COPD and other inflammatory diseases. The main objective of this project is to study the action and mechanism of ginseng and ginsenosides on cytokine mediators in inflammatory cells models.The key hypothesis is that ginseng and ginsenosides may target TNF-α, cAMP and NF-κB signalling pathway to exert their anti-inflammatory actions. In order to test this hypothesis, a cell inflammatory model was firstly set up using macrophage-like U937 cells induced by LPS or CSE, which then be used to evaluate the effects of ginseng and ginsenosides on cellular release of cytokines and activation of NF-κB and cAMP pathways.

Firstly, a cell inflammatory model was successfully set up using macrophage-like U937 cells induced by LPS and CSE. The model exhibited significant activation NF-κB pathway, supported by the findings of increased release of cytokine (NF-α, IL-1β and IL-6) and up-regulated expression of key NF-κB proteins (IKK, p-IKK, IκBα, p-IκBα, p65 and p-p65), as well as the transcriptional activity of NF-κB by LPS and/or CSE. In addition, a possible interaction between NF-κB and CAMP pathways was also demonstrated with findings of effects of NF-κB pathway inhibitor (BAY11-7082), cAMP

pathway activator and inhibitor (Foskolin and H89) on TNF-α release, and on the transcriptional activity of NF-κB by LPS (Fig. 6-1).

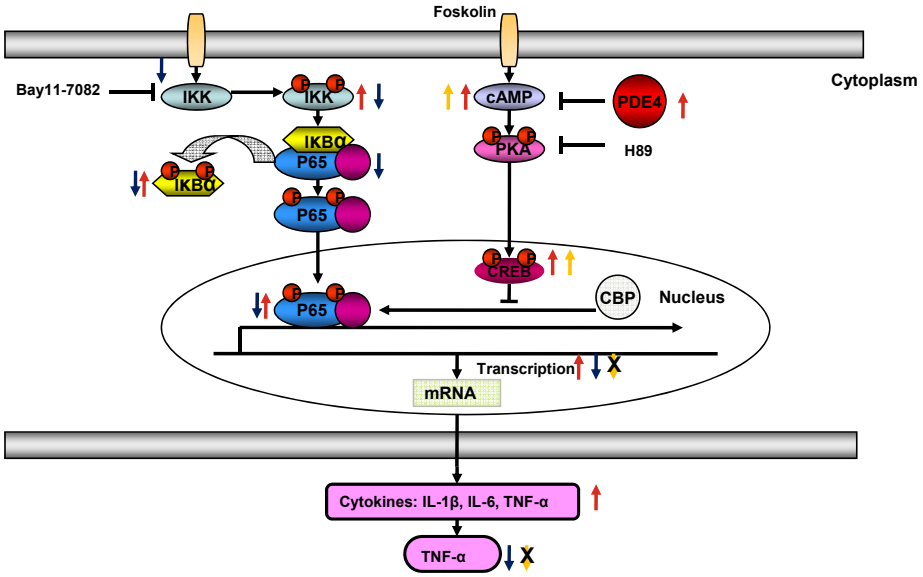


Figure 6-1: Establishment of LPS induced macrophage-like U937 cell models and the interaction between NF-κB and cAMP pathways in this model.
"↑" is used to display the up-regulating effect of LPS on the expression of p-IKK, p-IκBα, p-p65 and p-CREB, the level of cAMP, the activity of PDE4, the transcriptional activity of NF-κB and the release of IL-1β, IL-6 and TNF-α in macrophage-like U937 cells. "↓" is used to display the inhibitory effect of Bay11-7082 on the expression of p-IKK, p-IκBα, p-p65, IKK, IκBα and p65, the transcriptional activity of NF-κB and the release of TNF-α induced by LPS. "↑" is used to display the up-regulation of Foskolin on the level of cAMP and the expression of p-CREB induced by LPS; "↓" is used to display the down-regulation of Foskolin on the transcriptional activity of NF-κB and the release of TNF-α induced by LPS. "X" is used to display H89 abolished the inhibition of Foskolin on the transcriptional activity of NF-κB and the release of TNF-α induced by LPS.

Secondly, the effects of ginseng (G115) and several ginseng formulas (GHMFs) as well as ginsenosides on the release of cytokines were investigated. GHMF I&II, composed by

commercial herbal extracts from China, displayed the similar inhibition on the release of TNF-α, GHMF-III, composed by raw-herbal extracts without polysaccharide components, displayed more potent inhibition on the release of TNF-α than that of GHMF-IV which was prepared in the same way as GHMF-III except including polysaccharide components, indicating that the polysaccharide components in ginseng may weak the anti-inflammatory actions of ginseng.It was found that the release of TNF-α was significantly inhibited by GHMF-III, Rb1, Rg1, Rg3, CK and Rh1 in LPS induced cell model and G115and GHMF-III in CSE induced cell model. GHMF-III, G115, Rb1 and Rg1 also inhibited the release of IL-1β and IL-6 induced by LPS. Among the GHMFs tested, GHMF-III seemed to be most potent.

Thirdly, the effects of ginseng and ginsenosides on the activation of NF-κB signalling proteins were investigated. G115, GHMF-III, Rb1, Rg1, Rh1 and CK, but not Rh2 significantly inhibited the expression of IKK, p-IKK, IκBα, p-IκBα, p65 and p-p65 (Table6-1) and decreased the transcriptional activity of NF-κB induced by LPS. G115, GHMF-III and Rg1, but not Rb1, Rh1, CK and Rh2 also significantly increased the cellular level of cAMP and the expression of p-CREB, but inhibited the activity of PDE4 induced by LPS. These findings indicate that ginseng has a significant inhibition of cytokine release and activation of NF-κB pathway in LPS-induced macrophage-like U937 cells. In addition, ginseng and ginseng related products may also act as PDE4 inhibitor to regulate cAMP pathway.

Table 6-1 Regulation of Ginseng and Ginseng related products on the expression of proteins in RPs of NF-κB pathway

	p-IKK	IKK	p-IκBα	IκBα	p-p65	p65
G115	$\downarrow^{***}$	$\downarrow^{**}$	$\downarrow^{**}$	$\uparrow^{**}$	$\downarrow^{***}$	no
GHMF-III	$\downarrow^{***}$	$\downarrow^{***}$	$\downarrow^{**}$	$\uparrow^{*}$	$\downarrow^{**}$	no
Rb1	$\downarrow^{***}$	$\downarrow^{***}$	$\downarrow^{**}$	$\uparrow^{*}$	$\downarrow^{***}$	no
CK	$\downarrow^{*}$	no	$\downarrow^{***}$	no	$\downarrow^{**}$	no
Rg3	$\downarrow^{*}$	$\downarrow^{*}$	$\downarrow^{*}$	no	$\downarrow^{***}$	$\downarrow^{*}$
Rh2	No	no	no	no	no	no
Rg1	$\downarrow^{***}$	no	$\downarrow^{***}$	no	$\downarrow^{**}$	no
Rh1	$\downarrow^{*}$	no	$\downarrow^{*}$	no	$\downarrow^{*}$	no

*:$p<0.05$

**:$p<0.01$

***:$p<0.001$

Finally, as a separate research objective in the present project, a systemic review was conducted to evaluate the clinical efficacy of TNF-α inhibitors in the treatment of progression of JD in active RA. Data from randomised controlled trials were collected and a meta-analysis was conducted to compare the effects of TNF-α inhibitors on the radiological progression of active RA patients when used alone or combined with MTX, and to study the correlation between the degree of activity of RA and the efficacy of different types of TNF-α inhibitors. It was found that TNF-α inhibitors showed a better efficacy on the progression of JD in active RA than that of MTX, and TNF-α inhibitors in combination with MTX produced a better efficacy than TNF-α inhibitors used alone. Among different types of TNF-α inhibitors, infliximab in combination with MTX exhibited a better efficacy than other types of TNF-α inhibitors on the progression of JD in active RA patients. The markers of RA activity, including CRP, ESR and DAS28, were shown to be associated with the efficacy of TNF-α inhibitors on progression of JD in active RA. These findings may help to guide clinical use of TNF-α inhibitors to treat JD in active RA patients.

6.2 Inflammatory Cell Model

LPS and CSE induced macrophage-like U937 cells have been used as inflammatory cell models for studying the pathogenesis of human-COPD[394,396,474]. It has been demonstrated that inflammatory mediators generated by macrophage-like U937 cells in response to LPS and CSE, initiate and/or amplify abnormal inflammations in COPD[279,394,475]. Previous studies showed that LPS induced macrophages to secrete a broad-spectrum inflammatory mediators, including TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-12, TGF-β and MMP-1[474,475]. CSE, as a specific pathogen of COPD, also stimulated macrophage to release the IL-8, TNF-α, IL-1β, IL-6 and IL-17[279,280,476-478].The present study has focused on the regulation of ginseng and its related products on the release of TNF-α. TNF-α is powerful inflammatory mediator released by LPS and CSE induced cell models[279,394,396]. The demonstration of TNF-α release and activation of related NF-κB pathway are a clear indication of success of the cell model. In this regard, the present results are consistent with previous findings[279,394,394].,

6.3 Actions of G115 and Ginseng Formulas

G115 is a standardized extract of ginseng with ginsenosides as its main active components and a clinical trial has demonstrated its efficacy in moderately-severe COPD condition [220]. However, the pharmacological mechanism involved is not clear. The finding that G115 inhibited the release of TNF-α in LPS and CSE induced macrophage-like U937 cells indicate it may act through inhibition of inflammatory mediators. Thus the study provides experimental evidence to support the use of G115 for COPD. We also proved G115 and GHMF-III inhibited the release of TNF-α through down-regulating the activity of NF-κB

and up-regulating the activity of cAMP pathways to inhibit the transcriptional activity of NF-κB induced by LPS.

The present study has tested several ginseng formulas. All these formulas are developed based on clinical experience Guangdong Provincial Hospital in China. These formulas are three herb formulations with ginseng as main ingredient. The first type of GHMF is composed by commercial extracts of individual herbs. This type of GHMF is convenient for standardization of application of GHMF in clinic. This kind of preparation is more easily administrated than traditional oral decoction. In this project, we compared the effect of GHMFs from different pharmaceutical companies. GHMF-I is composed by extracts from Guangdong, and GHMF-II composed by extracts from Jiangsu. Although GHMF-I demonstrated more significant inhibition on the release of TNF-α than GHMF-II at low and high concentration, at middle concentration (the most effective concentration) of GHMF-I&II, there was no distinction between the release of TNF-α of them. The second type of GHMF is extracted from traditional mixture of raw herbs with different extracting techniques. The most significant difference between GHMF-III and GHMF-IV is polysaccharide components. The findings that GHMF-III exhibits a more powerful inhibition on TNF-α release than GHMF-IV indicates that polysaccharide component of ginseng may affect the anti-inflammatory actions of ginsenosides. Studies have shown that ginsenosides displayed inhibitions on inflammatory response, such as decreasing the release of cytokines, down-regulating the activity of inflammation-related pathway, etc[479,480]. On the other hand, the polysaccharides of many herbs have been used in the treatment of cancers, such as Ginsan which is the polysaccharide extract from ginseng. Previous studies demonstrated that Ginsan showed an anti-tumor effect through increasing the production of inflammatory factors, such as TNF-α, IL-1β, IL-6, IL-12, GM-CSF and IFN-γ to aggravate the inflammatory response[481-483]. Thus, the polysaccharide in GHMF-

IV may weaken the anti-inflammatory effect of GHMF-IV comparing with GHMF-III. This is consistent with the finding that GHMF-III, as only GHMF excluding polysaccharide, displayed the most potent inhibition on TNF-α release among all GHMFs tested. Thus, GHMF-III may have a potential for further development as ginseng-based formula for the treatment of COPD.

In the classical theory of Chinese medicine, there is usually the main herb in a formula which displays the pharmacological characteristics in the course of treatment of diseases, and other herbs in the formula usually play a supporting role. However, this theory although seems to have been verified in clinic, is difficult to be proved in the experimental setting. In this project, we chose TNF-α as a target, and tried to find the correlation between the ginseng and GHMF-III on the inhibition of the release of TNF-α induced by LPS. We found that there is a significant correlation between the GHMF-III and G115 on the inhibition on the release of TNF-α induced by LPS/CSEM, indicating the action of GHMF-III may mainly related to ginseng. However, further study is necessary to exclude the contributions of other herbs on this effect.

6.4 Actions of Ginsneosides

6.4.1 PDGs & PTGs

The main bioactive compounds in ginseng are ginsenosides, which can be classified into two types, dammarane and oleanane (Ro). The dammarane type ginsneosides includes two sub-types: protopanaxadiols(PDGs) and protopanaxatriols (PTGs)(see Section 1.2.2.1). PDGs and PTGs include the most commonly used ginsenosides, such as Rb1, Rb2, Rc, Rd and Rg3(PDGs) Rg1, Re and Rf (PTGs)[324]. Some studies have shown that PDGs and PTGs displayed different effects on the specific biological mechanisms. For example, Liu found

that PDGs inhibited the pancreatic lipase activity in a dose-dependent manner, but PTGs showed no such effect. In the present study, Rb1 and Rg3 were selected as the representative of PDGs and Rg1 as the representative of PTGs to test their effects on the release of TNF-α and NF-κB pathway in macrophage-like U937 cells induced by LPS.

Both ginsenoside Rb1 and Rg3 significantly inhibited the release of TNF-α induced by LPS, accompanied with down-regulations of the expression of IKK, p-IKK, p-IκBα and p-p65, as well as the decreased the transcriptional activity of NF-κB. Rb1 also increased the expression of IκBα and Rg3 also decreased the expression of p65. These results indicate that PDGs type ginsenoside Rb1, Rg3 inhibited the release of TNF-α through down-regulating the NF-κB pathway. Similarly, Ginsenoside Rg1 significantly inhibited the release of TNF-α induced by LPS, accompanied with inhibition of the expression of p-IKK, p-IκBα and p-p65and decrease in the transcriptional activity of NF-κB. In addition, Rg1 increased the level of cAMP, the up-regulated expression of p-CREB and CREB. The results indicate that Rg1 not only inhibited the activity of NF-κB pathway, but also up-regulated the activity of cAMP pathway to inhibited the release of TNF-α induced by LPS. Most likely Rg1 achieved the up-regulation of cAMP pathway through inhibition of the activity of PDE4. Since Rb1 had no effect on cAMP level, the results suggest that PDGs may act on mainly on NF-κB pathway, while PTGs may regulate both NF-κB and cAMP pathways to inhibit the release of TNF-α, which is partly through inhibiting the activity of PDE4. Such different actions of ginsenosides may help to understand the complex actions of ginseng.

6.4.2 Metabolites of Ginsenosides

The study on the effect of metabolites of ginsenosides is very important, as the absorption of some ginsenosides through the gastrointestinal tract is very low *in vivo*[326,327]. Most of metabolites displayed the analogous pharmacological actions to corresponding ginsenosides. Previous studies showed that both ginsenoside Rb1 and its metabolite, CK, significantly inhibited the expression of p-p65 and p-IκBα in LPS induced mouse peritoneal macrophages[340]. In addition, both ginsenoside Rg1 and its metabolit, Rh1, activated cAMP pathway and decreased the transcriptional activity of NF-κB. In the present study, rRg1 and Rh1 also showed the analogous regulation on the expression of proteins in NF-κB pathway, the transcriptional activity of NF-κB and the release of TNF-α induced by LPS. However, Rh2, the metabolite of ginsenoside Rg3 couldn't inhibit the expression of proteins in NF-κB pathway, the transcriptional activity of NF-κB and the release of TNF-α induced by LPS as Rg3, and increased the release of TNF-α induced by LPS at high concentration. The regulatory mechanism of Rh2 on TNF-α is still uncertain. It maybe involve in some specific mechanism.

6.5 Interaction between NF-κB and cAMP Pathways

It is known that NF-κB can be activated by a series of the PRs in NF-κB pathway, and then enter into nucleus to transform into promoter to classically transcribe the mRNA expression of TNF-α[484]. This is supported from findings of the present study ,as well as the effect of Bay11-7082, an inhibitor of NF-κB pathway (see Chapter 4). .

One of interesting finding is the interaction between NF-κB and cAMP pathways. It has been demonstrated that the transcriptional activity of NF-κB can be inhibited by activated cAMP pathway through its down-stream factor (p-CREB) competitively binding with

CBP[279,280]. The elevated the level of cAMP also decreased the release of TNF-α, which was accompanied with the activation of cAMP pathway[485]. However, there was no evidence on inhibition of release of TNF-α by activated cAMP pathway through down-regulating the transcriptional activity of NF-κB. In the present study, Foskolin, a cAMP pathway stimulator, had no effect on the activity of PRs in NF-κB pathway, but significantly inhibited the release of TNF-α induced by LPS[486], the effect accompanied with the increased levels of cellular cAMP and expression of p-CREB, and the decreased transcriptional activity of NF-κB. The effects of Foskolin were blocked by the cAMP pathway inhibitor H89. Thus, it is most likely that activated cAMP pathway cause down-regulate the release of TNF-α through inhibiting the transcriptional activity of NF-κB.

6.6 Inhibition by Ginseng and Ginseng Related Products on the Release of IL-1β and IL-6

IL-1β and IL-6 are the important inflammatory factors in COPD. A previous study in IL-1β type 1 receptor knockout mice showed that IL-1β was involved in the formation of emphysema[487]. IL-1β was also involved in the regulation on the production of MMPs[488]. The role of IL-6 in the phathogensis of COPD is still uncertain, however, it has been shown that IL-6 was increased in sputum, bronchoalveolar lavage, and exhaled breath condensate of COPD patients, especially during exacerbations[124,489]. The concentration IL-6 was also increased in the plasma of COPD patients[125,126,227], particularly during exacerbations[228]. In the present study, G115, GHMF-III, Rb1 and Rg1 significantly decreased the secretion of IL-1β and IL-6 induced by LPS, the effect was very similar with the inhibitions by these agents on the release of TNF-α. These results further support the anti-inflammatory effect of ginseng and ginseng products, and their actions on NF-κB pathway. [27,28].

6.7 Use of TNF-α Inhibitors for Treating JD in Patients with Active RA.

Controlling the progression of JD is the most important treatment consideration for managing RA. However, there was no effective drug available to control or slow the progression of JD in active RA until the recent application of TNF-α inhibitors[419]. Now there clear evidence showing that both TNF-α inhibitors used alone and combined with MTX significantly improved the progression of JD in active RA patients through valuing the radiological scores of these patients[437,446,448-452]. However, there is still no evidence to verify which kind of administration of TNF-α inhibitors in combination with MTX or using alone is better and which particular TNF-α inhibitors are more beneficial for the improvement of JD in active RA patients. In addition, we are also unclear that the affecting factor of efficacy of TNF-α inhibitors on the progression of JD in active RA patients. The present meta-analysis reveal that TNF-α inhibitors (used alone and combined with MTX) significantly improved the progression of DJ, comparing with MTX used alone, in active RA patients. Thus, the synergic action of TNF-α inhibitors and MTX should be considered as a preferred treatment option for managing progression of DJ in active RA patients. Among the TNF-α inhibitors, INF in combination with MTX seems to be more potent than others. In addition, the finding of a negative correlation between the inhibition of progression of DJ by TNF-α inhibitors and the increased level of CRP, ESR or DAS28 indicates that a better clinical outcome may be achieved for RA patients with higher level CRP, ESR or DAS28 than those patients with lower level CRP, ESR or DAS28. Thus, it is recommended that TNF-α inhibitors in conjunction with MTX should be used to treat the progression of DJ for active RA patients, especially for those with the high active risk.

6.8 Limitations of the Study

The present study has achieved the main research objective to identify the mechanism of anti-inflammatory actions of ginseng and ginseng related products. However, the study also has some limitations.

First, the nature of study is *in vitro* and limited to LPS and CSE related mechanisms. It is not clear if the observed actions of ginseng also occur in other inflammatory conditions and more importantly *in vivo*. Previous studies showed that RG, PDGs, Rg3, Rf and Rh2 significantly inhibited the release of TNF-α in diethyldithiocarbamate and LPS induced animal model[379],[383]; Rg1 increased the level of cAMP in hippocampus of both young and old rats, and Rb1 also increased the level of cAMP in rat liver compared with that in controls[354]. These results indicate that TNF-α as well as NF-κB and cAMP pathways may also be the target of ginseng and ginsenosides *in vivo*.

Second, the present study has used a commercial cell line U937. It is important to extend the study to primary cells. Previous study demonstrated that ginseng significantly inhibited the release of TNF-α in OMP, ConA or anti-CD3 induced lung cells from CBA/J mice [374]; Rb1 and CK down-regulated the expression of p-IκBα and p-p65 in mouse peritoneal macrophages induced by LPS[340]. These results show that ginseng and ginsenosides may also involve in the regulation on the release of TNF-α and the activation of the key proteins in NF-κB pathway in primary cells.

Thirdly, the present study only studied key proteins in NF-κB and cAMP pathways, It is not clear if other mechanisms, including PI3K/Akt and ERK1/2 and P38, are also involved, as all of them contribute the activation of NF-κB pathway (see chapter1).

Finally, it should be pointed out the concentrations tested *in vitro* may not apply to the situation *in vivo*, as It is not clear how the body metabolism process affects the absorption and fate of ginseng and ginseng products *in vivo*.

6.9 Future Directions

It is important to carry out further studies on ginseng. Based on the results of the present study, the following research work can be carried out in the future:

- Test the effects of ginseng, ginseng formulas and ginsenosides in smoke-induced COPD animal model *in vivo*.
- Extend the *in vitro* study to primary human cells and tissues
- Employ gene modification technique to study the role of particular proteins, e.g. CREB on actions of ginseng
- Conduct further studies on interactions between NF-κB and cAMP pathways on regulation of cellular cytokine productions *in vitro* and *in vivo*
- Conduct clinical studies to evaluate the efficacy and safety of ginseng and effective ginseng formula on managing COPD.
- Using Mass spectrum and HPLC to find activation compound of Ginseng and GHMFs.
- And more control experiments need to be down by using GHFs(Ginseng and HunagQi Extraction), GMFs(Ginseng and Maidong Extraction)and HMFs(Ginseng free, only HuangQi and Maidong extraction), to narrow down the activate compounds.
- To identify and the active some molecular compound as NFkB inhibitor or activator form GHMFs is the goal of future direction.

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Appendix

Appendix 1 : RIPA Buffer

For 250mL stock, to be stored at 4ºC:

- 65mM Tris = 1975mg
- 150mM NaCl = 2250mg
- Add dd H_2O to ~180ml, stir until clear and pH to 7.4 with HCl
- 5mM EDTA = 12.5ml of a 100mM stock
- 1% NP-40 = 2.5ml of a 100% stock
- 0.5% Na-deoxycholate = 12.5ml of a 10% stock
- 0.1% SDS = 2.5ml of a 10% stock
- 10% glycerol = 25ml of a 100% stock

On the day of use, to a total of 50ml of RIPA buffer, add the following protease and phosphatase inhibitors:

- 1µg/ml aprotinin = 24µl of a 2.1mg/ml stock (inhibits trypsin and related proteolytic enzymes)
- 1µg/ml leupeptin = 5µl of a 10mg/ml stock (inhibit cysteine, serine and threonine peptidases)
- 10mM NaF = 2.5ml of a 200mM stock (inhibits serine and threonine phosphorylation)

- 1mM Na_3VO_4 = 250µl of a 200mM stock (inhibits tyrosine phosphorylation)

- 1mM PMSF = 250µl of a 200mM stock (dissolved in 100% ethanol) (serine protease inhibitor)

- Prosease inhibitor cocktail and phosphatase inhibitor cocktail 250ul respectively.

Appendix 2: Bradford Protein Concentration Assay

- Dilute the extracts in d-H_2O
- Make up standards using Bovine Serum Albumen (BSA) at 3, 2, 1.5, 1, 0.75, 0.5, 0.25 mg/ml.
- Dilute the Biorad protein dye reagent 1:4 with d-H_2O and load this solution in a flat bottom 96 wells plate (200µl/well).
- Add 10ul standard or protein sample in each well. Perform all samples in duplicate.
- Include a room template for 30 minutes.
- Read plate at 595nm.

Appendix 3: The preparation of 10% Gel

- Thoroughly clean glass plates with detergent, wash with tap water, rinse with distilled water several times, and air dry.

- Assemble gel unit in the casting mode according to the instructions.

- Check for potential leaks by pipetting some distilled water into the assembled unit. Then drain away the water by inverting the unit.

- Prepare the separating gel

    - Acrylamidebi-acrylamide(30%) : 8ml

    - 3M Tris-Hcl, PH8.8: 3 ml

    - Dd H_2O: 12.56ml

    - 10% SDS: 240µl

    - 10% APS: 200µl

    - TEMED: 12µl

- Immediately pipette the separating gel mixture into the assembled unit up to level that is ~3 cm from the top.

- Load 1 ml isopropanol onto the surface of the acrylamide gel solution. The isopropanol layer will make the surface of the gel very even. After 20 minutes, a sharp gel-water interface can be visible after the gel has polymerized.

- Drain away theisopropanol layer by gently tilting the casting unit and rinse with dd H_2O, and dry it.

- Prepare the stacking gel
    - Acrylamidebi-acrylamide: 1ml
    - 2M Tris-Hcl, PH6.8: 500µl
    - Dd H_2O: 6.4ml
    - 10% SDS: 80µl
    - 10% APS: 200µl
    - TEMED: 4µl
- Pipette the stacking get mixture and insert the comb into the unit according to the instructions.
- Allow the gel to polymerize and sit for 20 minutes.

Appendix 4: The formulations of various buffers in Western blot

1. Running Buffer

   10x Running buffer, for 1L

   - Glycine – 144g
   - Tris Base – 30g
   - Add distilled H20 to 900ml
   - pH to 8.9
   - Add 50ml of 10% SDS solution
   - Add H_2O to 1L

2. Transfer Buffer

   1x Transfer Buffer, for 1L

   - 100 ml 10 x stock
   - 200 ml(20%) methanol
   - Add dd H_2O to 1L

3. TBS-T

   TBS-T (10X) pH: 7.5

- Tris base: 24.2 g
- Sodium Chloride (NaCl): 80.0 g
- Tween-20: 10 ml
- Add dd H_2O to 1L
- Stripping Buffer
- 62.5ml 1MTris-HCl, pH 6.7
- 100ml 20% SDS
- 837.5ml d- H_2O

On the day of use add 700ul 2-mercaptoethanol per 100ml

Appendix 5: NF-κB reporter assay

- Seed 2.5x 105 macrophage-like U937 cells per well of a 24-well plate in 500µl of an appropriate culture medium containing serum and antibiotics.

- Incubate the cells under normal growth conditions (typically 37°C and 5% CO_2) for 24 hours.

- Wash cells in the plate once with Dulbecco's PBS without calcium and magnesium.

- Refresh medium with 500ul Opti-MEM® containing 0.6 µg NF-κB promoter-firefly/Renilla luciferase (40/1) /Negative control/positive control (QIAGEN Pty. Ltd.) and 1.5ul attractene transfection reagent (QIAGEN Pty. Ltd.) each well

- Dilute 0.6 µg DNA in 493 µl Dilute 0.6 µg DNA in 60 µl culture medium without serum. Add 1.5 µl of Attractene Transfection Reagent to the diluted DNA and mix by vortexing.

- Incubate the samples for 10-15 minutes at room temperature (15-25°C) to allow the formation of transfection complexes.

- Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.

- After 18 hrs incubation, refresh the cell with normal culture medium for another 6hrs

- Refresh the medium with treatment drug.

- After 2hrs pre-incubation, treat 1µg/ml LPS for 6hrs.

- After the treatment, the cells were rinsed with cold PBS and lysed with reporter lysis buffer (Promega) 100ul/well. Place the culture plates on a rocking platform or orbital shaker with gentle rocking. Rock the culture plates at room temperature for

15 minutes. The lysate was centrifuged at 15,000 × g for 5 min at 4°C, and the supernatant was harvested. Transfer the lysate to a tube or vial for further handling and storage.

- Both firefly and Renilla luciferase levels were measured in a luminometer using the Dual-Glo® Luciferase Reporter Assay System (Promega Australia).

- Predispense 100µl of Luciferase Assay Reagent II (LAR II) into each well. Carefully transfer up to 20µl of cell lysate into the well containing LAR II; mix by pipetting 2 or 3 times. Do not vortex. Place the plate in the luminometer and initiate reading. Program the luminometer to perform a 2-seconds premeasurementdelay, followed by a 10-second measurement period for each reporter assay.

- Add100µl of Stop & GloR Reagent into each well and vortex briefly to mix. Replace the sample in the luminometer, and initiate reading.

Appendix 6: The protocol of cAMP assay

1. Buffer Preparation

    - EIA Buffer Preparation

      Dilute the contents of one vial of EIA Buffer Concentrate (10x) with 90 ml of Ultra-pure water. Be certain to rinse the vial to remove any salts that may have precipitated.

    - Wash Buffer Preparation

      Dilute the Wash Buffer Concentrate (400x) to a total volume of 2 L with Ultra-pure water and add 1 ml Polysorbate 20.

    - Note

      Water used to prepare all reagents and buffers must be deionised and free of trace organic contaminants.

      Store all diluted buffers at 4°C; they will be stable for about two months.

2. Preparation of Assay –Specific Reagents

    - cAMP AChE Tracer

      Dilute 100 dtn cAMP AChE Tracer with 6 ml EIA Buffer.

    - cAMP EIA Antiserume

      Dilute 100 dtn cAMP EIA Antiserume with 6 ml EIA Buffer.

    - Note

Store the reconstituted cAMP EIA Antiserume at 4C; they will be stable at least four weeks.

3. Sample Preparation

- Aspirate medium from 24 well-plate
- Add 55 ul of 0.1 M HCL for every well of surface area.
- Incubate at room temperature for 20 minutes.
- Scrape cells off the surface with a cell scraper.
- Dissociate the mixture by pipetting up and down until the suspension is homogeneous, and transfer to 1 ml centrifuge tube.
- Centrifuge at 1000*g for 10 minutes.
- Decant the supernatant into a new tube.
- The preparation of cAMP standards
- Reconstitute the cAMP EIA standard with 1 ml of EIA buffer. The concentration of this solution will be 7,500 pmol/ml. Store this solution at 4C; it will be stable for approximately six weeks.
- To prepare the standard for use in EIA

  Obtain eight clean 1ml tubes and number them 1through 8. Aliquot 900 µl EIA buffer to tube 1 and 600 µl EIA buffer to tubes 2-8. Transfer 100 µl of the bulk standard (7500pmol/ml) to tube 1 and mix thoroughly. The concentration of this standard, the first point on the standard curve, is 750 pmol/ml. Serially dilute the standard by removing 300 µl from tube 1 and placing in tube2; mix thoroughly. Next, remove 300 µl from tube 2 and place it into tube 3; mix thoroughly. Repeat this process for tube 4-8. The

concentrations of these standards are 750 pmol/ml,250 pmol/ml,83.3 pmol/ml,27.8 pmol/ml, 9.3 pmol/ml, 3.1 pmol/ml,1.0 pmol/ml and 0.3 pmol/ml separately. These dilupted standards should not be store for more than 24 hours.

4. Performing the assay

- Add 50 µl EIA buffer to Maximum binding(B0) each well.
- Add 50ul standard sample in standard well (1-8) and 50 µl sample in common well.
- Add 50ul cAMP AChE Tracer to each well.
- Add 50 µl cAMP EIA Antiserum to each well.
- Cover each plate with platicfil, and incubate 18 hours at 4C
- Reconstitute Ellman's Reagent immediately before use.
- Dilute 100 dtn vial Ellman's Reagent with 20 ml of Ultra-pure water.
- Empty the wells and rinse five times with wash buffer.
- Add 200 µl of Ellman's reagent to each well.
- Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate to develop in the dark for 120 mins.
- Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
- Remove the plate cover being careful to keep Ellman's reagent from splashing on the cover.

- Read the plate at 420 nm.

5. Calculations

    - Average the absorbance readings from all data.

    - Calculate the B/B0 (sample or standard bound/maximum bound) for the remaining wells.

    - Plot the standard curve

    - Plot %B/B0 for standards 1-8 versus cAMP concentration using linear(y) and log(x) axes and perform a 4-parameter logistic fit. Plot the data as logit (B/B0) versus log concentrations and perform a linear regression fit.

    - Determine the sample concentration

    - Determine the concentration of each sample using the equation obtained from the standard curve plot.