中國醫藥大學藥學院中國藥學研究所博士論文

組別:藥理學 組　　编號:**ICPS-D66**

指 導 教 授 ： 彭文煌　博士

共同指導教授： 徐士蘭　博士

以活體動物及離體細胞模式評估木犀草素治療肺炎及肺纖維化之療效及分子作用機轉

Luteolin alleviates experimental lung inflammation and fibrosis in vivo and in vitro

研究生： 陳 秋 媛

Chiu- Yuan Chen

中　華　民　國　九十七　年 七 月

中國醫藥大學
中國藥學研究所博士學位論文考試

以活體動物及離體細胞模式評估木犀草素治療肺炎及肺纖維化之療效及分子作用機轉

Luteolin alleviates experimental lung inflammation and fibrosis in vivo and in vitro

本論文係陳 秋 媛同學於中國醫藥大學中國藥學研究所完成之博士論文，經考試委員審查及口試合格，特此證明。

論文口試委員審定書

論文口試委員會 指導教授 彭文煌

委　　員 [illegible]

[illegible]

[illegible]

[illegible]

所　　長 [illegible]

中 華 民 國　九十七 年　七　月　十 日

謝　辭

感謝上天的安排，讓我在離開學校十二年後，重新再踏上學習的路程，在浩瀚無邊的知識之海悠游學習和汲取新知。感謝徐士蘭老師，提供良好的研究環境，耐心的指導我跨入原來並不熟悉的領域。感謝彭文煌老師和吳啟瑞老師提供研究方向的建議，感謝謝明村前校長、蔡輝彥院長、陳玉芳所長及張永勳老師細心審閱論文初稿並提供寶貴的意見，更感謝中興大學獸病所廖俊旺老師在疾病動物模式及組織病理切片部分詳盡的指導，使得論文內容能更加的完備。

感謝台中榮總分子腫瘤實驗室的研究同仁，旗志學長、俊錡，修竹、淑鐘和美君學姐提供寶貴的經驗，志學、宜瑾和建勳在動物實驗進行時的協助，使我的研究能非常順利的完成。此外，同樣在實驗室日以繼夜為了自己的理想不斷奮鬥的諸位學弟妹，有你們的相伴，研究工作不再是枯燥無趣的，勝利是屬於堅持到最後一刻的人，加油喔!

感謝我的父母在生活上的協助，使我能兼顧家庭並完成學業。更感謝外子及小女一路陪伴，你們的包容與鼓勵是我最大的動力。生命中所有愛護與提攜我的人，在此一併致上最誠心的祝福。

目　錄

略字表

2-ME	2-Mercaptoethanol
α-SMA	Alpha-smooth muscle actin
AEC	Alveolar Epithelial Cells
ALI	Acute lung injury
AP-1	Activating protein-1
ARDS	Acute Respiratory Distress Syndrome
BALF	Bronchoalveolar lavage fluid
BMP	Bone morphogenetic protein
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
DAPI	4’,6’-Diamidino-2-phenylindole
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-Linked Immune Substrate Asssay
EMSA	Electrophoretic mobility shift assay
EMT	Epithelial-mesenchymal transition
ERK	Extracellular-regulated kinase
EtBr	Ethidium bromide
FACScan	Flow cytometer
FITC	Fluorescein isothiocyanate
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin

iNOS	Inducible NO synthase
IPF	Idiopathic pulmonary fibrosis
JNK/SAPK	c-Jun N-terminal/stress-activated protein kinases
LAP	Latency-associated protein
LTBPs	Latent TGF-β binding proteins
LPS	Lipopolysaccharide
LOX	Lipoxygenase
MAPK	Mitogen-activated protein kinase
MMP	Matrix metarollproteinase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazdiumbromide
NFκB	Nuclear factor-kappa B
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor
PBS	Dulbecco's phosphatebuffered saline
PGE_2	Prostaglandin E_2
PI_3K	Phosphoinositide 3-kinase
PKB (Akt)	Protein kinase B
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SARS	Severe Acute Respiratory Syndrome
TIMP	Tissue inhibitor of metalloproteinases
TGF-α	Transforming growth factor-α
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitors of metalloproteinase
TNF-α	Tumor necrosis factor-alpha

附表及附圖目錄

圖表目錄

以活體動物及離體細胞模式評估木犀草素治療肺炎及肺纖維化之療效及分子作用機轉

研究生 陳秋媛

中國醫藥大學 藥學院 中國藥學研究所

摘 要

從許多動物體內及體外細胞的研究都證明木犀草素具有良好的抗發炎作用。然而，木犀草素對於肺炎及肺纖維化的作用機轉尚未明確。本研究利用活體動物及離體細胞模式探討木犀草素的抗發炎及抗肺纖維化作用及其分子機轉。 C57BL/6J 小鼠以氣管內注入 bleomycin 引發肺炎及繼發性肺纖維化，治療組以胃管投與木犀草素，並以投與類固醇作為治療對照組。在第 7 和 14 天取出老鼠肺臟，以肺沖洗液之細胞計數及分析發炎性細胞激素含量評估木犀草素的抗發炎效果，並在第 21 天取出老鼠肺臟作病理切片及染色、膠原蛋白含量測定及肺組織 TGF-β1 mRNA 表現等評估肺纖維化改善程度。 活體動物實驗結果顯示，早期(第 1 天開始)與後期(第 10 天開始)投與木犀草素都可以減少 bleomycin 引發小鼠的肺纖維化程度。在細胞培養部分，我們利用初代小鼠肺纖維母細胞和肺上皮細胞株 A549 探討木犀草素抗肺纖維化之機轉，結果發現木犀草素抑制 TGF-β1 刺激小鼠肺纖維母細胞產生 α-smooth muscle actin (α-SMA) 和 collagen 1 表現是透過抑制 Smad3 磷酸化及減少 Smad4 的表現。從細胞型態及相關分子的表現 (E-cadherin, fibronectin and collagen1)顯示木犀草素會抑制 TGF-β1 刺激 A549 進行 epithelial-mesenchymal transition (EMT)。 根據我們的結果顯示木犀草素的抗纖維化作用可能是透過阻斷肺纖維細胞和肺上皮細胞 TGF-β/Smad 的訊息傳遞，使纖維化相關分子表現減少。本論文也進一步以

巨噬細胞探討木犀草素調節免疫作用的分子機轉。 研究發現在老鼠肺泡巨噬細胞株 (MH-S)及周邊巨噬細胞株(RAW264.7)中，木犀草素可抑制由脂多醣體 (LPS) 引發產生一氧化氮(NO) 和前列腺素 E_2 (PGE_2)等發炎物質及腫瘤壞死因子-α (TNF-α)及介白素-6 (IL-6)等細胞激素，同時也抑制了 NO 及 PGE_2 上游的調控酵素 inducible NO synthase (iNOS)及 cyclooxygenase-2 (COX-2) 的蛋白質表現。從半定量反轉錄聚合酶鏈反應 (RT-PCR) 實驗中證明木犀草素可抑制由 LPS 引發 TNF-α、IL-6、iNOS 和 COX-2 mRNA 等的表現。在 NF-κB 路徑中木犀草素能抑制 IκB-α 的分解以及 NF-κB p65 subunit 進入細胞核與 DNA 結合。不僅如此，木犀草素也明顯地減少 AP-1 與 DNA 結合，進而調控其下游與發炎相關物質的產生。我們也進一步發現細胞前處理木犀草素可抑制由脂多醣體刺激產生活性氧化物 (ROS)，同時也減少 protein kinase B (Akt) 及 IKK 磷酸化。綜合上述結果顯示木犀草素抑制 LPS 刺激肺泡巨噬細胞產生發炎相關物質的機轉為經由抑制轉錄因子 NF-κB 與 AP-1 的活化，其抑制 NF-κB 與 AP-1 的活化可能與 ROS 有關。我們的研究結果顯示木犀草素在活體動物及離體細胞模式下有良好的抗發炎及抗肺纖維化作用。

Luteolin alleviates experimental lung inflammation and fibrosis in vivo and in vitro

Chiu- Yuan Chen

Graduate Institute of Chinese Pharmacrutical Sciences, College of Pharmacy, China Medical University

Abstract

Luteolin possesses anti-inflammatory property and effectively in treatment of lung diseases. The present study investigated the antifibrotic effect and its molecular mechanism of luteolin on lung fibrosis in both *in vivo* and *in vitro* models. C57BL/6J mice were administered a single intratracheal injection with bleomycin and then treated orally with luteolin. Lung inflammation was examined by direct counting inflammatory cell population and cytokine levels in the bronchoalveolar lavage fluid (BALF) at day 7 and day 14. Antifibrotic effect of luteolin was determined by investigating histological changes, collagen contents and induction of TGF-β1 mRNA expression at day 21. To elucidate the antifibrotic mechanism of luteolin, the expression of α-smooth muscle actin (α-SMA), collagen 1 and phosphorylated Smad3 protein were analysized by immunofluorescence staining and Western blotting in TGF-β1-stimulated primary mouse lung fibroblasts. The morphological changes as well as epithelial and mesenchymal marker were examined in TGF-β1-stimulated human lung carcinoma-derived alveolar epithelial A549 cell line. *In vivo* study showed that luteolin treatment ameliorated all these biochemical indices and histological alterations induced by bleomycin even delayed luteolin treatment. *In vitro* study showed that luteolin attenuated TGF-β1-induced α-SMA and collagen 1 upregulation and Smad3 phosphorylation in mouse lung fibroblasts.

Furthermore, TGF-β1 mediated E-cadherin downregulation as well as fibronectin and collagen 1 upregulation was significantly inhibited by luteolin in A549 cells. Our data suggest that luteolin may be useful as a therapy for pulmonary fibrosis and its antifibrotic effect at least partly through blockade of TGF-β–signaling pathway. We also verified the effects of luteolin-mediated immune modulation in macrophages when stimulated with lipopolysaccharide (LPS). We examined the effects of luteolin on the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2), as well as the expression of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) in mouse alveolar macrophage MH-S and peripheral macrophage RAW 264.7 cells. Luteolin dose-dependently inhibited the expression and production of these inflammatory genes and mediators in macrophages stimulated with LPS. Semi-quantitative reverse-transcription polymerase chain elongation reaction (RT-PCR) assay further confirmed the suppression of LPS-induced TNF- α, IL-6, iNOS and COX-2 gene expression by luteolin in a transcriptional level. Luteolin also reduced the DNA-binding activity of nuclear factor-kappa B (NF-κB) in LPS-activated macrophages. Moreover, luteolin blocked the degradation of IκB-α and nuclear translocation of NF-κB p65 subunit. In addition, luteolin significantly inhibited the LPS-induced DNA binding activity of activating protein-1 (AP-1). We also found that luteolin attenuated the LPS-mediated protein kinase B (Akt) and IKK phosphorylation, as well as reactive oxygen species (ROS) production. In sum, these data suggest that, by blocking NF-κB and AP-1 activation, luteolin acts to suppress the LPS-elicited inflammatory events in mouse alveolar macrophages, and this effect was mediated, at least in part, by inhibiting the generation of reactive oxygen species. Our observations suggest a possible therapeutic application of this agent for treating inflammatory disorders in lung.

第一章　緒 論

呼吸系統直接與外部環境接觸，因此對吸入的有害物質相當敏感。許多散布在空氣中的化學物質及病原菌都會引起肺的損傷。例如二氧化氮，臭氧或硫酸的吸入，會導致肺泡微血管的滲透性增加，並造成第Ⅰ型、第Ⅱ型肺泡上皮細胞和結締組織的損害。吸入性肺損傷的原因還包括細菌或病毒性肺炎、吸入胃內容物，以及肺部挫傷等，許多肺部疾病的發展過程都會產生氧化壓力與一連串的發炎反應，例如急性肺損傷 (acute lung injury; ALI)/急性呼吸窘迫症候群 (acute respiratory distress syndrome; ARDS)、敗血症(sepsis)、缺血再灌注(ischemia-reperfusion)、高血氧症(hyperoxia)、肺臟移植 (lung tansplantation)及慢性阻塞性肺炎 (chronic obstructive pulmonary disease; COPD) 等。這些急性或慢性的肺部損傷，均會造成細胞毒性物質的釋放，包括活性氧化物 (reactive oxygen species; ROS) 和活性氮化物 (reactive nitrogen species; RNS) 大量的產生以及細胞激素 (cytokines) 的釋放等，進而使得嗜中性白血球以及肺泡內巨噬細胞的活化，釋放促發炎物質，破壞上皮細胞，或導致微血管通透性增加，產生肺水腫現象，對肺組織造成傷害[(1)]。以急性肺損傷 (ALI) 造成的急性呼吸窘迫症候群 (ARDS) 為例，病人原先肺部正常，而在外傷或感染後出現急性呼吸困難，低血氧，肺順應性減低 (reduced lung compliance)，胸部 X光有兩側瀰漫性浸潤，以傳統呼吸治療方法無法矯正其低血氧等現象。病理上出現肺組織明顯的充血，肺泡及間質水腫，肺泡塌陷，炎性細胞浸潤，肺泡內有透明膜 (hyaline membrane) 之形成及纖維化病變[(2)] (附圖1)。

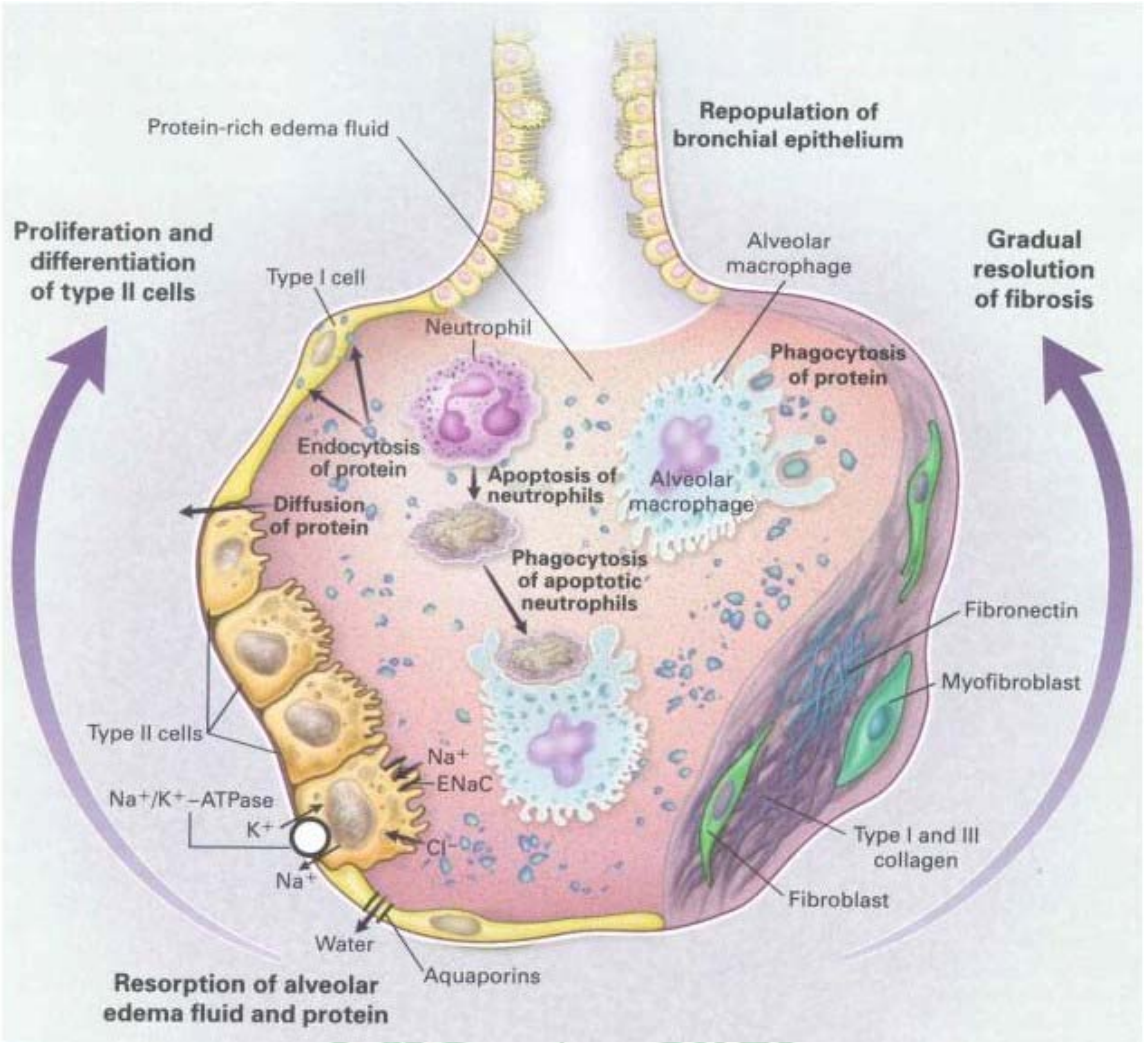
由一類新種冠狀病毒 (SARS-CoV) 所引發的嚴重急性呼吸道症候群 (SARS) 是本世紀新興傳染性疾病，從 2002 年 7 月到 2003 年 9 月流行期間，在全世界共造成八千多人感染，死亡人數高達 812 人[(3)]，典型的症狀

為發燒、咳嗽、呼吸困難、肺浸潤及肺實質化，並快速發展為瀰漫性肺泡損傷進而造成致命性的呼吸衰竭 (ARDS) 或繼發性的肺纖維化，平均致死率為 9.6%。急性期病理特徵為肺部單核球浸潤，肺泡塌陷，肺泡上皮及微血管內皮的損傷、以及滲出性的肺間質水腫，與 ALI 所造成之病理特徵類似[4]。研究顯示肺泡巨噬細胞和 T 細胞釋放出大量的 proinflammatory cytokines，如腫瘤壞死因子-α (tumor necrosis factor-α)、介白素-1 (interleukin-1) 和介白素-6 (interleukin-6)、血小板活化因子、前列腺素、thromboxanes 和活性氧化物 (ROS) 等，都參與了肺部損傷，也在病理機轉中扮演重要的角色。

中醫藥對抗疫疾已有三千多年的經驗，從典籍文獻的記載中，影響較大的疫疾包括鼠疫、傷寒、霍亂、流感、肺炎、腦炎、瘧疾、痢疾、天花、麻疹、寄生蟲等疾病。在中醫藥典籍中，記載許多對肺部疾病有療效的方劑或單味藥，如清熱解毒藥金銀花、板藍根、連翹、魚腥草等；還有清肺止咳藥如黃芩、山梔子、桑白皮、桑葉、沙參、麥冬、貝母、枇杷、桔梗等。在 SARS 流行期間，具有抗病毒及消炎作用的金銀花、黃芩、板藍根或是魚腥草等，更被認為有預防疾病的效用。這些沿用已久的中草藥雖有中醫臨床經驗的傳承，但卻缺乏科學化的實驗證明其療效及確實之作用分子機轉，因此無法受到國際醫學界普遍之認同及使用。因此，如何善用中藥的免疫調節作用以中西醫結合的方法來面對 SARS 的挑戰，並將中醫臨床應用上有確實療效之中草藥，以現代的科學知識及技術，探究其藥理分子機制，是將中醫藥推向國際社會之必行之路，也是中醫藥學界重要的研究方向。

由於化學合成藥物對正常細胞殺傷力大，近幾年來，歐美各國興起萃取藥用植物進行輔助療法，以減緩化學藥物對人體的危害。因此，從植物活性成分提取到各項生物活性的分析研究，都有不少的成果。以廣泛分佈

於植物體內的黃酮類 (flavonoids) 化合物為例,它是一類廣泛分佈於植物體內的低分子量多酚類 (polyphenols) 物質,在植物體內多以游離態或與糖結合成苷的形式存在,也是許多藥用植物的主要活性成分。現已發現數百種不同類型的黃酮類化合物具有廣泛的藥理活性。黃酮類化合物具有清除自由基、抗氧化、抗癌、抗菌、抗過敏、抗發炎、抗病毒等多種生物活性及藥理作用,對人類的腫瘤、衰老、心血管等疾病的治療和預防都具有重要意義。在傳統中醫藥中,金銀花是忍冬科 (Caprifoliaceae)多年生常綠纏繞灌木忍冬 (*Lonicera japonica* Thunb.)的乾燥花蕾,以其抗菌、抗病毒及清熱解毒、祛風熱、清咽喉熱痛等功效,廣泛地應用在預防及治療流行性感冒、上呼吸道感染、肺炎、細菌性痢疾等發炎疾病。在 SARS 流行期間,具有抗病毒及消炎作用的金銀花,被認為有預防及治療的效用,因而成為防治非典型肺炎處方中使用頻率最高的單味藥[(5)]。 從文獻中得知近幾年在探討天然物之純化物對人類之發炎、心血管疾病、以及癌症具有預防治療作用方面,有顯著的成果,亦可驗證於分子機轉。木犀草素 (luteolin)為金銀花主要成分之一,屬於四羥基黃酮類化合物,在許多的動物體內及體外細胞實驗中發現木犀草素具有明顯的抗氧化及免疫調節作用,然而針對木犀草素治療肺部疾病的相關研究則不多。以 bleomycin 誘導小鼠肺纖維化是目前國際上認為與人類肺纖維化病理改變最為接近的一種動物實驗模型,本研究透過 bleomycin 誘導小鼠產生急性損傷性肺炎及繼發性肺纖維化模型,評估木犀草素抗肺炎和抗肺纖維化作用,並深入的探討其作用路徑及相關的分子,以提升金銀花等中藥的應用價值,並提供此藥作為治療肺炎及肺纖維化疾病時之參考。此外,研究肺纖維化的機轉亦可提供其他纖維性疾病一些參考性的資料,例如 atherosclerosis、cirrhosis 和 connective tissue diseases 等,因為這些疾病的發展均有許多相似的過程,如發炎、免疫過度活化、細胞激素及分子機轉等。



附圖 1. Mechanisms important in the resolution of acute lung injury and the acute respiratory distress syndrome.[6]

第二章　總 論

第一節 金銀花之文獻探討

1. 本草資源考證

金銀花，又名忍冬花，具有清熱解毒、疏散風熱的功效，是常用的中藥之一。其名始見於本草綱目忍冬條下。忍冬始載於名醫別錄，列為上品，主治寒熱身腫。 陶弘景曰：“處處有之，藤生淩冬不凋，故名忍冬。”李時珍謂：“忍冬在處有之，附樹延蔓，黃微紫色，對節生葉。葉似薜荔而青，有毛。三四月開花，長寸許，一蒂兩花二瓣，一大一小，如半邊狀，長蕊。花初開者，蕊瓣俱色白，經二三日，則色變黃，新舊項參，黃白相映，故呼金銀花。”又謂：“莖葉及花，功用皆同。”在不同的歷史時期，金銀花的藥用部位發生了很大變化。宋代以前獨用莖葉，明代則莖、葉、花同等入藥，此後強調以花為主，其莖葉成為同一植物的另外一種藥物，即忍冬藤。忍冬藤具清熱解毒、通經活絡的作用。金銀花植物來源複雜，在中國分佈廣泛。中華中藥典記載金銀花 Lonicerae Flos 為忍冬科 Caprifoliaceae 植物忍冬 *Lonicera japonica* Thunb.、紅腺忍冬 *Lonicera hypoglauca* Miq.、山銀花 *Lonicera confusa* DC. 或毛花柱忍冬 *Lonicera dasystyla* Rehd. 之乾燥花蕾[(7)]。

2. 金銀花活性成分研究

金銀花是忍冬科同屬多種植物忍冬的乾燥花蕾，外形呈棒狀，上粗下細，長 2～3.5 cm ，外表淡黃色，久貯色漸深。金銀花為常見中藥，具有清熱解毒、抗菌消炎、保肝利膽的功能。臨床應用於治療瘡瘍、瀉痢、外感、熱病等，也用治療呼吸道感染、頭痛咽痛等疾病。傳統認為花蕾採收宜為初夏，莖枝於秋冬割取。現代研究證明:花蕾的採收期大都在清明季

節，此時成熟花蕾中綠原酸含量最高。對於葉的採收，有人認為以 8～9 月份含綠原酸最豐富，此時採收較為適宜。研究顯示其花中主要含有揮發油、黃酮類、有機酸、三萜類等活性成分[8, 9]。

2.1 揮發油類

金銀花乾品中含有揮發油成分 60 種以上，其中主要成分酸類化合物占揮發油總量的 59.76%，其次酮類化合物占15.58%，醇類化合物占12.85%，萜類化合物占1.57%，萜類氧化物占1.32%，醛類化合物占1.74%，烷烴化合物占7.18%，共占金銀花中揮發油總量的 98.44%。鮮品揮發油成分多為低沸點的不飽和萜烯類，其中以芳香醇為主，含量占揮發油的45. 5%以上。金銀花乾花蕾中多為單萜及倍半萜類化合物，含量較高的有香茅醇(citronellol)、芳樟醇(linalool)、α-松油醇(α-terpineol)、二十一烷醇(3-henen-1-ol)、辛烯醇(1-Octen-3-o1)和香葉醇 (geraniol)等[10]。

2.2 黃酮類

1995年高玉敏首次從金銀花中分離出4個黃酮類化合物，經鑑定為木犀草素- 7 - O -α - D - 葡萄糖苷(luteolin - 7 - O- α- D-glucoside)、木犀草素- 7 - O -β - D - 半乳糖苷(luteolin-7- O- β- D- galactoside)、槲皮素- 3 -O -β - D - 葡萄糖苷(quercetin- 3- O- β- D- glucoside)、金絲桃苷(hyperoside)[11]。

2.3 有機酸衍生物

綠原酸類化合物是金銀花的主要成分之一，包括綠原酸(chlorogenic acid)、異綠原酸 (isochlorogenic acid) 和咖啡酸 (caffeic acid)。其中異綠原酸為一混合物，它的異構體有七種，分別為 4,5-二咖啡酸醯奎尼酸、3,4 - 二咖啡酸醯奎尼酸、3 ,5-二咖啡酸醯奎尼酸、1,3 - 二咖啡酸醯奎尼酸、3-阿魏醯奎尼酸、4 -阿魏醯奎尼酸和5-阿魏醯奎尼酸[12]。

2.4　三萜及三萜皂苷類

1990年陳敏等[13]從金銀花中分離出一個新的含有 6 個糖基的三萜皂苷。1993年茅青等[14]也分離得到 3 個三萜皂苷， 1994年陳敏等[13]又分離出 2 個新的雙咖啡酸醯奎尼酸酯化合物。婁紅祥等[15]分離出 3 個三萜皂苷類，分別為3 - O - α - L- rhamno pyranosyl (1 →2) - α - L - arabinopy ranonosyl hederagenin 28 - O - β - D - xylpyranosyl (1→6) -β - D - glucopy ranosyl ester、3 - O -α - L -arabinopy ranosyl hedragenin 28 - O -α - L - rhamnopyranosyl (1→2) [β - D - xylpyranosyl (1→6) - β- D - glucopy ranosyl ester 和 3 - O - α - L - rhamnopy ranosyl (1 →2) - α - L - arabinopy ranosyl hederagenin 28 - O - α - L - rhamnopy ranosyl (1→2) [β - D - xylpyranosyl (1 →6) - β - D – glucopy ranosyl ester。

2.5　無機元素

金銀花含微量元素共 15 種，分別為Fe、Mn、Cu、Zn、Ti、Sr、Mo、Ba、Ni、Gr、Pb、V、Co、L i、Ca 。

2.6　其他

忍冬花蕾中還含有肌醇 (inositol)、β - 穀固醇 (β-sitosterol) 等。另外邢俊波等首次從該植物中發現 5 -羥基- 7, 4-二甲氧基黃酮，並首次從該植物花蕾中發現槲皮素、忍冬苷、齊墩果酸和胡蘿蔔苷[16]。

3.　金銀花之藥理作用

3.1　抗病原微生物

體外試驗顯示金銀花對金黃色葡萄球菌、溶血性鏈球菌、大腸桿菌、痢疾桿菌、霍亂弧菌、傷寒桿菌、副傷寒桿菌等均有抑制作用，對肺炎桿菌、腦膜炎雙球菌、綠膿桿菌、結核桿菌亦有抑制效果[17]。金銀花對變形

鏈球菌，具有較好的抑菌和殺菌作用，金銀花與青黴素合用，能加強青黴素對耐藥金黃色葡萄球菌的抗菌作用，兩者具有協同作用[18]。金銀花對流感病毒、孤兒病毒、皰疹病毒、猴免疫缺陷病毒 (SIV) 等多種病毒均有抑制作用，金銀花注射液對綠膿桿菌內毒素有對抗作用[19, 20]。對傷寒桿菌內毒素有減毒作用。此外，體外試驗顯示金銀花及其藤煎劑對鉤端螺旋體有抑制作用。

3.2 消炎及解熱作用

金銀花提取物對鹿角菜膠、蛋清等引起的大鼠足蹠腫脹有抑制作用，還有明顯的抗滲出和增生作用。對金銀花解熱、消炎、免疫等實驗研究結果顯示，其水煮液、口服液和注射液對鹿角菜、三聯菌苗致熱有不同程度的退熱作用，對蛋清、鹿角菜膠、二甲苯所致水腫有不同程度的抑制作用[21]，另外，還能明顯提高鼠腹腔巨噬細胞的吞噬百分率和吞噬指數，證明金銀花臨床用作清熱解毒治療感染性疾病，主要是透過調節機體免疫功能而產生的[22]。

3.3 保肝利膽作用

動物實驗顯示金銀花中的三萜皂苷對四氯化碳引起的小鼠肝損傷有明顯的保護作用，並明顯減輕肝臟病理損傷的嚴重程度。金銀花所含綠原酸能增進大鼠膽汁分泌，黃褐毛忍冬總皂苷皮下注射能顯著對抗四氯化碳和 D －半乳糖胺所致肝中毒小鼠血清谷丙轉氨酶升高，降低肝臟甘油三酯含量，明顯減輕肝臟病理損傷程度[23]。

3.4 其他作用

金銀花有提高免疫功能的作用，煎劑能增加白血球、炎性細胞的吞噬能力。有研究指出，金銀花及酒浸液對 S180 肉瘤及艾氏腹水癌有明顯細

胞毒殺作用。金銀花提取液口服對大鼠實驗性胃潰瘍有輕度預防效果。金銀花具有降血脂、興奮中樞神經系統、止血和降壓等作用。此外，綠原酸還能輕微增強腎上腺素及去甲腎上腺素對貓與鼠的升壓作用[24-26]。

第二節 木犀草素之獻探討

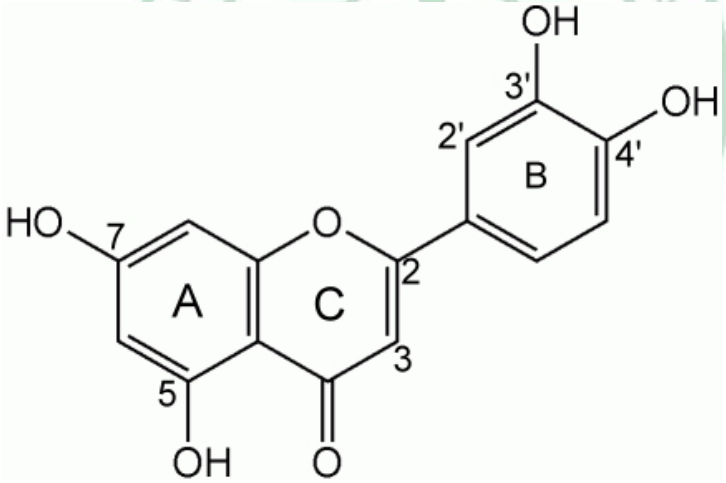
1. 木犀草素之結構與理化性質[27]

1.1 化學名： Luteolin (3', 4', 5, 7- tetrahydroxylflavone)

1.2 分子式及分子量： $C_{15}H_{10}O_6$；286.24

1.3 物理性質：木犀草素為黃色針狀化合物，微溶於水，溶於鹼中呈黃色溶液。熔點 328-330℃。

1.4 結構式：



附圖 2. Structure of luteolin.

1.5　來源植物

除了忍冬科植物忍冬(*Lonicera japonica*) 之外，木犀草素也在其它植物中被發現，如龍膽科植物濕生菡蕾(*Gentianopsis paludosa*)、敗醬科黑水纈草(*Valerlana amurensis*) 地上部分、豆科落花生(*Arachis hypogaea*)果實外殼、鷹爪豆 (*Spartium junceum*) 花中、玄參科金魚草(*Antirrhinum majus*) 花瓣中、十字花科植物薺菜(*Capsela jobursa*)[28]。

1.6　金銀花不同部位之木犀草素含量

金銀花之木犀草素含量隨著金銀花品種、部位不同而存在較大差異；金銀花同一品種，不同部位中木犀草素含量分布亦不相同，為葉、枝>花蕾>花。不同品種金銀花及不同部位中，木犀草素含量有很大的差異，從 27.89 μg/g 到 240.39 μg/g 不等，但在各個品種中，葉和枝中的含量都較高，因此有必要對這些資源加以開發利用[29]。

2.　木犀草素之藥理學研究

木犀草素屬於四羥黃酮類化合物，黃酮類化合物是一類多酚類化合物，廣泛分佈於植物界中，在植物體內大部分與糖結合成苷，一部分以游離形式存在，屬於植物的二次代謝產物。實驗證明其具有廣泛的生理和藥理活性，包括清除自由基、抗病毒、抗癌、抗氧化、抗發炎、抗衰老等，對人類的腫瘤、心血管疾病、老人癡呆等慢性疾病的治療和預防有重要意義。因此對該類化合物的研究已成為國內外醫藥界研究的熱門課題。而這許多生理活性多與其抗氧化作用有關，目前在抗氧化研究領域中黃酮類化合物是研究的熱門項目之一。黃酮類化合物泛指結構具有15 個碳原子的多元酚化合物。芳香環 (A 環、 B 環) 之間以一個三碳鏈 (C 環) 相連，用 C6 - C3 - C6 表示(附圖2)。其中 C 環部分可以是酯鏈，也可以與 B 環部分形成六元或五元的氧雜環。根據 C3 部分的成環、氧化和取代方式的差

異，黃酮類化合物可分為黃酮類 (flavones)、黃酮醇類 (flavonols)、異黃酮類 (isoflavones)、查爾酮類 (chalcones)、噢口弄類(aurones)、花青素類(anthocyanins) 等以及上述各類的二氫衍生物。黃酮類化合物抗氧化的作用機制是通過酚羥基與自由基反應生成共振穩定的半醌式自由基結構，從而終止自由基鏈式反應[30]。

除了具有優異的抗氧化能力之外，許多研究顯示黃酮類化合物在抗病毒、抗發炎、抗過敏或抗癌方面也都有很好的效果。從抗發炎相關的研究中發現，某些黃酮化合物的抗發炎作用優於其它種類。2001年 Xagorari 等人利用細菌內毒素(lipopolysaccharide;LPS)刺激 RAW264.7 巨噬細胞產生發炎細胞激素(proinflammatory cytokines)的實驗來評估一群黃酮化合物的抗發炎作用，細胞前處理 luteolin、luteolin-7-glucoside、quercetin 和 genistein可抑制 TNF-α 與 IL-6 的釋放，而 eriodictyol 和 hesperetin 只抑制 TNF-α 的釋放，luteolin 和 quercetin 的 IC_{50}分別為 1 μM 和 5 μM，糖苷 luteolin-7-glucoside 的效果則不如苷元 luteolin [31]。2004年 Chen 等人以 TNF-α 刺激肺上皮細胞釋放 ICAM-1的實驗，比較三種黃酮醇(flavonols): kaempferol, quercetin, myricetin及六種黃酮 (flavones): flavone, chrysin, apigenin, luteolin, baicalein, baicalin 的抑制效果，其中 kaempferol, chrysin, apigenin, luteolin 都可抑制 ICAM-1蛋白質及 mRNA的表現 (IC_{50} 分別為 12.1, 0.9, 0.82, 0.8 μM)，進一步的分子機轉探討中發現 luteolin 可明顯抑制 ICAM-1上游的轉錄分子 AP-1 與 DNA 結合，進而調控蛋白質的表現[32]。 2006年 Mo`nica Comalada 等人在primary bone marrow-derived mouse macrophages (BMDM) 所作的抗發炎實驗中也發現，在所有受試樣品(flavonols: quercetin, kaempferol; flavones: diosmetin, apigenin, chrysin, luteolin; isoflavones: genistein, daidzein; flavanones: hesperetin)中，quercetin 和 luteolin 對於 BMDM 的增生、IκBα 的磷酸化、cytokines及 NO 的釋

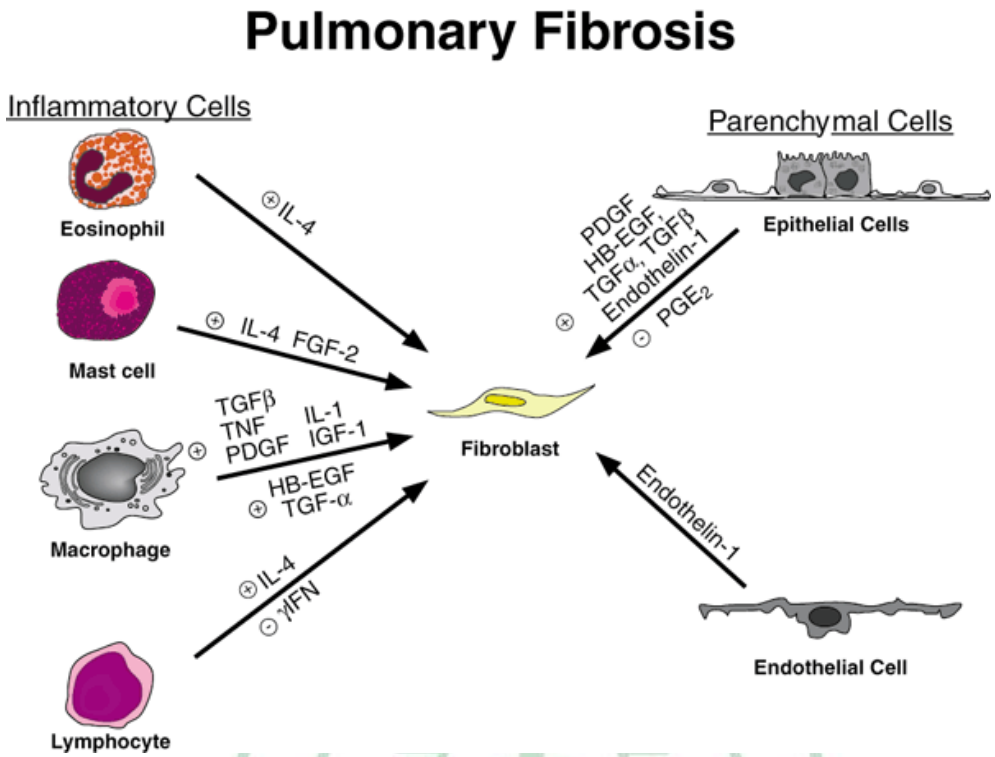
放都有顯著的抑制作用[33]。2006年 Kazuki Kanazawa 等人認為幾種黃酮化合物中 (quercetin, apigenin, baicalein, chrysin, luteolin, kaempferol)，只有 luteolin 與 quercetin 具有保護 HepG2 細胞免於氧化壓力造成細胞 DNA 傷害的能力[34]。2007年蔡汎修等人研究發現木犀草素可以改善類澱粉樣蛋白誘發大鼠學習記憶障礙，並對神經細胞有保護作用[35]。2002年 Hisashi Mastuda 等人在 Antigen-Induced RBL-2H3 Cells (mast cells) degranulation 實驗中發現，在幾種黃酮化合物中 luteolin 具有很強的抗過敏活性，因此作者歸納出黃酮化合物的抗過敏活性乃因其結構上連接了某些特殊的官能基[36, 37]。綜合以上所述，黃酮化合物的抗發炎作用與其結構之間存在著某種相關性 (structure-activity relationship; SAR)，而 luteolin 的抗發炎的效果是幾種黃酮類中最好的，因此在近幾年內有更多的學者投入了與 luteolin 有關的藥理及分子機轉研究。

2001年 Anastasia Kotanidou 等人在老鼠實驗發現 luteolin 可以降低由 LPS 引發的致死率以及相關 proinflammatory molecules 的表現[38]。2003年 M. Das 等人發現口服 luteolin 可以減少 ovalbumin 致敏小鼠的氣喘發作，降低血中 IgE 和 bronchoalveolar lavage fluid (BALF) 中 IFN-γ, IL-4 , IL-5 的濃度[39]。2005年 Liisa Tormakangas 在老鼠感染 *Chlamydia pneumoniae* 引發急性肺炎的實驗中發現，腹腔注射 luteolin 可降低老鼠的 infection load 及肺部發炎現象[40]。在分子機轉探討的部分，於不同的細胞株所進行抗發炎機轉的研究均認為 luteolin 抗發炎作用是透過調控轉錄分子 NF-κB 的活性，導致下游發炎相關分子的產量減少[41-44]。Luteolin 的抗發炎作用則可能是透過阻斷鈣離子通道及 PKC signalings 而抑制了下游 histamine、leukotrienes (LTs)、prostaglandin D_2 (PGD_2) 和 granulocyte macrophage-colony stimulating factor (GM-CSF) 等過敏物質的釋出[45]。

第三節、肺纖維化之病理機轉探討

一、 肺纖維化的定義

瀰漫性肺間質纖維化病變是一種由多種病因所引起的肺破壞性疾病，超過 200 多種的肺間質疾病 (Interstitial Lung Disease；ILDs) 都會導致肺纖維化，包括已知的成因如職業、環境、藥物、高血壓、感染、免疫系統的異常（如硬化症或特發性肺纖維化）等，每種病因其確切的發病機制尚未完全明瞭，但不同病種的肺間質纖維化改變極為類似，其共同的發展是以肺泡炎、間質性肺炎、肺泡上皮受損及膠原異常聚集為特徵的慢性炎性疾病，主要症狀為進行性呼吸困難，最終導致呼吸衰竭而死[46, 47]。其中特異性肺纖維化 (Idiopathic pulmonary fibrosis; IPF) 經確診後平均存活期為 2-4 年，5 年存活率為 30-50% [48, 49]。 肺纖維化過程包括肺組織的發炎性損傷、組織結構破壞以及隨後伴有肺間質細胞積聚的組織修復過程。在此過程中，肺發炎細胞 (主要為單核巨噬細胞)、肺泡上皮細胞、肥大細胞、內皮細胞和肺間質細胞 (如纖維母細胞、肌纖維母細胞)通過分泌細胞激素 (cytokines)、炎性介質等生物活性物質，發揮直接或間接的作用，活化了膠原蛋白的基因表現且製造大量的膠原蛋白堆積在細胞間質[50-53]。形態學上發現，纖維化病變內肺泡Ⅱ型細胞、纖維母細胞、巨噬細胞以及肥大細胞彼此之間緊密接觸。因此，參與肺纖維化的多種細胞共同構成了一個複雜的細胞網絡，彼此相互影響 (附圖 3)。研究結果顯示，其中以肺巨噬細胞、肺泡上皮細胞及肺纖維母細胞在纖維化起始及進展過程中扮演相當重要的角色。



附圖 3. Pathogenesis of pulmonary fibrosis.[54]

二、 急性呼吸窘迫症候群 (ARDS) 與肺纖維化

急性肺損傷 (ALI)，包括細菌性、病毒性引發之肺炎或是吸入性、放射性損傷等非炎性因素[55, 56]。其中最嚴重的病理反應為急性呼吸窘迫症候群 (ARDS)，即使施予支持性療法，其死亡率仍在 35-40 % 左右。以 2003 年引發人類嚴重呼吸道症候 (SARS) 的新型冠狀病毒為例，當此病毒進入宿主的下呼吸道後，激活人體的免疫系統，尤其是 CD8-T cell 對冠狀病毒感染的呼吸道上皮及單核球細胞產生過強的免疫反應，初期肺泡內吞噬細胞增加，第二階段為瀰漫性 ARDS，又稱為激素風暴期 (cytokine storm)，發炎細胞 (淋巴球、單核球和嗜中性球) 聚集、釋放趨化物質及微血管通透性上升，發炎細胞及血漿滲出至肺泡，引發肺水腫及造成肺泡上皮細胞及肺

泡微血管內皮細胞損傷，患者之換氣功能受阻，缺氧甚而呼吸衰竭致死。第三期為發炎細胞活化後釋放大量之細胞激素造成纖維母細胞 (fibroblasts) 增生，此後多發展成肺部纖維化病變[3, 57, 58]。

三、免疫細胞在肺纖維化扮演的角色

肺纖維化是許多原因的肺損傷後不正常的組織修復結果。任何的損傷刺激都起始於發炎、免疫系統啟動，到最後完成於組織修復。IPF 病人和實驗動物肺損傷模型的組織都發現有許多的免疫細胞出現在發炎部位，包括最先出現的 neutrophils 及被趨化而至的 monocyte/macrophage、lymphocytes 和 eosinophils (附圖4)。其中 macrophages 和 monocytes 是 fibrogenic cytokines、chemokines 和 growth factors (TNFα, TGFβ, PDGF 和 MCP-1) 主要的來源細胞[53]。這些免疫細胞本身及其所釋放的介質對肺上皮組織造成輕重不一的傷害，也與纖維化發展關係密切(附表1)。

附表1. Major mediators expressed by lung cells and their potential roles in repair and fibrosis.[59]

	Made By						Major Biological Activities			
Mediators	Endo	Epi	Fibroc	Fib	Myo	Major Targets	Proliferation	Chemotaxis	Collagen Synthesis	Fibrogenic
$TGF\beta_1$	Y	Y	Y	Y	Y	Fib	±	+	+ +	+
PDGF	Y	Y	Y	Y	Y	Fib	+	+	+	+
FGF2	N	N	Y	Y	Y	Fib	+	+	+	+
TGFα	N	Y	Y	Y	Y	Fib	+	0	+	+
TNFα	N	N	Y	Y	Y	Fib	+	0	+	+
IL-1	N	N	Y	Y	Y	Fib	+	0	+	+
IL-4	N	N	Y	Y	Y	Fib	+	+	+	+
IL-10	N	N	Y	Y	Y	Fib	−	+	−	±
IL-13	N	N	U	Y	Y	Fib	+	−	+	+
IFNγ	N	N	U	Y	U	Fib	−	−	−	−
IL-8	N	Y	Y	Y	Y	Mac	+	+	+	+
MCP-1	Y	Y	Y	Y	Y	Mac	−	+	+	+
M1P-1α	Y	Y	Y	Y	Y	Mac	−	+	+	+
Col-1	N	N	Y	Y	Y	Fib	+	0	+	+

Endo Endothelial cells, *Epi* epithelial cells, *Fib* fibroblast, *Fibroc* fibrocyte, *Mac* macrophages, *Myo* myofibroblast, *Y* yes, *N* no, *U* undetermined, ++ major role or strongly stimulatory, + stimulatory, − inhibitory, ± variable-dose-dependent, 0 no effect.

1. 嗜中性白血球 (Neutrophils)

在宿主免疫反應中，polymorphonuclear leukocytes neutrophils (PMNs) 通常是最快抵達感染部位執行吞噬功能的白血球。此外，PMNs 製造許多 cytokines, chemokines 和 growth factors，如 IL-1, IL-6, IL-8, MIP-2, TNFα, granulocyte macrophage colony-stimulating factor (GM-CSF), arachidonic acid metabolites, proteases, reactive oxygen metabolites (ROMs) 和 neutrophils-derived complement fragments 等[60, 61]。在 PMNs 細胞膜上的接受器 (如 heterotrimeric G protein-coupled seven-transmembrane receptor) 可與C5a, platelet-activating factor (PAF) 及CXC chemokines family 結合後引發一連串的趨化反應。PMNs 對 parenchymal cells 具有相當程度的破壞力，它會分泌 MMP9 (Gelatinase B), acid hydrolases, low-molecular-weight cationic proteins, ROMs 和 lipid secretory products，這些分子會破壞 parenchymal cell 並促使 ECM 降解[62]。在許多的研究中發現 IPF 病人肺沖洗液 (bronchoalveolar lavage; BAL) 中 PMNs 的含量與症狀嚴重程度有正相關性而降低 PMNs 的治療策略也可以延緩纖維化的發生。這些研究顯示 PMNs 在肺泡的發炎反應轉變為纖維化時扮演重要的媒介角色[63]。

2. 肺巨噬細胞 (Alveolar Macrophages)

血液單核球細胞遷移到肺部後分化成具防禦功能的肺巨噬細胞，近年來有許多研究在探討肺泡巨噬細胞的生理功能，包括肺部免疫失調、癌細胞毒殺作用、發炎及纖維化病變。分布在不同區域的肺巨噬細胞功能略有不同，在正常人的肺，分布在肺泡內、氣道及上皮表層與吸入空氣充分接觸的稱為肺泡巨噬細胞 (alveolar macrophages; AMs)，AMs 主要負則第一線的病原菌毒殺及分泌細胞激素以啟動後續免疫活化等功能，最後 AMs 也會分泌 IL- 10 以防止過度發炎及非必要的免疫反應，維持肺泡正常生理功

能。間質巨噬細胞 (interstitial macrophages; IMs) 分布在隔開肺泡的間質組織，IMs 體積較 AMs 小，吞噬力也較弱，但因細胞表面較多的 MHC Class II 和CD54 表現，對 T-cell 有較強的 antigen presenting capability [64]。肺損傷或發炎時，受單核細胞趨化，從幹細胞分化而來並大量增生的肺巨噬細胞會使肺泡間質增厚，活化的肺巨噬細胞分泌大量的促纖維化細胞激素 (TGF-β1, TNF-α, MCP-1 和 insulin-like growth factor; IGF-1)；刺激周圍 epithelial, endothelial 和 mesenchymal cells 釋放其它纖維化細胞激素，同時作用於纖維細胞增生和 collagen deposition [65, 66]。

研究指出肺巨噬細胞對細胞外間質形成/降解的平衡與氣喘或肺纖維化疾病有某些關聯，肺巨噬細胞分泌的降解酵素 (MMPs, serine, cystine protease 和 acid hydrolases；主要是 MMP-1, MMP-2 和 MMP-9) 可分解 collagen 和 elastin 等 ECM 蛋白[67]。肺巨噬細胞受 cytokines 和 growth factors (尤其是TGF-β)刺激時也會分泌降解酵素抑制劑 TIMP 以利組織重建[68]。通常在 IPF 病人 TIMPs 會有較高的表現。MMPs/ TIMPs 失衡是造成細胞外膠原蛋白過度堆積甚至纖維化的重要因素。直到近幾年對於肺巨噬細胞的功能才逐漸明瞭，目前已知肺巨噬細胞具有遷移到肺損傷或發炎部位並參與組織修復工作，但對於它如何參與纖維化的發展仍有許多問題有待進一步研究。

3. 淋巴球 (Lymphocytes)

淋巴球可區分自身物質和外來的物質。 T 細胞會附著在攜有抗原的細胞上，並且直接和這些細胞相作用；而 B 細胞藉著分泌抗體(antibodies) 間接地對付抗原，抗體由體液攜帶並以許多不同的方式破壞特定的抗原或攜有抗原的物質。免疫系統中有兩種主要 T 細胞，分別是毒殺性 T 細胞 (cytotoxic T cell)及輔助性 T 細胞 (helper T cell)，它們皆含有 α鏈及 β 鏈

所組成的 T 細胞接受器 (TCRs)，並用它來辨認細胞上的組織相容性分子及抗原所形成的複合體 (MHC-Ag)。二者所不同的是：毒殺性 T 細胞以 αβT 細胞接受器及 CD8 輔助接受器來辨認標的細胞 (target cells)上的第一型組織相容性分子 (MHC-I) 及抗原，並分泌各種因子 (例如perforin)來攻擊並破壞這些標的細胞(例如，受微生物感染的細胞或腫瘤細胞)。而輔助性 T 細胞則以 αβT 細胞接受器及 CD4 輔助接受器來辨認抗原呈現細胞 (APCs) 上的組織相容性分子 (MHC-II) 及抗原，並分泌介白質(interleukins)以刺激這些抗原呈現細胞(如 B 細胞或毒殺性 T 細胞)分裂現象並製造抗體及 T 細胞接受器，用以去除外來抗原[69, 70]。 近年來也有研究討論關於 T-helper (Th) 淋巴球分泌的細胞激素與纖維化進展的關係，T-helper (Th) 淋巴球可分為二群，Th1 細胞分泌 IL-2, IL-12, IL-18 和 interferon-γ (IFN-γ)；Th2 細胞分泌 IL-4 , IL-5, IL-10 和 IL-13。 Th1 clones 媒介 delayed-type 過敏反應並與自體免疫疾病有關，升高體內 IgG2a 濃度而非 IgE。 Th2 clones 刺激 B cells 產生IgE ，與氣喘等過敏反應及 IPF 等慢性纖維化病變 (chronic fibro-proliferative disorders) 有關。IPF 病人的肺組織相對有較高的 Th2 cytokines 表現及較少的 Th1 cytokines (例如 anti-fibrotic cytokines-IFN-γ 的表現減少) 造成 collagen deposition[71]。 因此，Th2 /Th1 的失衡也是與慢性纖維化病變進展的原因之一。

四、肺泡上皮細胞 (AEC) 與肺纖維化

除了免疫細胞之外，肺實質細胞(尤其是肺泡上皮細胞)除了作為肺損傷的主要標的，同時也參與發炎反應[72]。大量的組織學觀察發現慢性纖維性肺泡炎病人的肺泡上皮 (Type I 和 Type II) 細胞有非常明顯的變化，Type I 肺泡上皮細胞被破壞而減少肺泡表面活性物質的製造及運轉，導致肺泡萎縮和塌陷；Type II 肺泡上皮細胞增生並重新分布於裸露的基底膜之

上使肺泡壁間質變厚[73]。肺泡上皮屏障的損壞將使得造成肺纖維化的前驅物質更容易移位至肺泡內，最後上皮細胞會被大量的纖維母細胞及平滑肌細胞取代，使得修復的過程朝向肺纖維化發展。肺泡上皮細胞受到細胞激素及 ECM components 的調控，自己也會製造 IL-8, MCP-1, GM-CSF, adhesion molecules, proteases, surfactant proteins 和 ECM components。在實驗動物的觀察中發現，急性肺損傷初期嗜中性白血球劇增，隨後而至的巨噬細胞和淋巴細胞聚集在肺泡壁[72]，分泌大量的 mediator 刺激纖維母細胞增生及 ECM 沉積。如果發炎現象沒有結束或被抑制，這些發炎細胞及其分泌物就會對肺泡上皮造成傷害。在某些嚴重或大面積的損傷，肺泡上皮被取代而無法再生，正常的組織修復及結構復原便無法完成，也因此產生纖維化組織 (附圖4)。

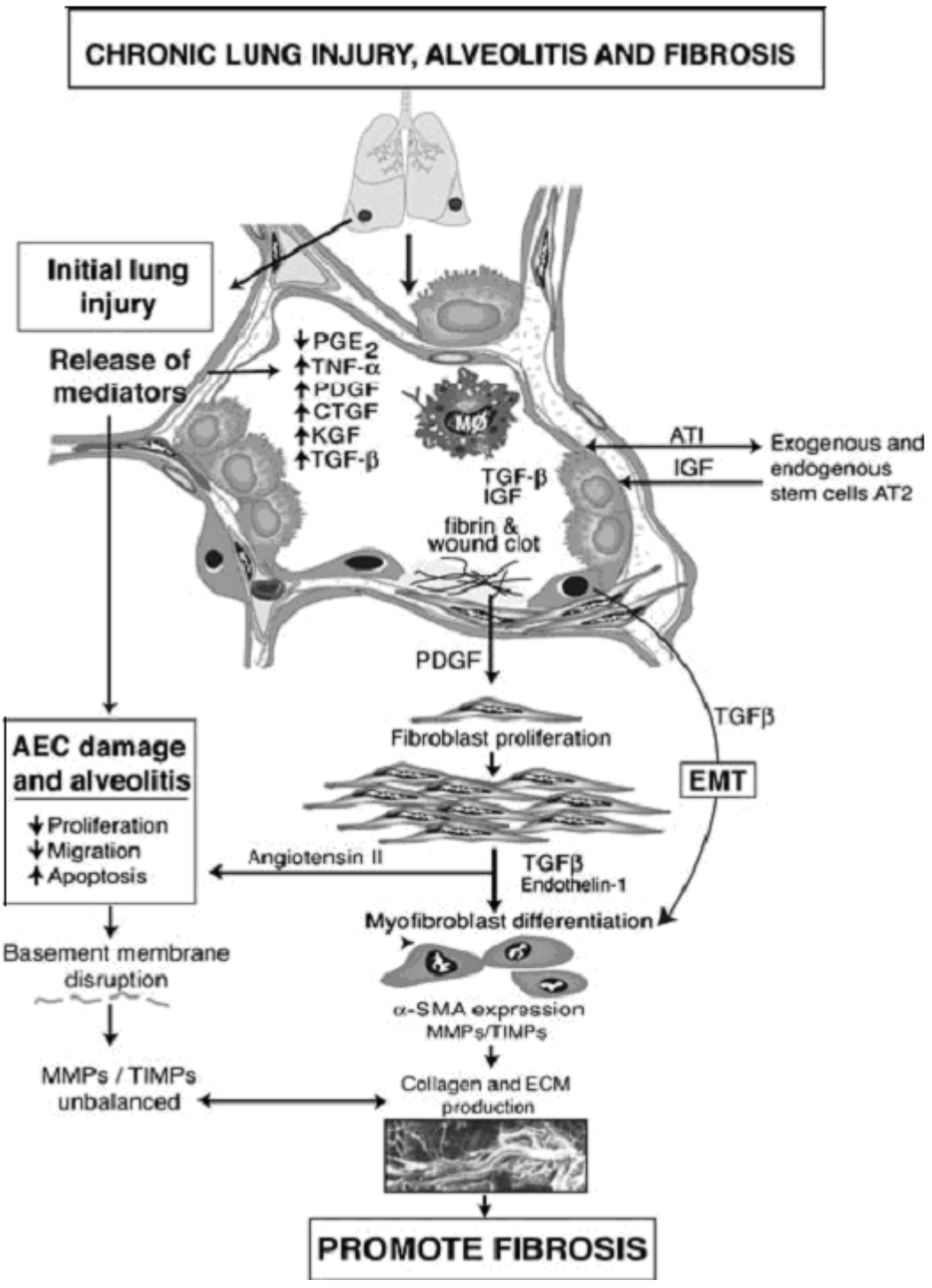
雖然大部分的肺纖維化研究都聚焦於發炎反應、纖維母細胞的活化和 ECM 沉積，近年來從解剖學及動物實驗都發現 Type II 肺泡上皮細胞增生為 IPF 的病理特徵之一[47, 51, 56]；許多 IPF 病人以抗發炎藥物或免疫抑制劑 (steroids and/or immunosuppressive drugs) 治療並無明顯改善。因此AECs在損傷/發炎之後的 dysregulated repair、 abnormal mesenchymal cell activation 都扮演重要角色。近來有許多研究都指出 AECs 具有 epithelial-mesenchymal transition (EMT) 的能力，在 TGF-β 刺激下會轉變為具 myofibroblast phenotype 的細胞，證明了纖維化時期 AECs 也可作為 myofibroblast 的來源細胞[74-76]。

在胚胎發育期間 EMT 促使細胞移動以形成新的器官[77, 78]。 EMT 的轉變代表細胞從原本上皮細胞(epithelial cell)分化成具有爬行能力的 mesenchymal cell。細胞進行 EMT 時，cell junctions 發生變化，原本緊密連接的細胞彼此脫離並失去上皮細胞該有的極性 (epithelial polarity)，新的結構蛋白(vimentin 和 α-smooth muscle actin; α-SMA) 產生，改變了細胞的

形態並增加爬行能力 (migration)，因此從外觀型態 (e.g. change from a cuboidal cell shape to an elongated or spindle-shaped form) 及一些 epithelial markers (E-cadherin) 和 mesenchymal markers (vimentin 和 α-SMA) 的表現即可觀察 EMT 的變化。在各種器官的纖維化也可觀察到 EMT 的進行，例如：在腎性糖尿病患者的腎小管間質部纖維化病灶發現 renal proximal tubular cells 的 α-SMA 表現明顯增加[79, 80]。

EMT 會在 epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factors (FGF)，尤其是 TGF-β 及 extracellular matrix components (通常是collagen) 刺激下進行。包括肺纖維化的在內的器官纖維化過程中，TGF-β 被認為具 "master switch" 的能力，是促進 EMT 及造成纖維化的主因[81, 82]。肺泡上皮細胞細胞膜表面的 TGF-β 接受器與活化態的 TGF-β 結合後會活化細胞內的轉錄因子 Smad proteins，引發下游基因轉錄調控或活化其它轉錄因子 (如 Slug, Snail, Scatter, lymphoid enhancing factor-1 和 β-catenin)[83]。這些轉錄因子都與 "EMT proteome" (junctional disassembly, cytoskeletal rearrangement 和 cellular motility) 的調控有關[84]。

綜合以上所述，肺泡上皮細胞在纖維化的過程中不再是被動的旁觀者，Adamson 等人認為持續的 AEC injury 和遲緩的上皮修復都是促進纖維病變的原因[72, 85]。分化後的 AEC 不但沒有促進組織再生及修復功能，還會製造多種的 profibrotic cytokines，與相鄰的纖維細胞組成聯繫的網路，促進彼此的 proliferation/survival 及功能。



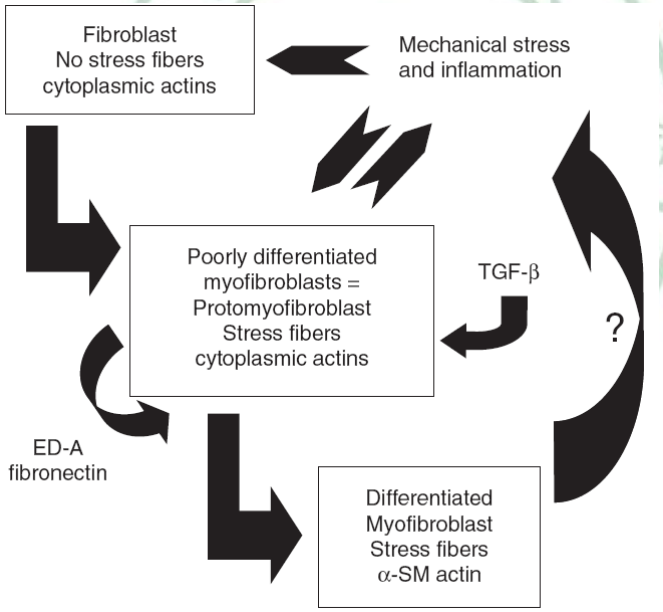
附圖 4. Summary of cellular and cytokine networks in chronic stages of lung injury and fibrosis.[59]

五、肺纖維母細胞 (Lung fibroblasts)、肌纖維母細胞 (Myofibroblasts) 與肺纖維化

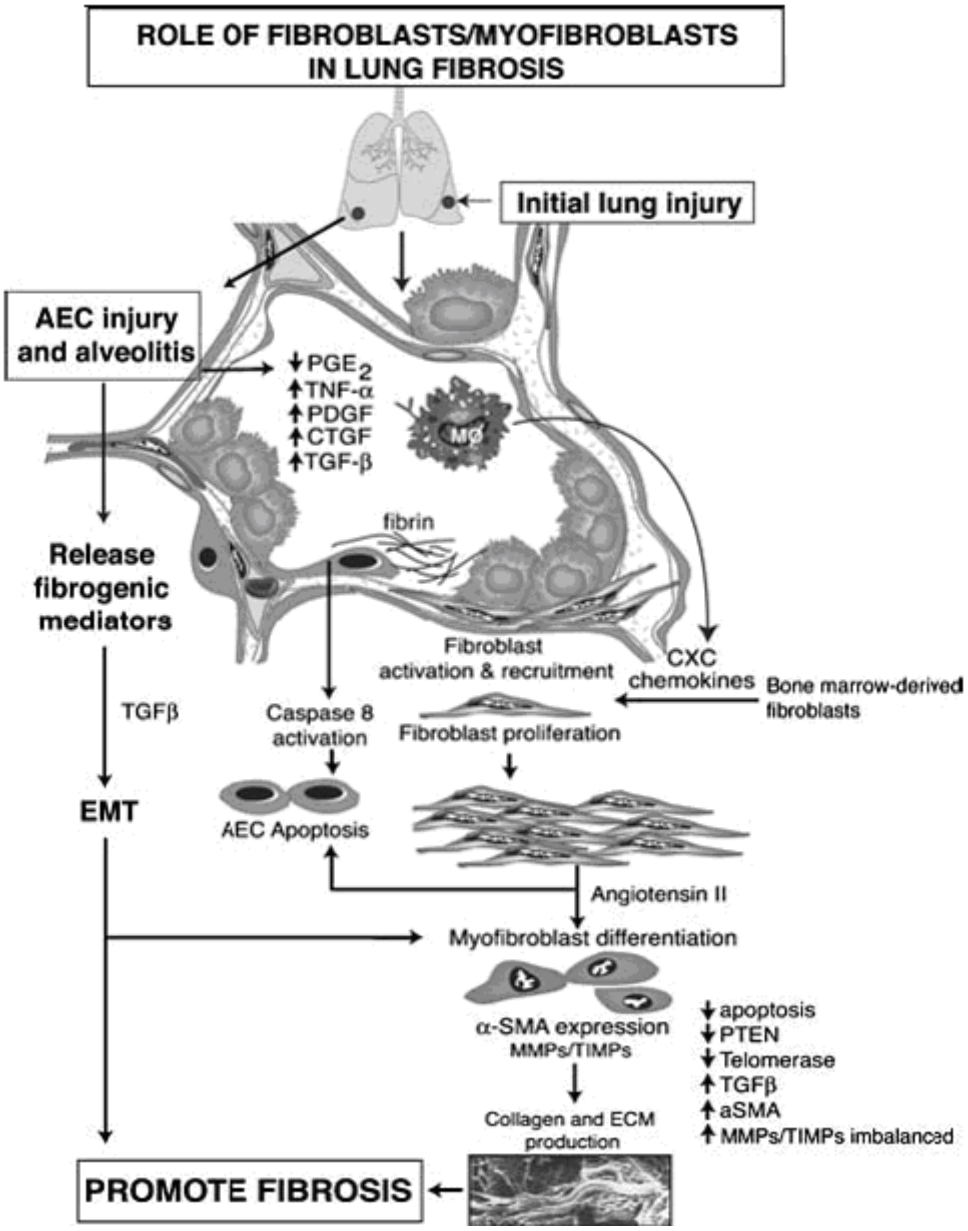
雖然肺纖維化的成因各異，但異常的 ECM 蛋白沉積破壞了正常的組織結構是共同的特徵，而這些 ECM 蛋白即是由佔據纖維化區域的一群 mesenchymal cell 所製造[86]。肌纖維母細胞是一種型態上介於平滑肌細胞和纖維母細胞並大量表現 α-SMA 的 mesenchymal cell，出現在損傷部位負責傷口的癒合，並製造大量的 ECM 蛋白以修補受損區域。正常狀態下，肺纖維母細胞少量地分布在組織間隙，損傷訊號或發炎激素的趨化會使附近或血液中的 fibrocytes 聚集到受損部位[87]，這些具有 proliferation marker-positive nuclei 的纖維母細胞快速增生或分化為具 stress fiber 的肌纖維母細胞(附圖5)。在不正常的組織修復及纖維化區域都有大量的 纖維母細胞和肌纖維母細胞的分布。肺纖維母細胞和肌纖維母細胞分泌 matrix degrading enzymes 以分解受損組織，也同時合成新的 matrix 填補空缺 (附圖6)。

相較於其它肺部細胞，肺纖維母細胞主要功能為損傷後的組織修復工作，因此較能抵抗各種傷害而存活。在正常的傷口癒合過程肌纖維母細胞會逐漸減少，肺纖維化的進展表示正常的傷口癒合動作無法停止，肌纖維母細胞持續地表現。在正常的情況下，肌纖維母細胞是經由 apoptosis 途徑消失[88]，*in vitro* 研究發現在纖維化老鼠的肌纖維母細胞由 IL1β-induced apoptosis 相關的 antiapoptotic protein Bcl-2/proapoptotic protein Bax 表現與正常老鼠的 肌纖維母細胞 不同，顯示它有較強的抗凋亡能力[89]。此外，TGF-β1 可促進肌纖維母細胞分化和增生，在 bleomycin-induced pulmonary fibrosis model 發現阻斷 TGF-β1 signaling 也會減少肌纖維母細胞的數量[90]。

許多 growth factors 和 cytokines 會調控 fibroblasts 的遷移、增生、分化和 ECM 的合成/分解，而這些 fibrogenic cytokines 多半也是 fibroblasts 自己活化後所分泌的。 纖維母細胞和肌纖維母細胞在纖維化的過程中扮演的角色越來越受重視，不僅如同發炎細胞一樣會分泌多種 fibrogenic mediator，而且是 ECM 的主要製造者。有許多的 fibrogenic cytokines，像 TGF-β, PDGF, GM-CSF, FGF, TNF-α, IL-4 和 IL-13，會增加 α-SMA 及 ECM 蛋白的表現，其中 TGF-β 是最重要的一種，而 IFNγ 和 IL-1β 則會減少 α-SMA 及 ECM 蛋白的表現[91-93]。



附圖 5. TGF-β1 induced myofibroblasts differentiation.[94]



附圖 6. Summary of the key pathogenetic mechanisms of fibroblast and myofibroblast in lung injury and fibrosis.[59]

六、胞外間質蛋白與損傷修復

肺組織的 extracellular matrix (ECM) 由 collagen (尤其是 types I, III), elastin, proteoglycan, fibronectin 及少量其它蛋白所組成。肺損傷會破壞 ECM 結構，之後的修復與重建 (repair and remodeling) 工作極為重要，包括巨噬細胞、肺泡和支氣管上皮細胞、內皮細胞和纖維母細胞都能製造 ECM components。為了填補損傷造成的空洞，AEC 快速的增生或分化並重新分布到裸露的區域形成新的障壁；同時也經由活化 anti-fibrinolysis 系統促進血塊形成。在正常的情況下，fibrinolytic 系統也會同時啟動以形成細胞爬行的通道。在肺纖維化時，AEC 高度表現 potent procoagulant factor 和 plasminogen activator inhibitor (PAI-1, PAI-2)，將不利於細胞的爬行及修補功能，不正常的組織修復會使傷口結痂及纖維化。此外，發炎細胞分泌的發炎激素、 chemokines、生長激素和間質分解蛋白酶 (例如 MMPs) 會促使纖維細胞聚集、增生和分化。 AEC 和肌纖維母細胞分泌的 MMP-2 和 MMP-9 (gelatinase A 和 B) 會加劇基底膜 (尤其是 type IV collagen) 的破壞，有利於 fibroblast/肌纖維母細胞 爬行、增生和新產生的 ECM 堆積[95]。在 IPF 病人肺組織可觀察到分布在基底膜損傷區域的肌纖維母細胞 有高度的 MMP-2 和 MMP-9 表現。不正常的組織修復造成纖維化也代表正常肺組織的功能被這些沒有功能的結締組織取代。

基質金屬蛋白酶 Matrix Metalloproteinases (MMPs；zinc dependent matrix degrading proteinases) 為分解 ECM 的一群具有共同基本型態的蛋白水解酶，在人類 MMPs family共有 23 個成員，MMPs 可根據其結構及受質類型被分類。最重要的一類包括: collagenases, gelatinases, stromelysin, matrilysin 和 membrane-anchored MMP [membrane- type (MT)-MMP] [96]。MMPs 會以 inactive proenzymes 被分泌，到了細胞外再被切成活性片

斷，細胞也會分泌 TIMPs1-4 抑制 MMPs 的活性，換言之 MMPs 的活性被 TIMPs 精密地監控著，以維持正常組織的恆定狀態[97]。包括 neutrophils, AMs, AEC 和 肌纖維母細胞 在內的許多細胞分泌不同功能的 MMPs /TIMPs 到細胞外，與 ECM, integrins, growth factors 和 parenchymal cells 密切的合作以維持正常狀態的肺組織結構與功能。肺在急性或慢性發炎發生後，MMPs 的表現增加對修復期的再上皮化(re-epithelialization)來說非常重要。最近的研究發現，在 IPF 病人肺組織 TIMPs 表現高於 MMPs，這使肺組織處於非分解性膠原纖維的微環境。此外， TGF-β 會透過抑制纖維母細胞的 MMP-1/ MMP-2、增加 TIMP 的合成與分泌以減少膠原纖維的分解，所以 MMPs /TIMPs 的調控失衡也是造成組織纖維化的原因之一[96, 98]。

七、TGF-β1 與肺纖維化

1. TGF-β 結構及活化的調控

乙型轉型生長因子(TGF-β) 是一群多功能的細胞激素，至今以有超過40個家族成員被發現[99, 100]，TGF-β superfamily 分為三大類：TGF-β family, bone morphogenetic protein (BMP) family和activin family，它們具有共同的結構、類似的訊息傳遞路徑和重疊的生物活性。在哺乳類生物有三種已知的 TGF-β isoforms (TGF-β1-3)被確認，其中TGF-β1與肺纖維化的關係最為密切。TGF-β1與 latent TGF-β binding proteins (LTBPs)結合成 latent precursors complex 分泌[101]，與 latency-associated protein (LAP)形成大複合物結合於胞外基質[102] (附圖7)，經過活化的 TGF-β1才能產生生理活性。 在體外，latent form TGF-β1 可經加熱酸化處理而被活化；在生物體中至少有三條活化路徑：(1)主要的活化路徑，是透過與 epithelial cell integrin αvβ6 結合造成結構變化而產生活化態的 TGF-β1，作用於鄰近細胞的 TGF-β1 receptors，(附圖8) (2) 與 matrix protein thrombospondin-1 (TSP-1) 結合，(3)

被蛋白酶 (plasmin, MMP2, MMP9, elastase, or cathepsins) 分解成活化態 TGF-β1 (附圖9)[103-105]。TGF-β1 分泌及作用的調節不僅取決於轉錄、轉譯、分泌和活化等環節，還受 TGF-β1 接受體的數量及其他細胞激素的相互作用等複雜因素影響。

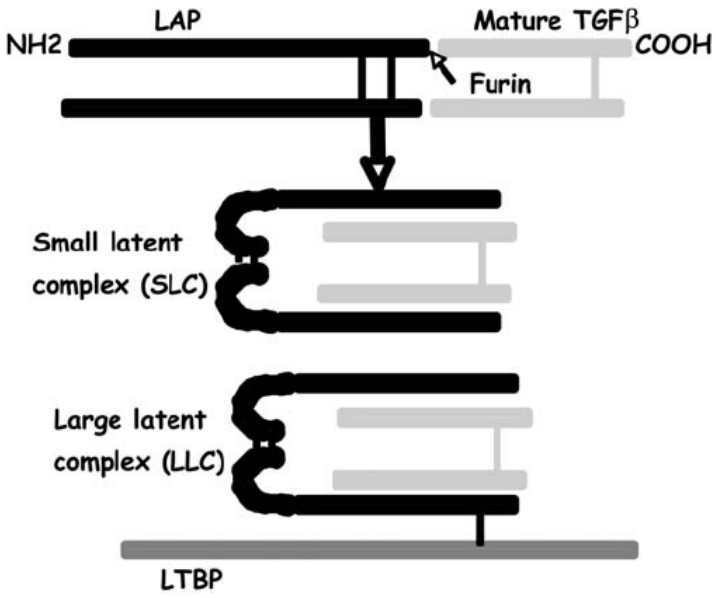
TGF-β1 能發揮各種調節功能有賴於其在細胞膜表面的專一性受體，至今已發現三種與 TGF-β1具高親和性的受體：TβRI、TβRII 和 TβRIII，TGF-β1 必需同時與 TβRI 和 TβRII 結合才能將訊息往下傳遞。TβRI 和 TβRII 均為穿膜的 serine/threonine kinase receptor ，TβRI 又稱為活化素受體激酶 (avtivin receptor like kinases, ALKs) (附圖10)，其位在細胞質內近膜區的 serine/threonine kinase domain 能被 TβRII 磷酸化，TβRII 則具有自我磷酸化的功能。當活化態的 TGF-β1 與標的細胞膜上的 TβRII 結合使其自我磷酸化，接著靠近並活化相鄰的 TβRI。被活化的 receptor complex接著將下游的receptor-regulated Smads (R-Smad; Smad2 and 3 for TGF-β/activin) 在 MH2 domain的C-terminal serine residues SXS (S465/S467) 磷酸化[106]，促使 common mediator Smad (Co-Smad; Smad 4) 與 R-Smad結合成 heterodimer，被活化的 R-Smad/Co-Smad complex 隨即 translocates 到細胞核，與 co-activators 或 co-repressors 結合共同調控下游分子的轉錄表現[107]。此外，inhibitory Smad (I-Smad; Smad6 and 7) 透過分解 TβRI 而減弱 TGF-β1 的訊號傳遞(附圖11)。雖然大多數學者公認 Smads 蛋白是細胞內負責將 TGF-β1 刺激所產生的訊息傳遞至細胞核的傳遞者，但也有其它的傳遞路徑被討論過，例如在 primary intestinal epithelial cells 和 breast cancer cells lines，TGF-β1 會活化 Ras, extracellular signal-regulated kinase 1/2(ERK1/2) 和 c-Jun N-terminal kinase (JNK)；在murine mesengial cells，TGF-β1 會活化 protein kinase A[107]。

2. TGF-β 的生理功能

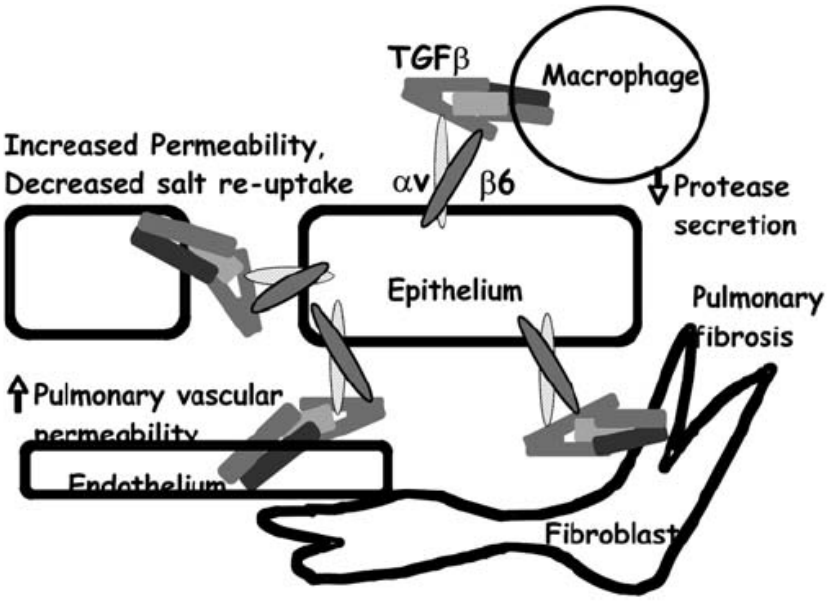
TGF-β 家族成員對許多的組織和器官有著廣泛的生理作用，它們參與細胞各項的活動包括細胞生長、認知、分化、凋亡、胚胎發育期及成熟組織的特化[100, 108]，TGF-β 成員中以 TGF-β1 與組織癒合的關係最為明確，很多不同種類的細胞都能製造 TGF-β1來調控細胞的生長、分化和增加胞外間質蛋白的分泌。在正常的生理環境中，TGF-β1 經由調控細胞週期相關分子的表現而抑制細胞生長，例如：cyclin kinase inhibitors $p^{15INK4b}$, p^{21CIP1}和 p^{27KIP}[109]；此外 TGF-β1 也會經由Smad-dependent 或 Smad- independent pathway (包括MAPK pathway 和 PP2A/p70S6 kinase pathway) 抑制細胞生長[109]。在正常細胞，TGF-β1 通常會透過增加胞外間質分泌及抑制其分解而促進細胞的黏著[99]。TGF-β1 在癌細胞發展的不同時期有不同的調控 [105, 110]，在早期，TGF-β1 扮演抑制者的角色抑制癌細胞分裂；當癌細胞進入生長抑制抵抗期而快速發展時，TGF-β1 則會製造利於癌細胞增生及轉移的微環境，一旦進入惡性期，TGF-β1 就對癌症的發展與轉移產生很大的影響。在許多器官的癌細胞都可發現 TGF-β1 大量的表現，大部分的研究也認為 TGF-β1 透過增加 MMP 和 uPA 的表現促進癌細胞的侵潤。 例如在癌化的上皮細胞發現，TGF-β1 透過活化 Smads [83] 或MAPKs [111] 使 MMP2 和 MMP9 的表現增加；在人類卵巢癌細胞發現，TGF-β1 透過活化 Src 或 Smad 增加 uPA 和 PAI-1 的表現 [112, 113]。

3. TGF-β1 與肺纖維化

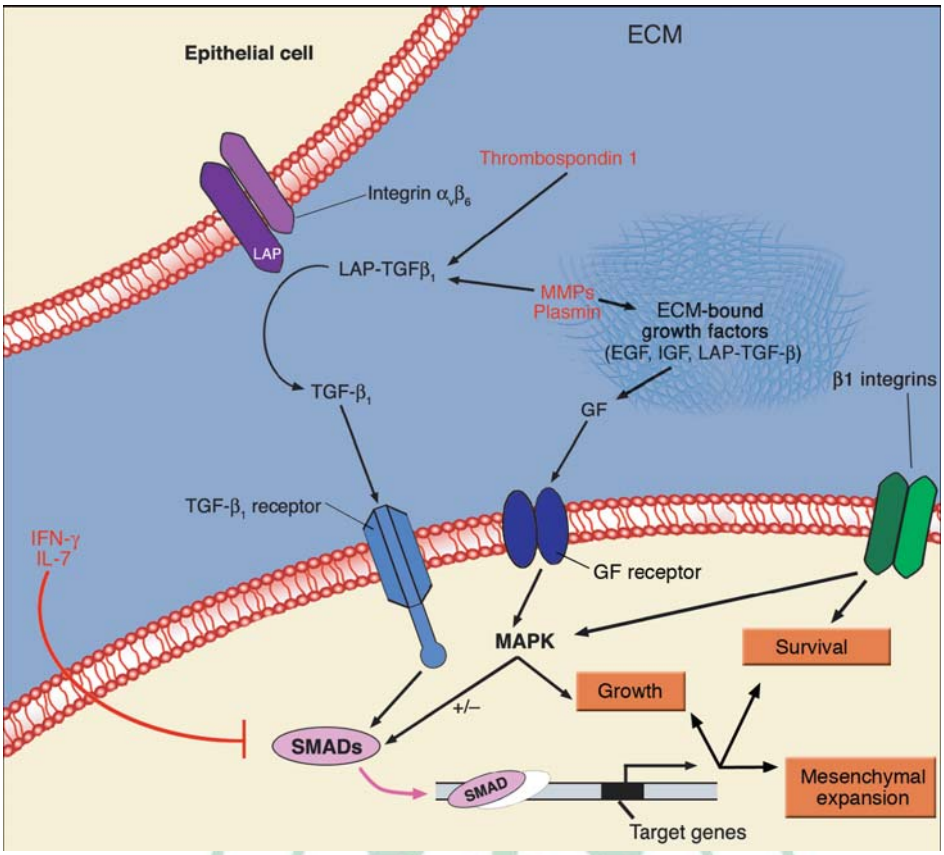
TGF-β1 由血小板、巨噬細胞和內皮細胞等產生，它能促進纖維母細胞趨化、產生膠原 (collagen I, III, IV, V) 和纖維連接蛋白並抑制膠原降解，對器官纖維化有重大的影響[114]。 TGF-β1 刺激周圍細胞分泌 fibrogenic cytokines (例如 TNF-α, PDGF, IL-1β 和 TGF-β1)，TGF-β1 本身可促使肌纖維母細胞分化，並透過 PDGF 刺激纖維母細胞增生；透過 IL-1β 抑制纖維母細胞凋亡。TGF-β1 造成胞外膠原沉積的原因如下：(1)增加纖維母細胞 collagen、fibronectin、proteoglycans 和其它 ECM components 的mRNA表現，(2)抑制 plasminogen activators、 MMPs 和 elastase 的表現並增加 TIMPs、PAI-1 和 PAI-2 的表現以減少新產生的 ECM 降解 [98, 115] 。 從肺纖維化病人及實驗動物的肺組織都發現活動病灶內有有高度 TGF-β1 表現；給予 TGF-β1 抗體可減緩纖維化的程度[116, 117]，且不會引起明顯的免疫干擾[118]。因此，以 TGF-β signaling pathways 為靶點的治療策略將是研發抗纖維化藥物很重要的一個方向 [119]。



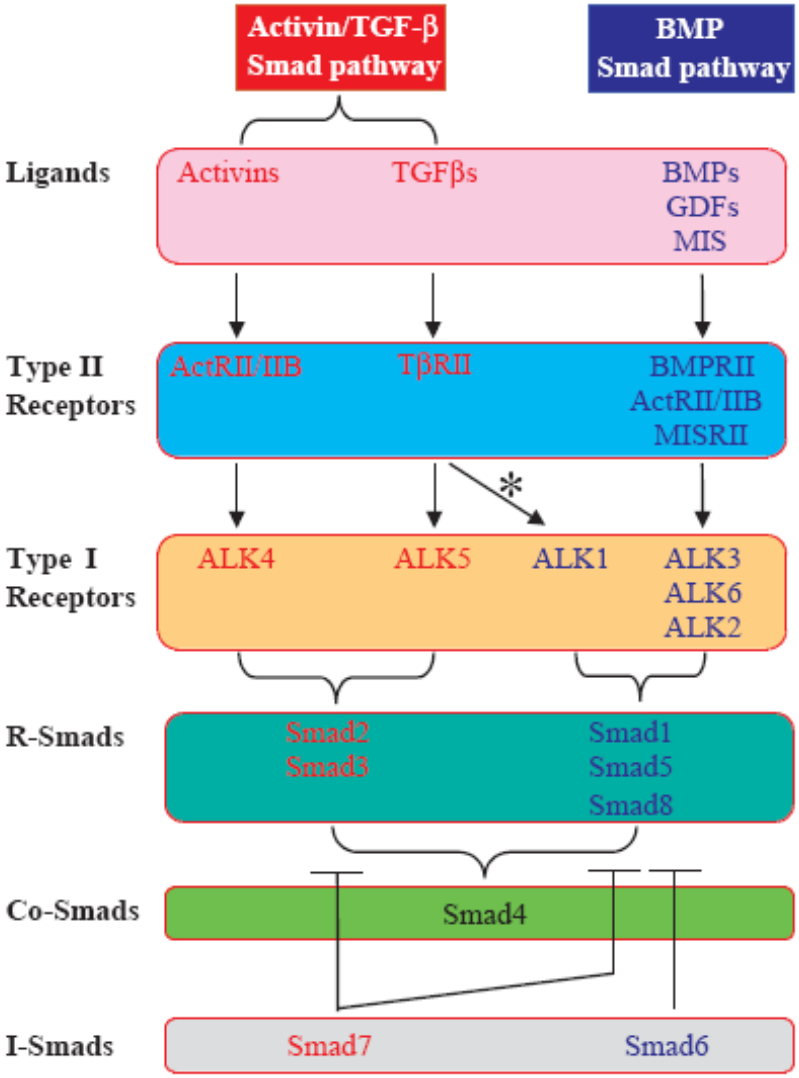
附圖 7. Synthesis and organization of latent complexes of TGF-β. [102]



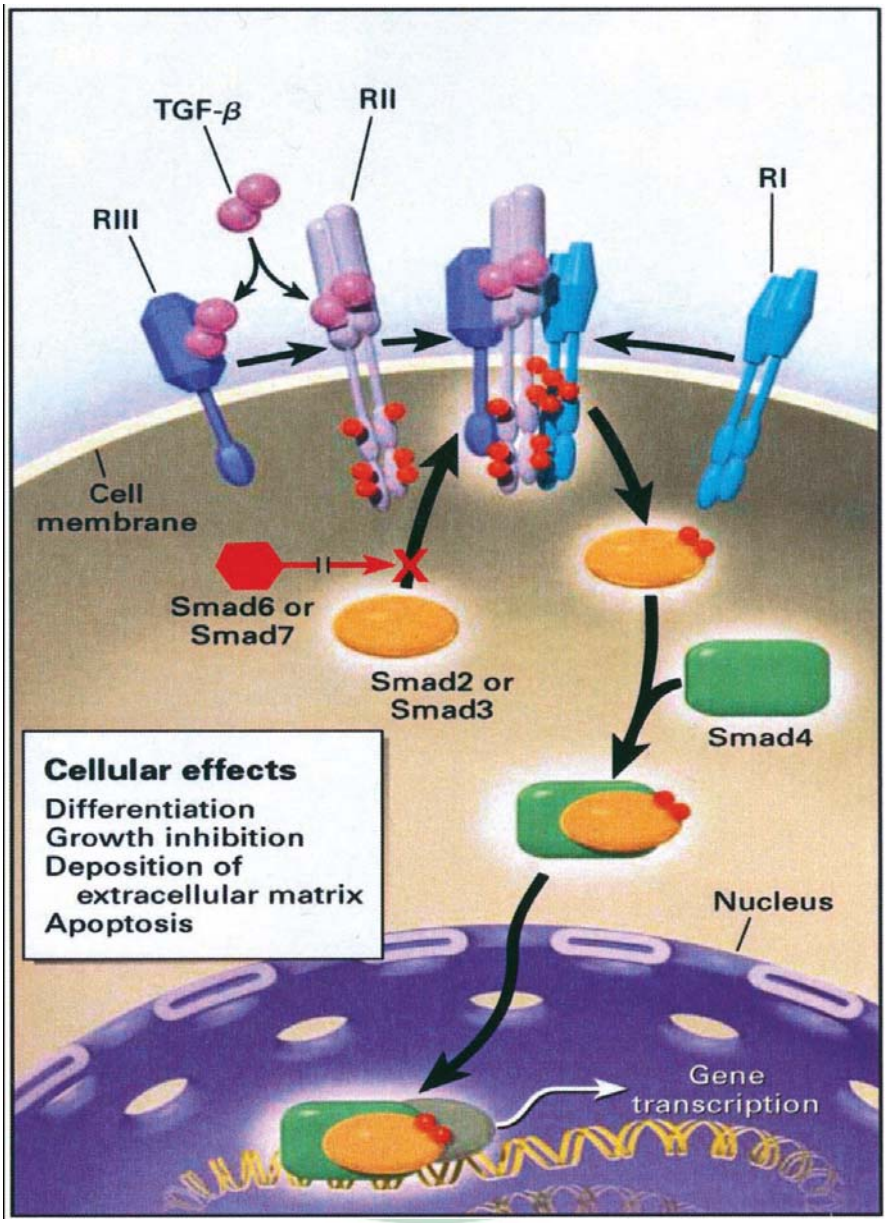
附圖 8. Model of the consequences of αvβ6 integrin-mediated activation of TGF-β by alveolar epithelial cells.[102]



附圖 9. Pre- and postreceptor regulation of TGF-β1 signaling. Latent TGF-β1 can be activated by proteases (such as MMPs, plasmin) and by binding to TSP-1 or the integrin avb6, expressed on epithelial cells.(120)



附圖 10. Signaling specificity in the TGF-β superfamily. Classification of the mammalian Smad signaling cascade into activin/TGF-β and BMP pathways. [121]



附圖 11. Mechanism of signal transduction mediated by TGF-β.[122]

八、 國內外有關肺纖維化治療之研究

1. 中醫對肺纖維化的研究

中醫認為本病屬於肺痹的範疇，“痹”有痹阻之義，本病是正氣本虛反復外感，邪舍於肺，肺氣痹阻而成。肺痹一病，其病名始見於黃帝內經，認為皮痹不愈，反復感邪，內舍於肺，發為肺痹，素問·痹論：“肺痹者，煩滿，喘而嘔。“淫氣喘息，痹聚在肺”。現代中醫對肺痹進行了較為深入的研究，對肺痹的概念、證候特點、病因病機、診斷依據、治療方法進行了較為系統的論述。該病呈慢性進展，臨床表現出急性發作期和緩解期；感染常為急性發作的誘因又是病情加重的條件。急性發作期(肺泡炎期)病程較短，以肺泡炎症滲出為主，中醫辨證多屬實證，以痰、瘀、熱、毒等阻滯肺絡最為常見。在間質纖維化期(慢性遷延期)，肺泡間隔多有纖維結締組織增生和纖維化形成，病程日久，遷延不愈，病機多屬因實致虛，絡虛不榮，虛實夾雜，以氣虛血瘀痰阻之證最為常見，辨證治療常選擇現代藥理中具有明確的逆轉肺纖維化的藥物及具有調節免疫功能的藥物相結合。通肺活血主要應用生黃耆、金銀花、當歸、丹參、鬱金、旋覆花等藥，傳統藥性認為生黃耆“補五臟諸虛”，在這裏還取其“能通調血脈，流行經絡…”的作用。 現代藥理研究還指出生黃耆、當歸皆可調節免疫功能，其中生黃耆、丹參有逆轉肺、肝纖維化的作用。臨床可結合不同患者的病情隨症加減治療。中醫認為分期論治及活血化痰通絡是肺間質纖維化的根本大法，肺絡痹阻是肺間質纖維化的基本病機特點，但在不同時期其病機發展重點則有所區別。 **急性期**：常因外感六淫誘發，以痰、瘀、熱、毒等阻滯肺絡最為多見。因此，解表化痰通絡和清熱利濕解毒、活血化痰通絡為肺纖維化急性期常用之法。 (1) 解表化痰通絡：肺纖維化常因外感六淫誘發、加重和惡化，尤以風寒襲肺、風熱犯肺、風燥傷肺之證最為多見。“絡以辛為泄”，因此若因風寒襲肺誘發者，治以辛溫解表、散寒通絡，

小青龍湯加減，藥如炙麻黃、桂枝、白芍、乾薑、 細辛、五味子、制半夏、厚朴、杏仁、甘草等。若因風熱犯肺誘發者，治以辛涼解表、化痰通絡，銀翹散加減，藥如金銀花、連翹、荊芥、薄荷、牛蒡子、淡豆豉、桃仁、杏仁、漏蘆等。 (2) 清熱利濕解毒，活血化痰通絡：肺纖維化急性期痰濕內阻，鬱而化熱，蘊久成毒，毒瘀阻絡而為肺痹。因此，清熱利濕解毒，活血化痰通絡為肺纖維化急性期常用治法。藥如全栝樓、黃芩、清半夏、當歸、浙貝母、苦參、連翹、郁金、牡丹皮、丹參等。**慢性遷延期**：絡虛不榮，虛實夾雜為慢性遷延期病機特點。“大凡絡虛，通補最宜”（葉天士引臨證指南醫案），故通補兼施，寓通於補為肺纖維化慢性遷延期總的施治原則[123, 124]。 (1) 益氣活血，化痰通絡：肺纖維化反覆發作，遷延不愈，終致氣虛、血瘀、痰阻，本虛標實，虛實夾雜。氣虛血瘀痰阻為肺纖維化慢性遷延期最常見證候之一，故益氣活血、化痰通絡法為肺纖維化慢性遷延期的主要治法。藥如黨參、黃耆、赤芍、川芎、地龍、桂枝、法半夏、旋覆花、皂角刺、白芥子等[125]。(2) 益氣養陰，化瘀解毒通絡：放射性肺炎以及彌漫性間質性肺病長期應用糖皮質激素後易出現氣陰兩虛，瘀毒阻絡證候。因此，益氣養陰，化瘀解毒通絡為其常用治法。藥如太子參、沙參、麥冬、五味子、百合、當歸、丹參、牡丹皮、浙貝母、海蛤殼等[126]。中醫重視整體治療，善於通過調整整個機體功能治療疾病。對於肺纖維化，在採用”活血化瘀”法的同時，兼顧 ”扶正、補虛、健脾、益氣” 和 ”補腎” 等措施，往往能提高抗纖維化的療效。

2. 西醫治療藥物

肺纖維化是一種進行性且不可逆的疾病，至今尚未有任何藥物可有效阻止其病程進展。肺纖維化的傳統治療包括類固醇單獨或併用免疫抑制劑，類固醇在肺泡炎性期可能有效，但在廣泛間質纖維化期則難有改觀[127]。目前關於肺纖維化的治療研究最多的是針對肺泡巨噬細胞所分泌的細胞激素及拮抗劑，如 IL-1、 TNF-α、 TGF-β 和 IFN-γ 等[128, 129]。另外，直接抑制纖維母細胞增生的藥物，如 pirfenidone， 在 bleomycin 引發的肺纖維化實驗中可減輕實驗老鼠的肺纖維化，*in vitro* 實驗中可降低纖維母細胞產生 TGF-β1 並減少細胞外膠原蛋白的沉積[130, 131]。 由於西方醫學對肺纖維化複雜的過程及機轉越來越清楚，也認為不同的病理階段可能需要不同的治療策略，與中醫講求的”分期論治”之治療原則不謀而合。近年來也有許多的中草藥被用在治療肺纖維化的研究，例如，從中藥雷公藤抽取出之成分 PG-490-88 具有免疫抑制的作用，在發炎期或發炎後期治療實驗動物的肺纖維化均有成效，同時有抗發炎及抗纖維化的作用[132]。因此，經由對疾病機轉充分的瞭解將有助於證明傳統中醫藥的有效性。

第四節 人體的免疫系統與發炎反應

一、 先天性免疫

人體的免疫系統依其功能可分成二大類: 先天性免疫 (innate immune response) 和後天性免疫 (acquired immune response)，先天性免疫是保護人體的第一道屏障，它是由非特異性作用來達到防禦各種不同感染性病原的功能。先天性免疫有二道防線，第一道防線由皮膚、黏膜及其分泌物組成，均具有抵制病原體等外來異物侵犯的能力，有助於摧毀一部分病原體。第二道防線則包含了吞噬細胞的吞噬作用以及發炎反應。吞噬細胞可分為單核球 (monocytes)及多形核顆粒球 (polymorphonuclear leukocytes; PMN)。單核球則是指：「專業性」之吞噬細胞－巨噬細胞 (macrophage) 及抗原呈現細胞 (antigen presenting cells; APC); 多形顆粒球則包括嗜中性球 (neutrophils)、嗜鹼性球 (basophils)、嗜酸性球 (eosinophils)、肥大細胞 (mast cell)及血小板 (platelets)。吞噬細胞的功能是藉由 pattern recognition receptors 辨識外來病原體的表面受體作用分子，並釋放出一些發炎反應相關物質直接毒殺外來的病原體,例如:細胞激素、溶酶體、過氧化物 (peroxide) 及氮化物 (NO)，或是引起更多連鎖反應來攻擊病原體，如果說第一道防線還不足以消滅外來病原體時，此時，就會啟動第二道防線：後天性免疫系統，活化 T 細胞和 B 細胞。細胞激素風暴 (cytokine strom) 是當人體受到病毒感染時,免疫系統的 T 細胞會產生細胞激素(cytokine,包括干擾素 IFN-γ 以及腫瘤壞死因子 TNF-α)，使巨噬細胞活化並且具有吞噬外來的病毒的能力。巨噬細胞完成任務之後，又會釋放細胞激素，活化 T 細胞。巨噬細胞與 T 細胞在細胞激素的作用之下，雖然成功抵禦外來的病原體，但是過度的免疫反應也會傷害人體細胞，造成身體發燒、發炎、血管擴張、組織細胞急劇的壞死、血球總數低下與血球吞噬現象等症狀。此時人體自然的抗病毒系統不僅不能保護人體免受病毒的侵襲，反而還成為致死的因素。

二、肺泡巨噬細胞

為了防止入侵者的傷害，肺部有一套嚴密的防禦機制以排除吸入的粒子和微生物體。以結構上來區分，肺部的防禦機制可分為上呼吸道區及肺泡部分，上呼吸道及主要支氣管透過機械障壁、咳嗽反射、纖毛運動和分泌免疫球蛋白 (Ig A) 等防護保護肺部，黏膜層之下佈滿 dendritic cells 抓住任何侵入生物體並且將它們帶到附近的淋巴結。逃避過氣道防禦機制病原菌進入到肺泡時，啟動宿主細胞性免疫反應，病原菌會被充滿在 IgG、補體、surfactant 和 fibronectin 環境下的肺泡巨噬細胞所吞噬，並且隨時可啟動嗜中性球及淋巴球的聚集。從 bronchoalveolar 灌洗出的細胞中，肺泡巨噬細胞占了85%，他們來自於骨髓母細胞分化成周邊單核球，循環到不同組織後特化成組織巨噬細胞。肺泡廣大的表面積與外界空氣接觸時也增加了感染的機會，相較於其它組織的巨噬細胞，肺泡巨噬細胞具有較強的吞噬能力及反應性[133]。肺泡巨噬細胞通常是在下呼吸道內唯一的吞噬細胞，除了數目相當多之外，也具有廣泛的生理功能，它能夠自由移動，吞噬進入肺部的微小顆粒、侵入的微生物和死細胞的碎片，也能作為抗原呈現細胞來活化其他免疫細胞，並且防止一些不正常細胞生長和修補肺部的實質組織等，可說是肺部免疫反應的第一線防禦細胞，不單具有先天免疫功能，同時也可以啟動後天性免疫[134]。

肺泡巨噬細胞在活化後透過分泌酵素、補體、發炎媒介物、細胞激素等物質引發一連串的發炎反應。發炎反應起始於 IL-1α、IL-1β 或 TNF-α 的釋放並刺激肺泡周圍釋放chemokines (IL-8, MIP-1α/β, RANTES or MCP-1/3)、生長激素 (GM-CSF, G-CSF or M-CSF)，促使內皮細胞及上皮細胞黏附分子表現，發炎激素活化了臨近的細胞並吸引血液中的免疫細胞至發炎部位，同時活化 cyclooxygenase 將 arachidonic acid 轉變成脂質分子

(thromboxane A_2, LTB_4, 5-HETE, PGE_2 和 PGD_2)，增大血管通透性及誘發嗜中性白血球的趨化反應 (chemotaxis)。導致嗜中性白血球和血漿蛋白如抗體 (antibody) 及補體 (complement) 等滲出血管到達受感染的地方。發炎細胞也會分泌一些抑制分子以調控發炎反應，例如 IL-1 receptor antagonist (IL-1Ra) 或 TNF-soluble receptors (TNFsR55 or TNFsR75)，巨噬細胞也會分泌 IL-10 以抑制 IL-1 與 TNF 的釋放，避免產生過度的發炎反應。巨噬細胞製造 lysozyme 可將吞入的病原菌分解。 Defensins是一帶正電蛋白質，可殺死大部分的格蘭氏陽性菌、格蘭氏陰性菌和黴菌。巨噬細胞分泌的活性氧化物 (Reactive oxygen intermediates; superoxide anion, hydrogen peroxide, hydroxyl radicals) 或活性氮化物 (Reactive nitrogen intermediates ; nitric oxide, nitrites or nitrates) 也參與了微生物甚至是癌細胞的毒殺作用[135] (附表2)。

在細胞激素的調控下，肺泡巨噬細胞分泌 metalloelastases, collagenase, metalloproteases (MMP1, MMP9) , TIMPs 和 urokinase 等細胞外間質蛋白分解或抑制分解的酵素以參與肺部損傷後的重建與修復，同時也分泌乙型轉型生長因子(TGF-β) 有助傷口癒合。

附表 2. Macrophage-secretory products.[135]

Cytokines IL-1β/IL-1ra TNF-α/TNFsRs IL-6 IL-10 IL-12	Defensins and lysozyme Reactive oxygen intermediates Reactive nitrogen intermediates Enzymes Metalloprotease/TIMPs Macrophage metalloelectase Urokinase Acid hydrolases
Chemokines IL-8 MIP-1α/β RANTES MCP-1-MCP-3	Bioactive lipids Cyclooxygenase Products of arachidonate
Complement proteins Most components of complement pathways and the inhibitor C1q	IL-1ra = IL-1 receptor antagonist.

三、LPS與 Toll-like receptors

巨噬細胞的免疫調體作用 (opsonization) 是促進病原菌吞噬作用即引發免疫反應最主要的機轉，三種不同功能的接受器： Fc receptors, complement receptors 和 lectin receptors 扮演著重要的角色。(1) immunoglobulin G (Ig G) receptors： FcγRI , FcγRII, FcγRIII， (2)肺泡巨噬細胞有三種 complement receptors 其中最重要的是CR1 (CD35) receptor，它對 C3b 具高度親和性而與 iC3b 及 C4b 為低親和性；CR3 (MAC-1, α-chain CD11b, β-chain CD18) 它對 iC3b 具高度親和性而與 C3dg 及 C3d 為低親和性；CR4 (α-chain CD11c, β-chain CD18) 與 iC3b 結合。 (3)在肺泡巨噬細胞為數眾多的 phagocytosis-associated receptors (lectin-binding receptors)[66] (附表3)。除此之外還有接受 autocrine 活化的cytokine receptors，例如 interleukin-1 receptors (IL-1R) 和 TNFR。

附表 3. Ligands recognized by alveolar macrophages *via* receptors.[66]

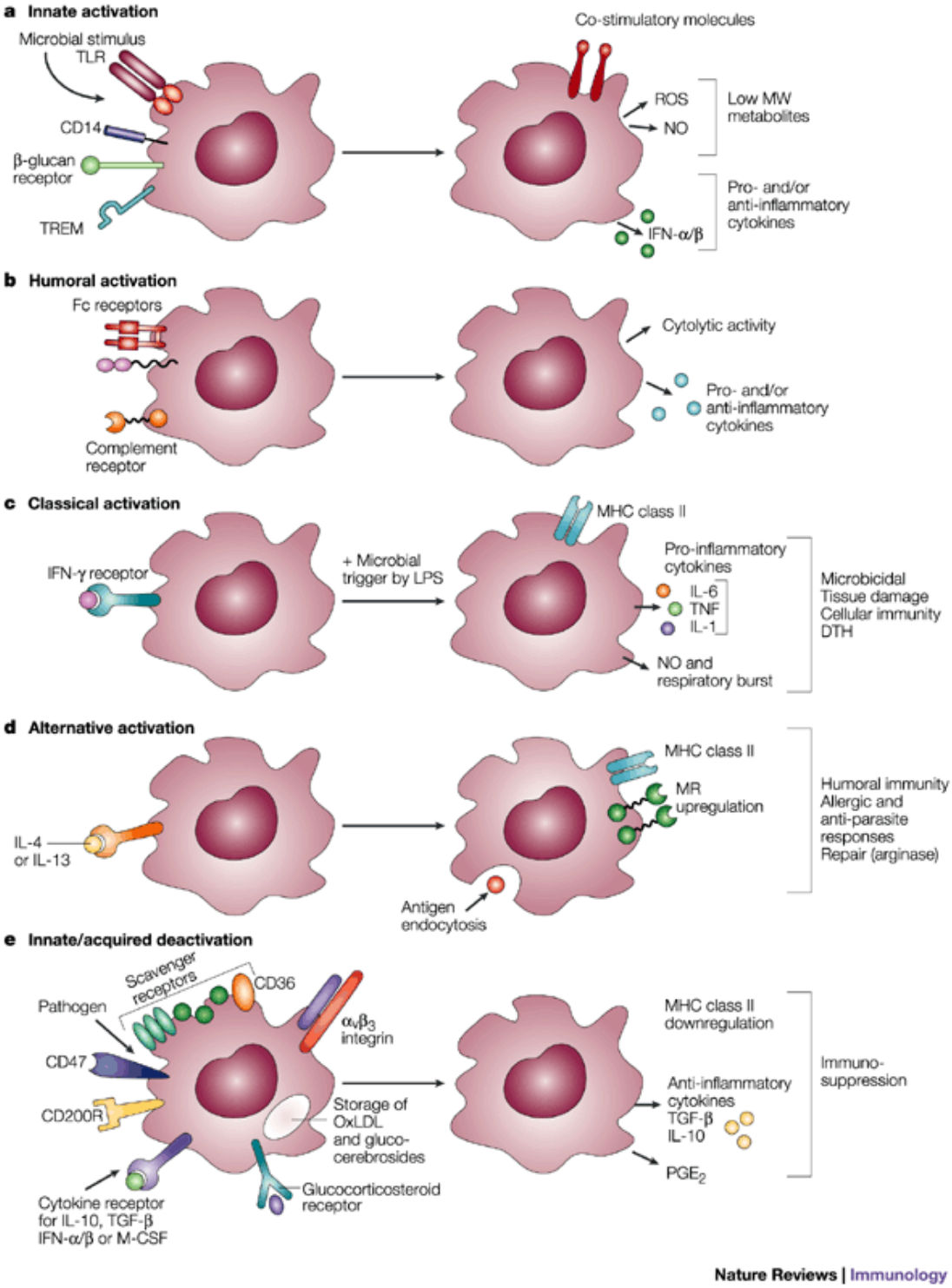
Immunoglobulins	**Complement**
IGg1, IgG2a (murine)	C3b, iC3b, C4b, C3d, C5a
IgG2b, IgG3 (murine)	
IgG1, IgG3 monomers (Human)	**Lipoproteins**
IgG complexes (human)	Low density lipoprotein
IgE, IgA (murine and human)	β-very low lipoprotein
Proteins	**Lectins with specificity for**
Fibronectin, fibrin	α-linked galactose residues
Lactoferrin, transferrin	N-acetylgalactosamine residues
GM-CSF, CSF-1	N-acetylglucosamine residues
Interferon-γ, IL-4, IL-1Ra	α-linked fucose residues
IL-2, insulin	Mannose residues
	N-acetylneuramine residues
Surface markers	
Class II molecules, CD11a, CD11b, CD11c, CD14, CD18, CD54	
Molecules recognized by monoclonal antibodies:	
25F9 (mature macrophages), 27E 10 (inflammatory macrophages), Ki M2, Ki M8 (mature macrophages), RM31 (inflammatory macrophages), RFD1, RFD7, RFD9	

Ig: immunoglobulin; GM-CSF: granulocyte-macrophage colony-stimulating factor; IL: interleukin.

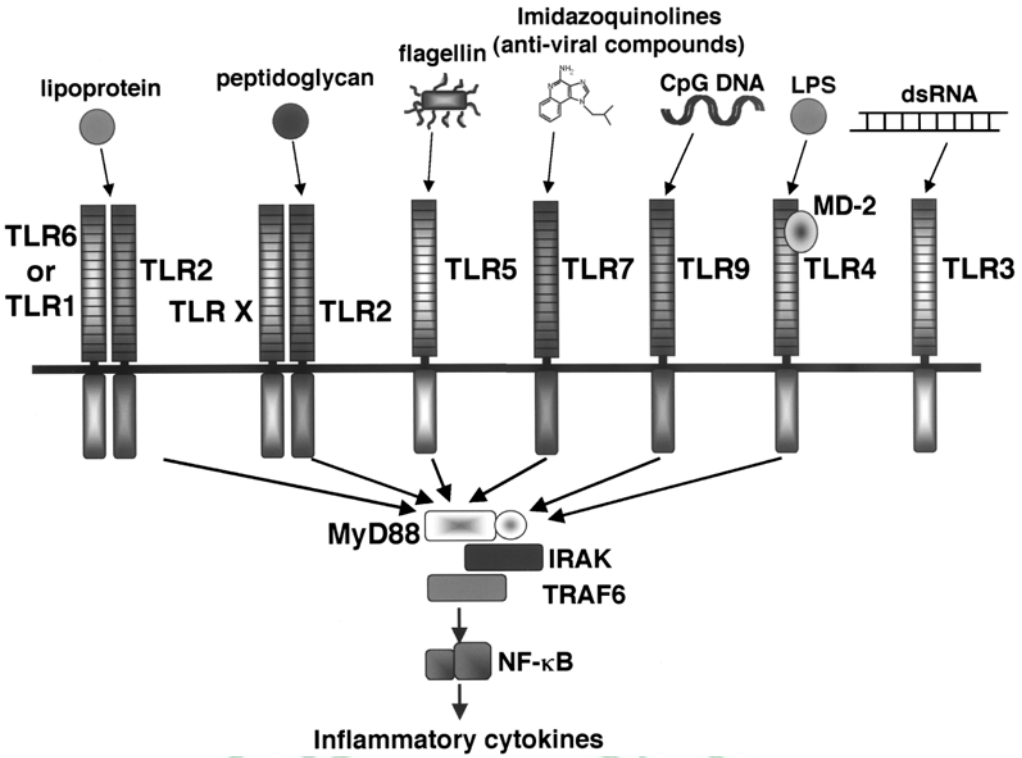
巨噬細胞靠著細胞表面的 lectin-binding receptor 來辨識病原菌。其中包含了 macrophage mannose receptor, macrophage scavenger receptor 及 CD14。Macrophage mannose receptor 利用其結構上的 cysteine rich domain 與病原菌的mannosylated antigens 結合，將它帶往次級淋巴器官處。Macrophage scavenger receptors 可與帶電荷的 ligand 結合，並且會修飾細胞內的蛋白質與低密度脂蛋白 (low density lipoprotein)。所有細菌細胞壁主要成份為肽聚醣 (peptidoglycan)，格蘭氏陽性菌外膜帶有磷壁酸 (teichoic acid)，格蘭氏陰性菌外膜帶有脂蛋白(lipoprotein) 和脂多醣類 (LPS)。吞噬細胞表面具有的分子圖案辨識受體 (pattern recognition receptor)，可以和病原菌上不同的分子圖案結合，例如 CD14 可與細菌的脂多醣 LPS 結合。當這些細胞表面的 receptor 辨識到病原菌時，巨噬細胞會產生吞噬作用，將病原菌包圍起來形成 phagosome，接著與細胞內的 lysosomes 融合在一

起形成 phagolysosome，存在 lysosomes 內的抗微生物蛋白質及酵素會將病原菌分解。另一方面，巨噬細胞受到不同的刺激 (內生性或細胞外來) 會造成不同訊息傳遞分子的活化(附圖12)。

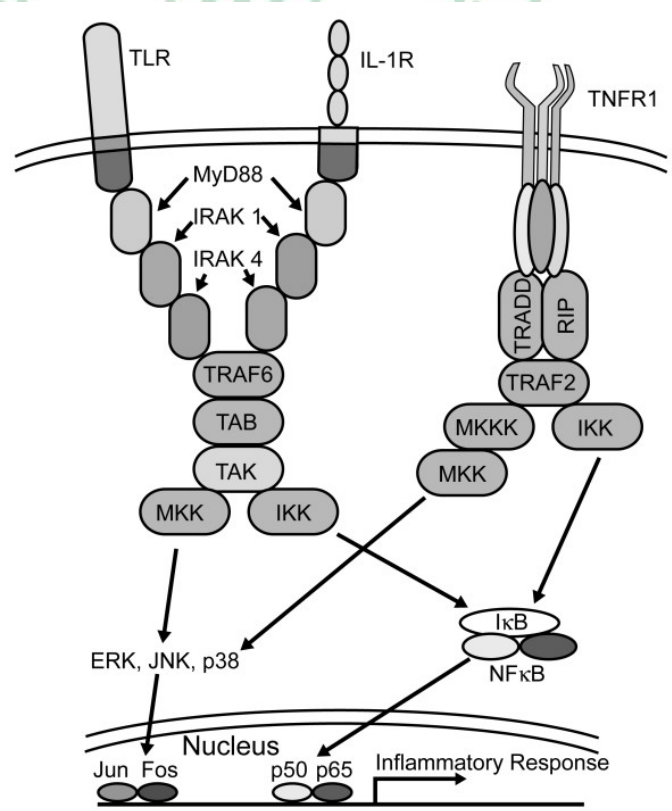
LPS 會與LPS binding protein (LBP) 形成複合物。LBP 在人體血液中的正常濃度為 3-10 μg/ml。當體內經過急性反應 (aute phase response) 後，血液中的 LBP 濃度會上升，LBP-opsonized particles 會與巨噬細胞表面的 CD14 結合。在哺乳類細胞中，LPS 也會被一種 toll-like receptor 4 (TLR4) 辨認，將訊息往下傳遞啟動共同調適蛋白 MyD88 和 IRAK (IL-1R-associated kinase)，接著活化 NF-κB, AP-1與 IRF3，啟動許多發炎相關基因的轉錄，引發後續的免疫反應。 TLR4 會與一胞外附屬蛋白 MD-2 在巨噬細胞的表面形成複合物，此複合物具有 LPS signaling receptor 的功能。TLRs 是屬於穿膜蛋白 (transmemberane proteins)，目前在人類發現有 10 種 TLRs 分子，這些分子都有一個共同的細胞膜外 leucin rich domin 和細胞內 domin，每一種 TLR 與特異的 ligan 結合會誘發特定的基因表現，因此宿主對於不同的感染原才能產生適當的反應[136] (附圖13)。 Katsuhisa Oshikawa 等人的研究指出肺泡巨噬細胞在 LPS, TNF-α 和 IL-1β 的刺激下 TLR2 的表現會明顯增加而 TLR4 則無變化，推測 TLR2 的表現會使肺泡巨噬細胞與更多的 TLR2 ligands 結合，加速肺部對細菌性感染時的先天性免疫反應。 TLRs 的活化可以引起一個共同的訊息傳遞路徑 (common signaling pathway)以活化蛋白激酶 (protein kinase)，及轉錄因子 nuclear factor-κB (NF-κB) (附圖14)、activating protein 1 (AP-1)等，誘發許多發炎媒介物的基因表現，包含TNF-α、IL-1 及 IL-6、granulocyte colony stimulating factor (G-CSF)、GM-CSF、M-CSF、IL-8、monocyte chemotactic protein 1 (MCP-1) 及 iNOS 等[137] (附圖15)。



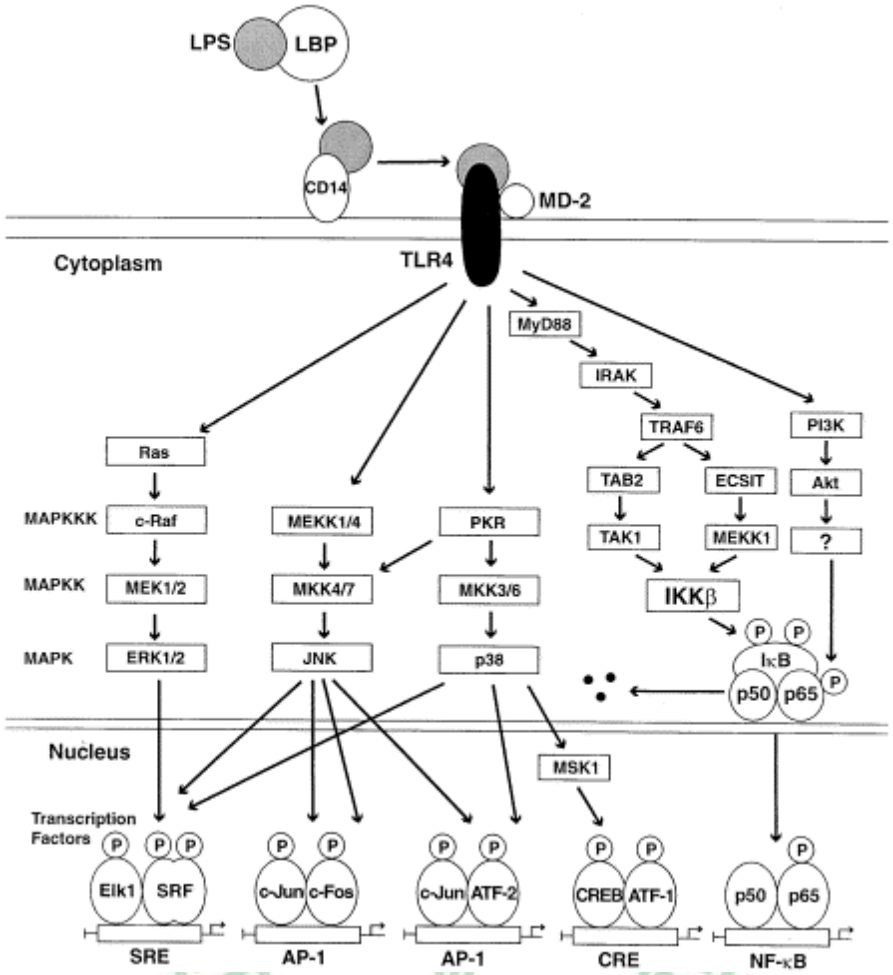
附圖 12. Innate and acquired immune activation of macrophages.[134]



附圖 13. TLR and their ligands.[138]



附圖 14. TLR signaling pathway.[139]



附圖 15. LPS stimulation of monocytes activates signaling pathway.[137]

四、巨噬細胞所釋放的細胞激素

1. TNF-α

1985 年，L. Old's 在動物體內 (*in vivo*)及體外 (*in vitro*)的實驗都發現，當老鼠經過 LPS 的注射後，血液中產生的一種蛋白質會造成某些老鼠的腫瘤壞死，因此開啟了腫瘤壞死因子的研究。後來的科學家 O'Malley 等人証實 LPS 對於壞死腫瘤的能力不是直接的，而是血清中的某種被誘導出的物質所造成的，隨後Carswell等人將其命名為腫瘤壞死因子[140]。體內大部分的細胞都會產生 TNF-α，如巨噬細胞 (macrophage)、淋巴球

(lymphocyte)、肥胖細胞 (mast cell)、多型核白血球(PMN) 等，其中又以巨噬細胞為最大宗的來源[141]。TNF-α 是一種很重要的促發炎激素(proinflammatory cytokine)，許多因素都可誘發細胞產生 TNF-α，像是寄生蟲、細菌、病毒或腫瘤細胞產生之外源性或內源性物質，都會活化細胞產生 TNF-α 來引發局部或全身性的發炎反應。在急性發炎期，TNF-α 能增加血管內皮細胞的附著因子，進而吸引嗜中性球和巨噬細胞到被感染部位，也能促進吞噬作用和清除病原菌及細胞碎片，TNF-α 也會透過活化其他細胞釋放更多的TNF-α 和 IL-1, PGE_2, NO 等物質以放大或延長發炎反應。正常情況下在局部傷害反應過後，TNF-α 的濃度會降低以限制發炎反應。在急性發炎期，局部性產生的 TNF-α 是有利於宿主，然而系統性的過度表現可能會造成全身性的水腫 (systemic capilliary leaking syndrome)、低血壓、敗血症性休克(sepsis) 及多重器官衰竭，最後死亡；而持續性的表現，則對於局部的組織造成慢性發炎，導致其他疾病，像是器官纖維化的發展。TNF-α 的另一功能是經由 Fas ligand 參與細胞凋亡 (apoptosis) 的訊息傳遞。

在許多肺部疾病的研究中證實 TNF-α 在病理過程中扮演著重要的角色。例如: interleukin (IL)-1β, IL-2, IL-3, IL-4, IL-5, granulocyte macrophage colony stimulating factor (GM-CSF), interferon-γ (IFN-γ), TNF-α 等，都參與了氣喘的發炎反應。其中，TNF-α 是一種促發炎細胞激素，同時也會促使相關的附著分子 (adhesion molecule)，如：E-selectin, vascular cell adhesion molecule-1 (VCAM-1) 以及 intercellular adhesion molecule-1 (ICAM-1) 的表現，對嗜中性白血球及嗜酸性白血球這兩種與發炎反應有關的細胞而言，也是一種趨化性的細胞激素 (chemotactic cytokine)。 1997 年 Olivenstein R 等人在動物實驗的模式中發現，經由 ovalbumin 致敏 (OVA-sensitized) 的老鼠，在其支氣管肺泡灌洗液 (bronchoalveolar lavage

fluid, BALF) 中，TNFα 濃度的上升會刺激平滑肌細胞分泌 endothelin-1 (ET-1)，進而使得纖維母細胞中 GM-CSF mRNA 表現增加，促進肌纖維母細胞增生而導致氣道壁纖維化[142]。在肺纖維化病變患者 BALF 中可發現大量的 neutrophils 和 eosinophils，這些會分泌 proteases 和 oxidants 破壞肺實質組織的白血球細胞，主要是受到肺泡巨噬細胞分泌的細胞激素 (IL-1 和 TNF-α) 的趨化作用而聚集在肺組織[143]。 發炎細胞與肺實質細胞之間的多重交互作用也是造成纖維化病變的原因。

2. IL-6

如同 TNF-α，IL-6 是發炎反應的一個關鍵的仲介因子，它是一種多功能的 (pleiotropic) 細胞激素，其功能包括調節人體的免疫反應、造血作用、急性期蛋白的合成及發炎反應[144]。 IL-6 也能夠促進血小板的增生、活化及促進凝固作用。活化的吞噬細胞、T 細胞、B 細胞、纖維母細胞、角質細胞、內皮細胞及神經膠細胞等細胞都可分泌 IL-6 [145]。其它的細胞激素，如：淋巴毒素 (lymphotoxin)、IL-1、TNF-α、血小板生長因子 (platelet-derived growth factor；PDGF)、血小板活化因子(platelet-activating factor；PAF) 和顆粒球抑制蛋白 (granulocyte-inhibitory protein) 等也會誘導 IL-6 的合成[146, 147]。除此之外，被病毒或細菌感染的細胞、受傷害的細胞及腫瘤細胞也會分泌 IL-6。過量的 IL-6 表現通常和多種免疫功能及發炎相關疾病有關，例如：風濕性關節炎 rheumatoid arthritis (RA)病人關節液中的 IL-6 濃度約為血清中的 30-1000 倍[148]，而血液循環中IL-6 濃度的升高通常可作為敗血性休克病患癒後的一項指標。

3. IL-10

Mosmann 等人於 1989 年發現一種由 T helper 2 (Th2) 細胞所分泌的新型細胞激素，具有抑制 T helper 1(Th1) 細胞產生IFN-γ 的功能，稱為 cytokine synthesis inhibiting factor (CSIF)，這個具有強力免疫調節功能的細胞激素就是 interleukin-10 (IL-10)[149]。體內許多細胞都會產生 IL-10，最主要還是以巨噬細胞及 Th2 細胞為主。一般而言 IL-10 可藉由減少顆粒性白血球的數量及發炎性 cytokines 及 chemokines 的產生以達到免疫抑制的作用。IL-10 抑制包括樹狀細胞在內的抗原呈現細胞 (APC) 活化及 MHC class II 的表現，並透過抑制發炎反應時 NF-κB 的活化，減少吞噬細胞產生 TNF-α、IL-6、IL-8、IL-1β和 IL-12等促發炎細胞激素 (proinflammatory cytokines)。在許多發炎反應、自體免疫實驗的動物模式中，例如：胰臟炎 (pancreatitis)、自體免疫腦炎(autoimmune encephalomyelitis)、糖尿病 (diabetes mellitus)、類風濕性關節炎(rheumatoid arthritis)、敗血性休克 (septic shock)和器官移植 (organ transplantation)等，給予 IL-10 有顯著療效[150]。IL-10基因剔除小鼠很容易發生致死腸道發炎，補充 IL-10 可以減少致死率[151]。在肺部發炎的研究發現 IL-10 可抑制肺泡巨噬細胞製造 TNF-α 與 IL-1β，因此可減少免疫複合體造成的肺損傷，也可以減少對白血球的趨化作用。K Nakagome,等人在 bleomycin 致老鼠肺纖維化實驗中發現，給予 IL-10 基因治療可減少膠原蛋白沉積及 αvβ6 integrin 表現而改善肺纖維化，其機轉可能是透過IL-10 對 TGF-β 的抑制作用[152]。近年來的研究指出 IL-10 會透過化活STAT3 以增加 soluble type II tumor necrosis factor receptor (TNFR) 和 IL-1 receptor antagonist (IL-1Ra) 的表現，也是抗發炎機轉之一[153]。

4. IL-1 和 IL-1Ra

IL-1基因家族可分為幾類: 二種致效劑 (IL-1α, IL-1β)、一種拮抗劑 (IL-1Ra) 和二種接受器 (IL-1RI, IL-1RII)[154]。 IL-1是當細胞受到傷害、感染或抗原刺激時所產生的一種常見的細胞激素。主要的分泌以巨噬細胞最多，其他包括內皮細胞、纖維母細胞與 B 細胞等。 IL-1主要有兩種形式：IL-1α 及 IL-1β，二者都會活化 T 細胞與 B 細胞，並產生發炎反應。IL-1會加速肝臟急性期蛋白 (acute phase protein) 的製造。幾乎全身所有的細胞都有 IL-1 的接受器，其對免疫細胞的作用與 TNF-α 類似。如:活化顆粒細胞 (granulocyte) 的代謝作用，並產生趨化作用 (chemotaxis) 移動到發炎部位；增加自然殺手細胞 (natural Killer cells, NK) 的毒殺能力；促使內皮細胞產生粘附因子 (adhesion molecules)，並增加其通透性，以利於免疫細胞的進出；活化巨噬細胞並加強其毒殺細胞的活性，增加其趨化作用；增強 T 輔助細胞的增生，與刺激其分泌細胞激素。

IL-1Ra 屬於 IL-1 家族之一，它的基因位置與 IL-1 非常接近，同樣位於第二對染色體上，所產生的蛋白主要有兩類型，第一種是所謂細胞內的 IL-1Ra (intracellular IL-1Ra；icIL-1Ra)，主要產生 icIL-1Ra 的細胞為角質細胞與上皮細胞，另外像一些活化後的單核細胞與巨噬細胞也可以產生此類蛋白質[155]。 icIL-1Ra 可以直接在細胞內就調控了 IL-1 的生成，並且擔任組織遭破壞後第一線消炎反應的功能。第二種則是所謂分泌性的第 IL-1Ra (secreted IL-1Ra; sIL-1Ra)，此種 IL-1Ra 的基因上多了一段可以合成訊息胜肽的序列，所以在蛋白質合成後，可被運送到細胞外，主要產生此類蛋白的細胞是週邊血液細胞與上皮細胞。它們被認為是扮演體內發炎反應負回饋作用中最重要的調節因子。體內一些可以促進發炎反應的細胞激素，例如：IL-1、IL-6、IL-8、IFN-γ 與 TNF-α 等都可以刺激 sIL-1Ra 的生成，使進行中的發炎反應得到負向的調控[156]。 維持體內 IL-1 和 IL-1Ra 之間的平衡是各個器官組織執行正常生理功能所必需。現今已有非常多利

用注射 IL-1Ra 蛋白或基因療法治療發炎疾病的例子，像敗血病、慢性風濕性關節炎、外科手術傷口、急性發炎與紅斑性狼瘡等，幾乎所有與發炎反應有關的疾病都可以經由透過 IL-1Ra 調控 IL-1 來達到治療的效果，所以研究如何提升免疫細胞製造 IL-1Ra 來抗發炎是一個非常有潛力的方向。

5. PGE_2 與 NO

人類和其它動物的肺泡巨噬細胞都有二種花生四烯酸 (arachidonic acid; AA) 的代謝途徑：cyclooxygenase pathway (thromboxanes and prostaglandins) 和 lipoxygenase pathway (leukotrienes and hydroxyl eicosatetraenoic acids HETEs)，這些代謝產物都與發炎反應的調控有關[157]。Cyclooxygenase 產物有 TxA_2, PGE_2, PGD_2 和 PGF_2；lipoxygenase產物有 LTB_4 和 5-HETE。其中以 TxA_2 的量最多(ng/ml)，高於第二大量的 LTB_4 達 5 倍之多。目前已知 cyclooxygenase 有兩種 isoforms：分別是COX-1 和 COX-2，COX-1在組織中是屬於持續性 (constitutive) 表現，主要作用於維持體內恆定 (homeostatis) 反應，例如分泌黏液 (mucus secretion)；COX-2 則是一種誘導型 (inducible) 酵素，主要參與調控發炎反應，當前列腺素被製造之後會快速地從細胞中釋放，透過和 plasma membrane 上特定高親和力的接受器 (receptor) 結合而作用在鄰近區域。肺泡巨噬細胞會受 IL-1β、TNF-α、cross-linking of Fc receptors 和 LPS 等發炎刺激物的誘發活化 COX-2 而產生 PGE_2；而 silica 的刺激則是活化 lipoxygenase[158]。

一氧化氮 (NO) 是一個作用短暫的雙原子 (diatomic) 分子，許多不同的細胞會釋出一氧化氮。一氧化氮和它的代謝物在調節許多生理功能及病理狀態中扮演著重要的角色，例如產生宿主的防禦系統 (抗微生物及抗腫瘤的功能)、神經的傳導、神經毒性及血管擴張作用[159, 160]。在內皮細胞所產生的一氧化氮又稱為 endothelium-derived relaxing factor，具有調節血管擴

張的作用。一氧化氮是細胞利用 L-arginine 經由一氧化氮合成酶 (nitric oxide synthase, NOS) 的作用產生。一氧化氮合成酶可分為三類，即神經型 (neuronal) 一氧化氮合成酶 (nNOS, type I NOS, NOS-1 或 NOS-1)、內皮細胞 (endothelial) 一氧化氮合成酶 (eNOS, type II NOS, NOS-II 或 NOS-2)、誘發型 (inducible) 一氧化氮合成酶 (iNOS, type III NOS, NOS-III 或 NOS-3)[161]。它們是從不同的基因被表現出來，坐位在不同的 chromosomal location ，在不同的細胞展現不同的調節作用。iNOS 在正常狀態下是不存在於大多數種類的細胞中。當細胞受到葛蘭氏陰性細菌的 LPS 或 cytokines 刺激，會誘發細胞表現出 iNOS[162]。 iNOS是發炎反應和病原菌清除最重要的一氧化氮來源，研究 iNOS knockout 老鼠可以清楚地證明在免疫反應中，一氧化氮對於特定微生物的防禦扮演重要的角色。在一氧化氮和氣喘之間的研究發現，氣喘病患呼吸道上皮細胞 iNOS 表現增加[163]，且偵測呼出的氣體中也有高量的一氧化氮[164]，但對於病人呼吸道中一氧化氮產生增加和氣喘致病機制之間的關聯仍不明瞭。當細胞持續增加 iNOS 的表現，並製造出大量的一氧化氮時，會引起細胞毒性及低血壓休克等現象[165, 166]。因此抑制 iNOS 生成一氧化氮，可作為開發抗發炎藥物的一個重要的治療標的。

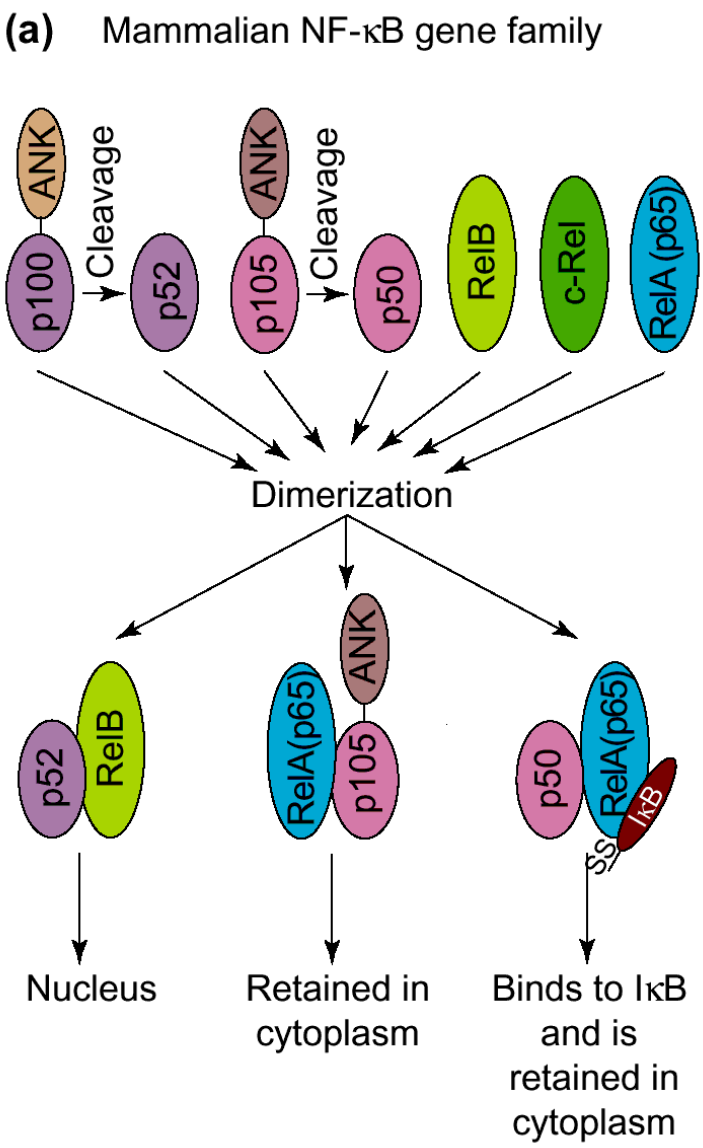
五、 NF-κB 及 AP-1 的訊息傳遞路徑

1. NF-κB/IκB kinase (IKK)路徑

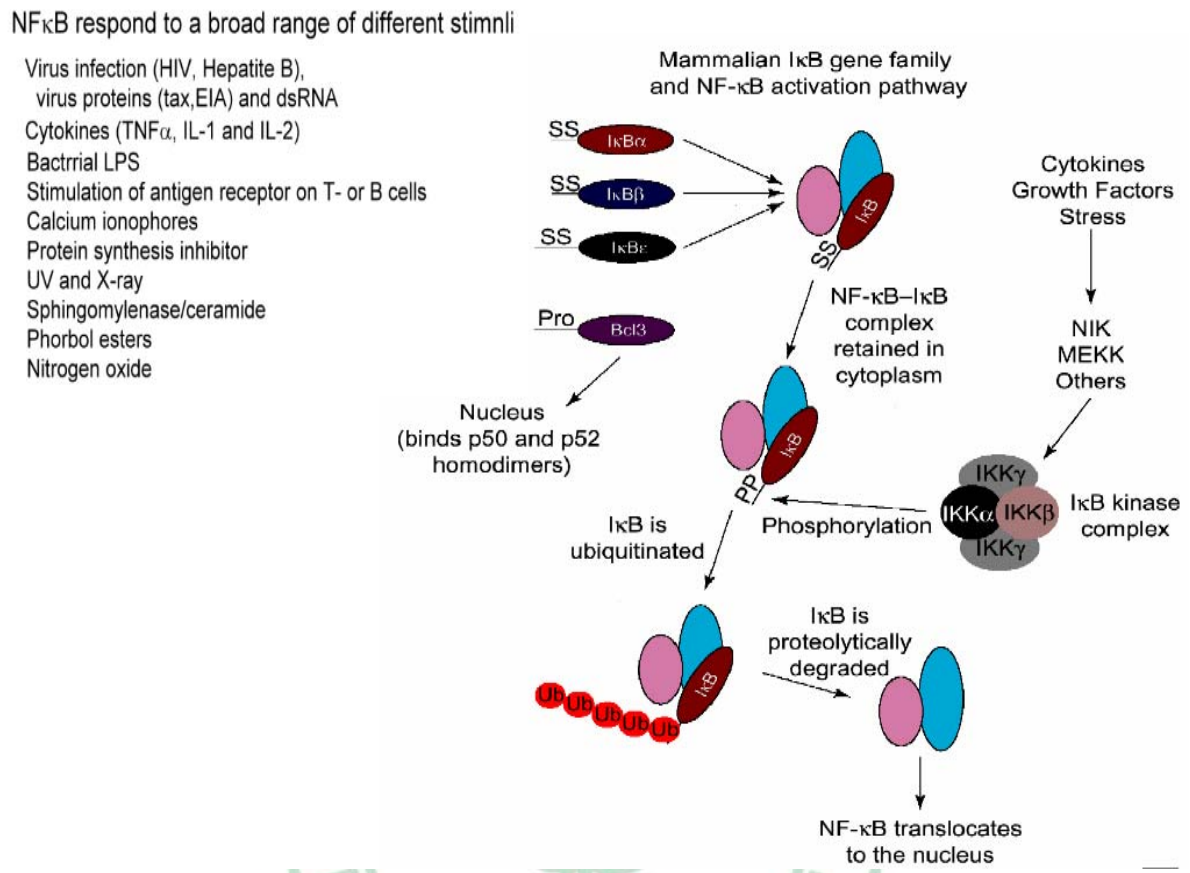
當巨噬細胞上的 receptor 與特定 ligan 結合後，會活化細胞內的訊息傳遞，此過程會誘導轉錄因子 NF-κB 的活化，以增加 cytokines 的基因表現[167, 168]。NF-κB 可在數分鐘之內被許多物質所活化，包括發炎性細胞激素 TNF-α、IL-1 和 T 細胞活化訊號、生長因子及氧化壓力等。在細胞核內，NF-κB 結合至標的 DNA，調節包括免疫及發炎反應在內的各種基因轉錄。已知 NF-κB 可以調節 cytokines 如 IL-1、IL-2、IL-6、IL-12、TNF-α、lymphotoxin α (LTα)、LTβ 和 GM-CSF；chemokines 如 IL-8、macrophage inflammatory protein-1α (MIP-1α) 、MCP-1 、RANTES 和 eotaxin; 黏著分子 (adhesion molec ules) 如 ICAM, vascular cell adhesion molecule (VCAM), E-selectin; acute phase proteins 如 SAA; 誘發型酵素如 iNOS 和 COX-2 基因表現。由於 NF-κB 在發炎過程中扮演重要的角色，因此也成為抗發炎藥物研發的標的。 NF-κB 家族有五個成員，分別為 Rel (c-Rel)、RelA (p65)、RelB、NF-κB1 (p50/p105) 和 NF-κB2 (p52/p100) [167] (附圖16)。 而 p105 與 p100 為 p50 及 p52 的前驅物，經過 C-端磷酸化和在 C-端的 IκB-like 部分進行 ubiquitin 依存性分解後產生 p50 及 p52 的[169]。NF-κB 以 homo- 或 heterodimers 形式存在。在未刺激的細胞中大多數的 NF-κB dimer 會與抑制性蛋白 (inhibitor of κB；IκB) 結合存在細胞質中。 IκB 家族的成員有 IκB-α、IκB-β、IκB-ε、IκB-γ、Bcl-3。 IκB 具有六個或更多的 ankyrin repeat，這些序列為與 NF-κB 產生結合所需，並藉由 RHD 與 NF-κB 結合。 IκB 抑制 NF-κB 的活性主要來自於其結構上的 C-端，而 N-端則包含具有調節功能的 domain。 在未受刺激的細胞中，IκB 會遮蔽掉 NF-κB 的 nuclear localization signal (NLS) 部分，形成不活化的 NF-κB/IκB 複合物，而停留在細胞質內[170, 171]，當 IκB 磷酸化並被分解後，就無法遮蔽 NF-κB 的 NLS，而使自由態的 NF-κB dimers 進入到細胞核

內與 DNA 結合，活化 NF-κB 所調節的基因表現。另外在 NF-κB 活化的過程中，除了會快速造成 IκB-α 的分解之外，也會快速合成新的 IκB-α。因 IκB-α 含有 leucine-rich nuclear-export signal (NES)，所以新合成的 IκB-α 能夠由細胞核回到細胞質中，並帶回與 DNA 結合的 NF-κB [172]。

IκB-α 之磷酸化主要是受到 IKK 的活化的刺激，IKK 是由 IKKα、IKKβ、及 IKKγ dimer 組成的形成一個 tetramer[173, 174]。其中 IKKα 和IKKβ 為 catalytic subunit，而 IKKγ (或稱 NF-κB essential modulator，NEMO) 為 regulatory subunit[175](附圖17)。當細胞受到TNF-α、IL-1、double-stranded RNA 或 endotoxin 刺激時會活化 IKKγ。在 IKK 的 catalytic subunit 中，IKKβ 與 IκB-α 的磷酸化比較有關係，而 IKKα 被認為並不是這條路徑所需要的 [176, 177]。 被活化的 IKK 會在 IκB-α 分子 N-端上的兩個 serine (S32 與 S36) 進行磷酸化作用，造成 IκB-α 的 polyubiquitination ，並在 26S proteosome 進行分解[173]。 Mitogen-activated protein kinase kinase kinase (MAP3K) 家族中的 NF-κB-inducing kinase (NIK) 以及 MEKK1 也會活化 NF-κB。 NIK 會磷酸化並活化 IKKα，而 MEKK1 則會優先磷酸化 IKKβ [178]。



附圖 16. NF-κB/Rel proteins : Homo- and hetero-dimeric TFs that in resting cells are retained in the cytoplasm in complex with IκB.[179]



附圖 17.　Multiple signaling pathways activate NF-κB.[179]

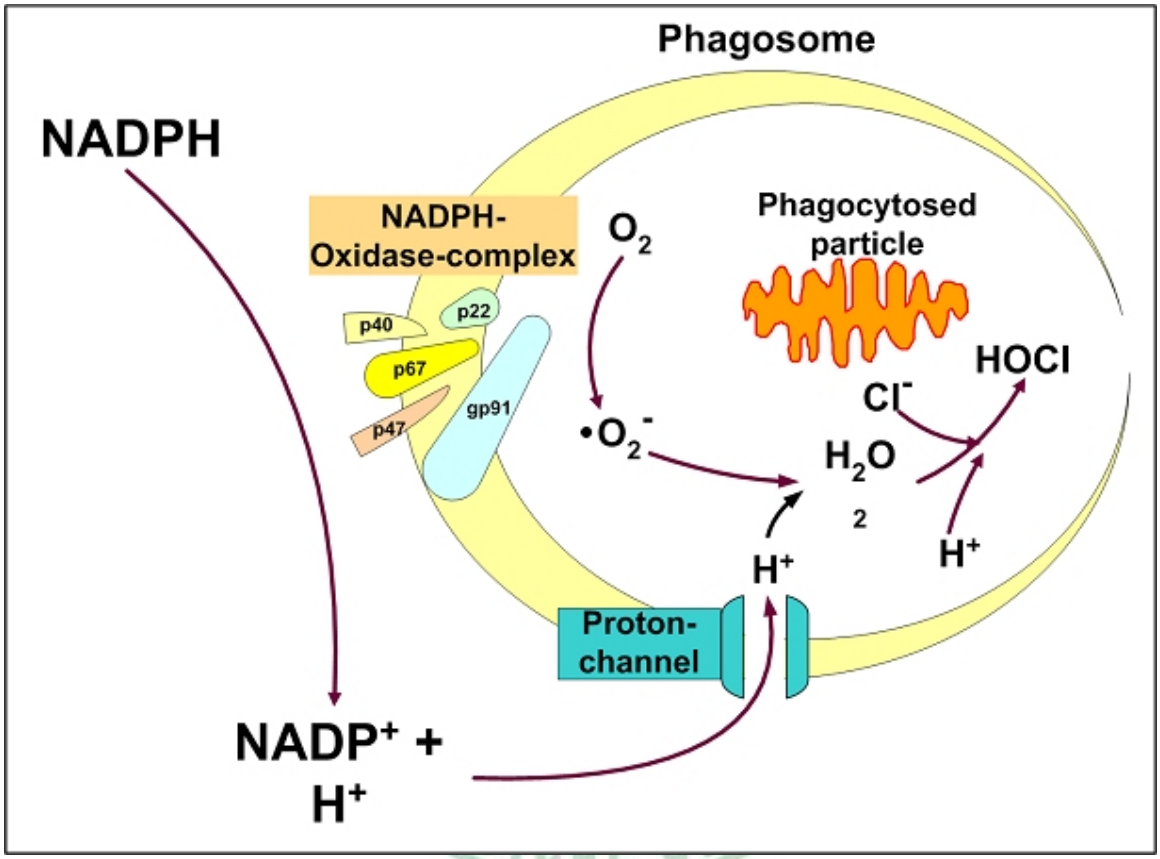
2. AP-1 路徑

除了 NF-κB 之外，AP-1也是一個與發炎相關的轉錄因子 (proinflammatory transcription factor)[180]。AP-1的 promoter region 有許多細胞激素 (cytokines)、黏附分子和細胞生長激素的結合位置[181, 182]。 AP-1是由 Jun 蛋白質家族 (c-Jun, JunB, and JunD) 及 Fos 蛋白質家族 (c-Fos, FosB, Fra1, and Fra2) 所組成，其相互間組合成同形二聚體 (homodimer) 或異形二聚體 (heterodimer)。當給予 LPS、細胞生長因子和細胞激素的刺激時，可藉由 MAPK 之訊息傳導路徑，如: ERKs, c-Jun-N-terminal kinases (JNKs) 和 p38 kinase 來活化 AP-1，進而調控下游基因[183](附圖15)。

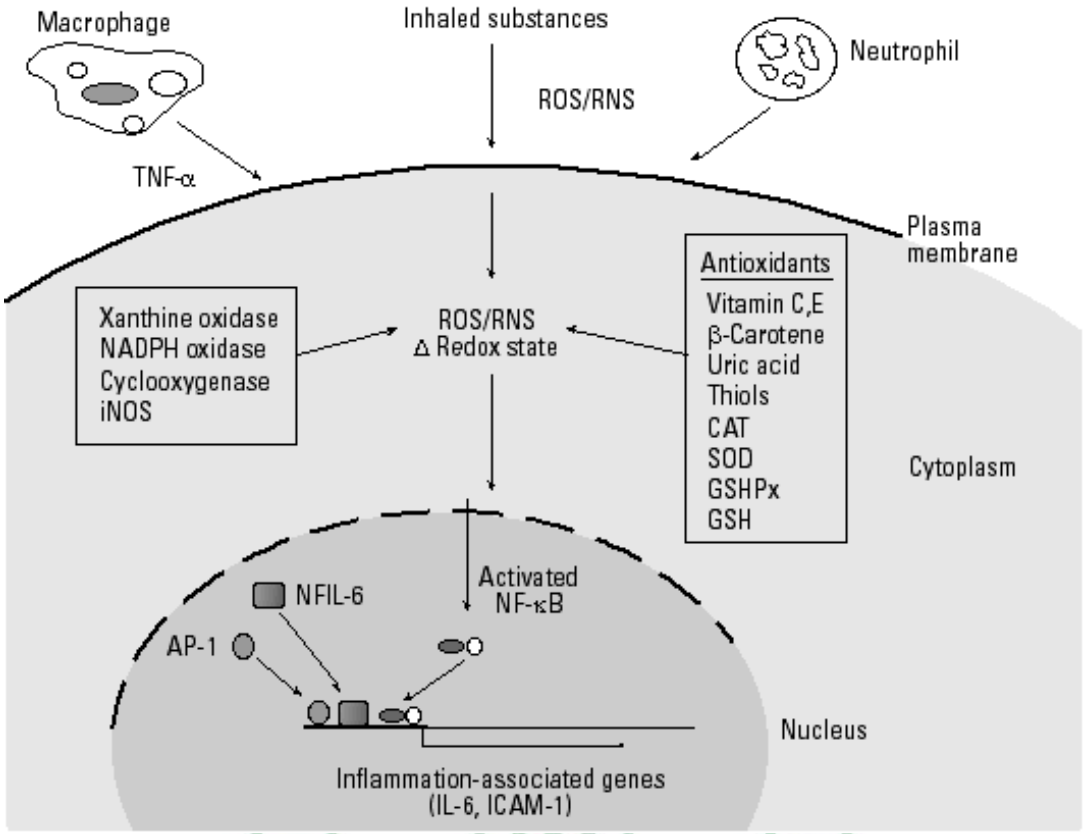
六、 Reactive oxygen intermediates 與訊息傳遞路徑

肺泡巨噬細胞在進行胞噬作用時同時也會釋放出氧代謝產物 (Reactive oxygen intermediates)，例如: superoxide anion ($O2^-$)、hydrogen peroxide (H_2O_2) 和 hydroxyl radical (·OH)，此機制又稱為呼吸爆發 (respiratory burst) (附圖18)。 此外，當細胞受到 phorbol myristate acetate (PMA), IFNγ, GM-CSF, TNF, LTB_4, zymosan 和 IgG immune complexes 的刺激時也會促進 ROIs 釋出。呼吸爆發作用與吞噬細胞之吞噬小體內及其細胞外的殺菌作用有密切的關係，卻也對鄰近的組織造成了傷害。細胞內可藉由粒腺體呼吸作用、NADPH oxidase 和 xanthine/xanthine oxidase 等系統形成活性氧化物 (ROS) 和活性氮化物 (reactive nitrogen species；RNOS)，其中 NADPH oxidase 是主要形成 ROS 的酵素。在肺部發炎反應中，活化的巨噬細胞、噬中性球、噬酸性球和肺部上皮細胞都會產生 ROIs 。 ROIs 能作用在蛋白質、DNA 和脂質，會直接造成肺部實質的損傷，包括肺部細胞的重組和增生。ROIs 也作為訊息傳遞者，藉由磷酸化作用來活化 MAPK family，包括 ERK, JNK,

p38 kinase 和 phosphoinositol-3 kinase，造成一些轉錄因子 (transcription factor) 如 NF-κB 和 AP-1 活化，促使一些促發炎媒介物 (pro-inflammatory cytokines)的基因轉錄作用(gene transcription) 增加[184, 185](附圖 19)。



附圖 18. Phagocytosis and oxidative burst. During phagocytosis, intracellular reactive oxygen species rapidly increase, known as oxidative burst.[186]



附圖 19. ROS/RNS and intracellular signaling molecules in airway epithelial cells.[185]

第三章 材料與方法

第一節 實驗材料

一、實驗試劑

1.生工有限公司(Taipei, Taiwan)

RT-PCR Forward and Reverse Primer (mouse TNF-α, IL-6, iNOS,COX2, IL-10, IL-1Ra, GAPDH)； 5'-biotinate double-stranded oligonucleotide probes containing a consensus binding-sequence of NF-κB and AP-1

2.內外化學工業股份有限公司(Taipei, Taiwan)

Prednisolone 5 mg/tab

3.波士特公司(Taipei, Taiwan)

RNA-Bee isolation of RNA

4.榮民製藥(Taipei, Taiwan)

Penicillin, Streptomycin

5. BD Biosciences (Franklin Lakes, NJ, USA)

Human TGF-β1, FITC-Mouse Anti-E-cadherin, Anti-Vimentin, E-cadherin antibody, ELISA OptEIA Set for TNF-α and IL-6

6. BioColor Ltd. (Newtownabbey, U.K.)

Sircol Collagen Assay Kit

7. BIO-RAD (BioRad, Hemel Hampstead, UK)

N,N'-methylene-bis-acrylamide (Bis), Acrylamide, Ammonium persulphate (APS), Glycine, N,N,N',N'-Tetramethylethylenediamine (TEMED)

8. Cayman Chemical (Ann Arbor, Mich, USA)

EIA kit for PGE_2

9. Cell Signaling (Danvers, MA, USA)

   Anti-phospho-Smad3 antibody , Anti-IKKα/β, Anti-phospho-IKKα/β antibody

10. Clontech (Palo Alto, CA, USA)

    Random hexamer primer, Oligo (dT) primer, dNTP mix, 5X reaction buffer, Recombinant RNase inhibitor, MMLV reverse transcriptase

11. Extrasynthese (Genay Cedex, France)

    Luteolin (HPLC test: > 99.0%)

12. GeneMark (Taipei, Taiwan)

    Trisolution Reagent Plus

13. Gibco BRL (Gaithersburg, MD, USA)

    Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Trypsin, Fetal Bovine Serum (FBS), RPMI 1640 Medium

14. HyClone Laboratories Inc. (Logan, UT, USA)

    Fetal Bovine Serum (FBS)

15. MERCK (Merck, Germany)

    EDTA, Postassium chloride (KCl), Sodium hydroxide (NaOH), Methanol, Dimethylsulfoxide (DMSO), Sodium dodecyl sulfate (SDS), Sodium chloride (NaCl), HCl, 2-Mercaptoethanol (2-ME), Formalin, Giemsa solution

16. Molecular Probes (Eugene, OR, USA)

    2', 7'-dichlorofluorescin diacetate (H_2DCFDA,)

17. NEN Life Science (Boston, MA, USA)

    L-glutamine, Non-Essential amino acid solution (NEAA), Polyvinylidene fluoride (PVDF) transfer membrane

18. Nippon Kayaku (Tokyo, Japan)

    Bleomycin

19. Oncogene (Cambridge, MA, USA)

    Anti-α-SMA antibody

20. PerkinElmer Life Science, Inc. (Boston, MA, USA)

    Horseradish peroxidase-conjugated rabbit anti-mouse or anti-rabbit IgG, ECL detection kits, nitrocellulose transfer membrane, streptavidin-horseradish peroxidase conjugate, SuperSignal chemiluminescent substrate

21. Santa Cruz (Santa Cruz, CA, USA)

    Anti-Smad3, Anti-Smad4, Anti-β-actin, Anti-COX-2 and IκBα antibody, Anti-p65 antibody

22. Sigma-Aldrich (St. Louis, MO, USA)

    Lipopolysaccharide (*Escherichia coli* 055:B5), Methylene blue, Collagenase, Trypsin (1∶250), Triton X-100, propidium iodide (PI), EGTA, Dithiothreitol (DTT), Leupeptin, ATP, Sodium pyruvate, LY294002, 4',6'-diamidino-2-phenylindole (DAPI), 7–bis (4-hydroxy -3-methoxy-phenyl)-1, 6-heptadiene-3, 5-dinone (curcumin), ammonium pyrryolidinedithio-carbamate (PDTC), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water), anti-iNOS, FITC-conjugated goat anti-mouse IgG antibody

23. Vector Labs (Burlingame, CA, USA)

    Vectashield

二、實驗動物

C57BL/6J 品系公鼠購自財團法人國家實驗研究院實驗動物中心。所有實驗使用 10-11 週齡，體重約 25-30 g 的小鼠。飼養管理：動物購入後，經隔離檢疫一週後，經由獸醫師核可移入 1 號代養室之無菌飼養籠，餵養於台中榮總動物中心的 1 號代養室，飼養室溫度控制在 21℃，濕度為 60 ± 10%，在 12 小時 light / dark 循環條件下，使用福壽牌大鼠飼料，飲水經過逆滲透處理，採不限飼料、不限水之飼養模式。實驗人員進入動物室時需換隔離衣並經 air shower 除塵處理，以確保動物於潔淨、無塵的環境進行試驗。。

三、細胞株來源及培養條件

本研究使用之初代培養之小鼠肺纖維母細胞取自 C57BL/6J 10-11 週齡公鼠之肺臟。A549 cells (human lung carcinoma-derived alveolar epithelial cell line; ATCC, CCL-185)、巨噬細胞株 MH-S (ATCC number: CRL-2019) 及 RAW 264.7 (ATCC number: TIB-71) 購自食品工業發展研究所，培養條件如下:

【A549 cells 】5 % FCS RPMI 和100 U/ml penicillin/streptomycin。

【MH-S (半附著型細胞 half-adherent cell) 】

RPMI1640 (10% FCS), 2 mM L-Glutamine, 4.5 g/L glucose, 1.0 mM Sodium pyruvate, 10 mM HEPES, 1.5 g/L $NaHCO_3$, 0.05 mM 2-mercaptoethanol, 1% Penicillin/Streptomycin

【RAW264.7 (附著型細胞 adherent cell) 】

DMEM (5 % FCS), 2 mM L-Glutamine, 1.0 mM Sodium pyruvate, 1.5 g/L $NaHCO_3$, 1% Penicillin/Streptomycin

第二節 實驗方法與步驟

Part A 以活體動物及離體細胞模式評估木犀草素抗肺纖維化作用及分子機轉探討

一、 活體動物模式藥效測定

1. 建立小鼠肺纖維化模式

小鼠以乙醚麻醉後，以 5 mg/kg 單一劑量氣管注入溶於 200 μl 注射用生理食鹽水的 bleomycin，對照組注入等量的注射用生理食鹽水(NS)。木犀草素以 1 mg/ml 濃度溶於 50 % ethanol，餵食劑量為 10 mg/kg。

2. 實驗設計與分組

2.1 木犀草素抗發炎作用之評估

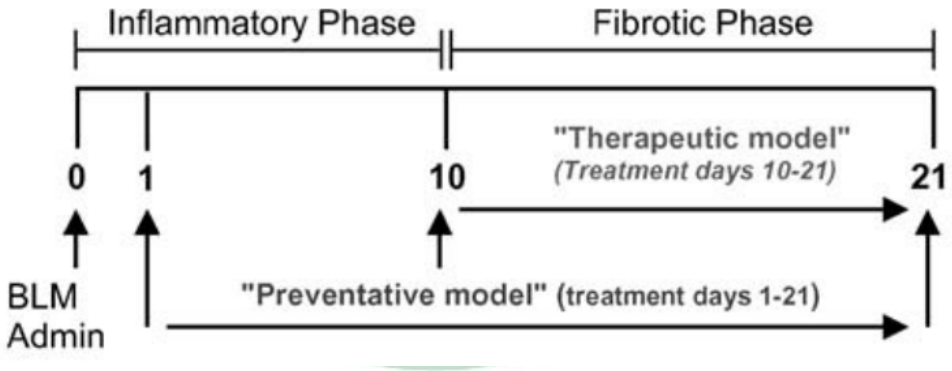
除對照組 (NS) 外，bleomycin 造模小鼠採隨機分組，分成 bleomycin (BLM) 組、木犀草素治療組 (Lut) 及類固醇治療組 (Pred) 共四組，每組 10 隻小鼠。木犀草素治療組於造模後隔天每日以胃管餵食 10 mg/kg 木犀草素， 類固醇治療組於造模後隔天每日以胃管餵食 10 mg/kg prednisolone。小鼠在餵食藥物後第 7天及第 14天以二氧化碳犧牲，剖胸取肺臟，從氣管注入 1 ml 福馬林固定組織，包埋，切片，脫蠟，hematoxylin & eosin (H&E) 染色，觀察組織病理變化。另外一批同樣分組的小鼠在餵食藥物後第7天及第14天以二氧化碳犧牲，取肺灌洗液 (bronchoalveolar lavage fluid, BALF)，小鼠剖胸取肺臟，從氣管注入 1 ml 生理食鹽水 (4℃) ，稍加按摩後回吸，重複灌洗四次，約可收集 3 ml 的 BALF，4℃ 離心後收集上清液於 -70 ℃貯存，待測 BALF 中 cytokines 含量； 離心後之下層細胞以 1 ml NS (4℃) 打散後，以 cytospin 將細胞打在載玻片上，以 Giemsa solution 染

色，顯微鏡下作細胞計數以觀察不同時期發炎細胞比例之變化 (differential cell counts)。上清液以免疫酵素聯結法 (ELISA) 測定 TNF-α 和 IL-6 活性。

2.2 肺纖維化的時程變化及實驗設計

2.2.1 肺纖維化的時程變化 (*Time course of bleomycin-induced lung fibrosis)*

為了確認 bleomycin 造模小鼠肺纖維化的病理變化時程，於造模後第 0, 3, 7, 10, 14, 21 天分別取各 3 隻 bleomycin 造模小鼠作病理切片，以 Masson's Trichrome 染色，觀察肺組織病理變化及膠原沉積產生的時間。



2.2.2 早期治療 (*Early treatment*)

除對照組 (NS) 外，bleomycin 造模小鼠採隨機分組，分成 bleomycin (BLM) 組、木犀草素治療組 (Lut) 及類固醇治療組 (Pred) 共四組，每組 10 隻小鼠。木犀草素治療組於造模後隔天每日以胃管餵食 10 mg/kg 木犀草素， 類固醇治療組於造模後隔天每日以胃管餵食 10 mg/kg prednisolone。

小鼠在餵食藥物後第 14 天及第 21天犧牲，剖胸取肺臟，從氣管注入 1 ml 福馬林固定組織，包埋，切片，脫蠟，H&E 和 Masson Trichrome (MT)染色，觀察組織病理變化。另外一批同樣分組的小鼠犧牲後，剖胸取左側肺葉作膠原含量分析；右側肺葉抽取 RNA 作 RT-PCR分析。

2.2.3 後期治療 (*Delayed treatment*)

另外一批同樣分組的小鼠在於 BLM 造模後第 10 天才開始餵食藥物，木犀草素治療組於造模後造模後第 10 天每日以胃管餵食 10 mg/kg 木犀草素，類固醇治療組於造模後造模後第 10 天每日以胃管餵食 10 mg/kg prednisolone。小鼠在餵食藥物後第 14 天及第 21 天犧牲，其餘實驗步驟同上。

3.以組織病理切片的結果評估藥物的療效

肺組織以 10% formalin 溶液固定 24 小時，每個檢體均取相同的肺葉並修片，石蠟包埋, 以 4 μm 厚度作連續切片, 待玻片乾後以 H&E 或 MT 染色，光學顯微鏡下觀察肺組織纖維化的程度。組織纖維化程度之判定參考 Tanino 及 Ashcroft 等人的方法並稍作修改[187, 188]，將纖維化程度輕重分為 0 到 8 級：grade 0-正常肺組織；grade 1-肺泡壁或細支氣管輕微增厚; grade 3-中度肺泡壁增厚但無明顯結構上的破壞; grade 5-明顯的膠原蛋白增生並破壞肺泡結構，膠原纖維集結成束; grade 7-肺泡結構嚴重破壞，大面積的膠原纖維分布; grade 8-膠原纖維充滿全部視野。 在 20x 物鏡下，每片檢體隨機選取 20 個視野計分，合併 20 個視野的分數並作統計分析。

4. 膠原蛋白含量測定

肺組織纖維化程度亦可以 Sircol Collagen Assay 偵測 collagen 含量作為評估項目。Sircol Collagen reagent 含有 Sirius Red (in picric acid) 會與

檢體中 type I-V collagens產生鍵結態的紅色沉澱物。方法如下：小鼠左側肺組織稱重後剪碎與溶於 0.5 M acetic acid/500 µl的 pepsin (EC 3.4.23.1；1:10 ratio of pepsin: tissue wet weight) 室溫下作用 over night， 每管檢體加入 1.0 ml Sircol dye reagent， 蓋緊上蓋後混合均勻，室溫下反應 30 分鐘，另以 1 mg/ml Collagen acid soluble type I standard 溶於 0.5 M acetic acid 製備標準濃度之溶液。檢體與標準溶液離心後去除上清液，沉澱物以 100 µl Sircol alkali reagent 回溶，取 200 µl 於 96 孔盤，在 560 nm 波長下測吸光值並以標準曲線及組織實重換算每毫克肺組織之膠原蛋白含量 (µg/mg of lung tissue)。

5. 反轉錄聚合酶鏈反應 (Real-time RT-PCR)

5.1 RNA extraction

小鼠右側肺組織置於滅菌過研缽中邊加入液態氮邊磨碎，加入 2 ml Trisolution Reagent Plus 與組織充分混合後取出置於無菌試管中反應 5 分鐘，加入 0.5 ml chloroform 室溫下作用 15 分鐘，之後在 4℃下以 12000 rpm 離心 15 分鐘，取約 0.5 至 0.6 ml 的上層液加入含 0.5 ml isopropanol 的 eppendorff 沉澱作用 10 分鐘後，再 4℃下以 12000 rpm 離心 10 分鐘，離心後吸去上層液體， 以 75% ethanol 清洗，最後在 4℃下以 12000 rpm 離心 5 分鐘，吸去上層液體後風乾，待 pellet 成透明狀後加入 30-50 µl 的 ddH_2O 置於 4℃冰箱隔夜儲存。 Muose TGF-β1 primer (524 bp)序列如下：Foward 5-TGGACCGCAACAACGCCATCTATGAGAAAACC-3 ， Reverse 5-TGGA GCTGAAGCAATAGTTGGTATCCAGGGCT-3。

5.2 反轉錄反應 (Reverse transcription)

RNA 以 ddH_2O (2 : 98)作50 倍稀釋後，從波長 260 nm 的吸收值換算成 RNA 濃度，每一處理取 2 µg RNA、20 µM random hexamer primer 0.5 µl

和 20 μM Oligo (dT) primer 0.5 μl，以 ddH_2O 調整體積至 13.5 μl 後，於 85℃下 denature 15 分鐘，之後加入 4 μl 的 5 倍 reaction buffer (250 mM Tris-HCl pH 8.3、375 mM KCl、15 mM $MgCl_2$)、1 μl 的 10 mM dNTP mix、0.5 μl Recombinant RNase inhibitor (40 units/μl)、1 μl 的 MMLV Reversetranscriptase (200 units/μl) 於 42℃下 extension 1 小時，94℃下作用 5 分鐘，加入 30 μl ddH_2O 稀釋成 2 倍 cDNA，置於 -20℃ 下儲存。

5.3 聚合酶鏈反應 (Polymerase chain elongation reaction)

取 1.5 μl 的 2X cDNA、2 μl 50% glycerol、1 μl 10 X PCR reaction buffer、0.25 μl dNTP mix (10 mM/μl；0.25 mM/reaction)、0.3 μl DNA polymerase (5 units/μl；0.2 units/reaction)、20 μM primer (forward& reverse primer各 0.5 μl)，最後以 ddH_2O 調整體積至 10 μl 後，以 Bio-Rad PCR mach ine 作聚合酶鏈反應(反應條件: 95℃/4分鐘→ 95 ℃/ 45 秒 25 cycle、60℃/1分鐘、72℃/2分鐘) → 72℃/ 7 分鐘→ 4℃ 保存)。

5.4 DNA 電泳膠實驗

利用微波爐加熱含有 2 % agarose 的 TBE buffer，待稍微冷卻後，加入 5 μl/100 ml 比例的 ethidium bromide (10 mg/ml)，混合均勻後倒入水平電泳凝膠模型中，盡量避免氣泡的產生，凝固後，置於 4℃ 冰箱冷藏一下或放置隔夜， 倒入 0.5 倍的 TBE buffer 於電泳槽中，並使 buffer 液面稍為高於膠面。將 PCR 產物與 6 倍 DNA gel-loading dye 混和均勻之後，注入 agarose gel 的凹槽中。利用 50 伏特的電壓進行 DNA 電泳，分離後，再以 EverGene 數位影像擷取系統(gel analysis system)分析。實驗試劑: 5 倍 TBE buffer : Tris 54g, Boric acid 27.5 g, 0.5 M EDTA /pH 8.020 ml，以 ddH_2O 調整體積至 1L。6倍 DNA gel-loading dye: 0.125 g Bromophenol Blue，0.125 g Xylene Cyanol FF, 15 ml Glyerol, ddH_2O 加至35 ml。

二、 離體細胞模式分子機轉探討

1. 木犀草素抑制小鼠肺纖維母細胞增生及分化

1.1 小鼠肺纖維母細胞之分離及培養

本實驗每次取 8 隻 8-10 週齡之 C57BL/6J 成鼠，犧牲後取出肺臟，之後步驟均於無菌操作台操作。以 1 ml無菌 HBSS 溶液沖洗肺臟3次，剪碎肺葉，以 collagenase (1 mg/ml) 和 0.25% trypsin (in 1 mM EDTA) 37℃攪拌作用6分鐘，以 22-μm nylon mesh 過濾並加入 DNAase，濾液加入等體積含血清培養液終止酵素作用，網篩上殘餘組織重複上述消化步驟，合併所有濾液以 300 x *g* 離心 5 分鐘，細胞沉澱以 9/10 體積的 ddH_2O 溶解紅血球隨即以 1/10 體積 10x HBSS 恢復等張，離心 5 分鐘，細胞沉澱以 10 ml 培養液打散，培養於 10 cm dish。培養液為 DMEM 10% FCS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 100 μM non-essential amino acid (NEAA), 20 μM HEPES buffer (pH 7.3), 1.5 % Fungizone。每 2 天換一次培養液，約 10 天左右肺纖維母細胞長滿培養盤即可以 trypsin 打下進行繼代分盤，之後每 2 天繼代一次，至第 5 代即為可進行實驗之純化肺纖維母細胞。以 α-smooth muscle actin 染色確認肺纖維母細胞純度為 95%以上。進行 cell proliferation 和 TGF-β1-induced myofibroblastic differentiation 實驗時細胞以不含 FCS 的 DMEM 培養。

1.2 木犀草素抑制小鼠肺纖維母細胞增生

以不同濃度木犀草素 (10, 25, 50 μM; DMSO:EOH=1:1) 處理肺纖維母細胞，分別於 24, 48, 72 小時以 Trypsin 染色法計數細胞並作出生長曲線圖。

1.3 免疫螢光染色 (Immunofluorescence imaging)

肺纖維母細胞以 3 X 10^4 cell/well 種於置有圓蓋玻片之 12 孔盤中，以木犀草素 (25 μM) 前處理 30 分鐘後，以 TGF-β1 (5 ng/ml) 處理不同組別的細胞，72 小時後取出玻片進行細胞免疫螢光染色。將種有細胞之玻片取出置於新的 12 孔盤中，PBS 清洗 10 分鐘 3 次，以 2 % paraformaldehyde 固定 30 分鐘，PBS 清洗 10 分鐘 3 次，加入 0.1% Triton X-100/in PBS 作用 20 分鐘，使細胞膜產生孔隙得以讓染劑進入，細胞在室溫下使用 5% bovine serum albumin /PBS 作用 60 分鐘，以 mouse anti-α-SMA (1:100) antibody 4℃作用24 小時，PBS 清洗 10 分鐘 3 次，接著以 FITC-conjugated goat anti-mouse IgG antibody (1:200) 37 ℃用 1 小時，PBS 清洗 5 分鐘 3 次。 待乾後覆蓋於載玻片上以 Vectashield /Tris buffer (pH 8.4) 封片。使用 Leica laser scanning confocal 顯微鏡照相記錄。

1.4 木犀草素抑制小鼠肺纖維母細胞分化及分泌 ECM

細胞以木犀草素 (25 μM) 前處理 30 分鐘後，以 TGF-β1 (5 ng/ml) 處理不同組別的細胞，72 小時後收取細胞蛋白以西方點墨法分析 α-SMA, vimentin 和 collagen 表現量。

1.5 蛋白質之定性及定量分析 (Western blot analysis)

蛋白質的萃取 (Protein Extraction)

不同時間點收集經藥物處理或未經處理之細胞，在 4℃ 以 1600 rpm 離心 5 分鐘，並用冰的 PBS 將細胞清洗一次後，於 4℃下再離心 5 分鐘，倒掉上清液並用棉棒將多餘的水吸乾，將細胞 pellet 均勻打散，加入適量的 RIPA buffer【50 mM Tris (pH 7.4) , 150 mM NaCl , 1% Triton X-100 , 0.25% Na Deoxycholate, EDTA (pH 8.0), 1 mM EGTA (pH 8.0), 1 mM DTT, 5

μg/ml Leupeptin , 0.2 mM PMSF, 5 μg/ml Aprotinin , 1 mM Na Vanadate , 1 mM NaF】，置於冰上作用 20 分鐘，4℃下將細胞液以 55000 rpm 超高速離心 30 分鐘，收集上清液，以一系列已知濃度的 BSA 做成之 standard curve 換算蛋白質濃度 (μg/μl)。將蛋白質分裝並用 RIPA buffer 調整成相同體積，接著再加入 1/3 量的 4 倍 protein loading dye (8 % SDS, 0.04 % serva blue R-250, 40% glycerol, 200 mM Tris pH6.8, 10% 2-mercaptoethanol)，以 95℃ 乾浴加熱變性 10 分鐘後，即可置於 -80℃中保存。

聚丙烯醯胺膠體電泳法 (SDS-PAGE Assay)

利用 SDS-PAGE 將蛋白質依分子量大小予以分離。首先配置 1.5 mm 厚的 discontinuous acrylamide gel，下層 separating gel 其 acrylamide 的百分比，視分析蛋白質分子量而定，上層的 stacking gel 則含有 4 % acrylamide。配置完成的膠體放至於電泳槽內，加入電泳緩衝液(runnung buffer : 25 mM Tris, 192 mM glycine, 0.1 % SDS)。接著將萃取出的蛋白質 sample 及標示標準分子量的 Multimaker 依序注入膠體的孔槽中， 通以電壓 80 伏特，待樣品通過 stacking gel 後電壓調整為 100 伏特，視其分子量大小斟酌電泳時間。

西方點墨法 (Western blot)

將 PVDF membrane 浸於 methanol 數秒後以 Milli-Q water 浸濕，接著將裁好的濾紙與 PVDF membrane 先浸泡在 transfer buffer (25 mM Tris , 192 mM glycine)中。取出電泳膠以濕式轉漬器轉漬至 PVDF membrane，於 4℃通以 100 Voltage電 壓 1小時。將 membrane 取出，浸泡於 5% non-fat milk /TBST中，於室溫下搖晃一小時。以 TBST buffer (24 .22 g Tris , 87.75 g NaCl , 10 ml Tween20，加水調到1 L) 清洗 membrane 10 分鐘 3次，加入一

級抗體於 4 ℃下作用 over night。隔日先以 TBST buffer 清洗 membrane 10 分鐘 3 次，加入二級抗體，使其在室溫下搖晃作用 1 小時之後，再用 TBST buffer 清洗10 分鐘 3 次， 接著在暗房中將membrane 與 ECL (Enhance chemi-luminescence) 反應後，裝於透明塑膠袋內並置於壓片夾中，以 X-ray film 感光顯影，再以自動沖片機沖片。

1.6 木犀草素抑制小鼠肺纖維母細胞之 TGF-β1 /Smads 訊息傳遞

細胞以木犀草素 (25 μM) 和 TGF-β1 inhibitor (SB431542, 10 μM) 前處理 30分鐘後，以 TGF-β1 (5 ng/ml) 處理不同組別的細胞，30分鐘後收取細胞蛋白以西方點墨法分析 p-Smad3, Smad3 和 Smad4 的表現量。

2. 木犀草素抑制肺上皮細胞株 A549 進行 EMT

A549 cells (human lung carcinoma-derived alveolar epithelial cell line; ATCC, CCL-185)培養於 5 % FCS RPMI 和100 U/ml penicillin/streptomycin。當細胞長至八分滿時換成 0.1% FCS medium (starved for 24 hours)，以木犀草素 (25 μM) 前處理 30分鐘後，以 TGF-β1 (5 ng/ml) 處理不同組別的細胞，48 小時後收取細胞蛋白以西方點墨法分析 E-cadherin, fibronectin 和 vimentin 表現；E-cadherin 細胞免疫螢光染色方法同前述。

Part B 木犀草素對於肺泡巨噬細胞的免疫調控

一、細胞存活率測定 (Cell viability test)

1. Trypan blue dye exclusion 染色法

存活之細胞可將 Trypan blue dye 排除於細胞外，而死亡之細胞則無此能力，故可將死亡的細胞染色。首先先將細胞以每孔 5×10^4 個細胞分種於 24 孔細胞培養盤中，均勻搖散細胞後，將細胞培養於37℃、5% CO_2 的培養箱中，培養 over night 後給予不同濃度 (5、10、25、50 μM) 木犀草素處理，經24小時後，以 Trypsin-EDTA 將細胞打下，再用 0.4% Trypan blue dye 染色，以血球計數器 (hemocytometer)計算各孔盤內的細胞數，此實驗至少重複三次，取平均值作成細胞存活曲線。

2. MTT 法

活性細胞中粒腺體酵素 (mitochondria succinate dehydrogenase) 會將 3-(4,5-dimethylthiaxol-2-yl）-2,5-diphenyl- tetrazoliumbromide (MTT) 還原成紫色 formazan 結晶，將結晶溶解後分析光學密度 (optical density) 值以代表活性細胞之數量。將細胞以每孔 5×10^4 個細胞分種於 24 孔細胞培養盤中，均勻搖散細胞後，將細胞培養於37℃、5% CO_2 的培養箱中，培養 over night 後給予不同濃度(5、10、25、50 μM) 木犀草素處理，經 24小時後，將細胞培養液吸除後以 PBS 洗 3 次，每孔加入 200 μl 溶於 PBS的 MTT (100 μg/ml)，於 37℃ 恆溫培養箱中避光培養 2 小時後，培養液倒棄，加入 200 μl DMSO 均勻混和，取 100 μl 至 96 孔盤中，以酵素免疫連結測定儀 ELISA Reader 偵測 550 nm 之吸光值(OD_{550})。相對存活率(%)=(藥物處理組OD_{550}/對照組OD_{550})×100%。

二、細胞激素 (Cytokines) 的 ELISA 分析

本實驗是以 mouse TNF-α 和IL-6 Enzyme-Linked Immune Substrate Asssay (ELISA) kit 進行酵素連結免疫分析，分析細胞培養液中 TNF-α 及 IL-6 的含量。將細胞以每孔 5× 10^4 個細胞分種於 24 孔細胞培養盤中，均勻搖散細胞後，將細胞培養於 37℃、5% CO_2 的培養箱中，前處理不同濃度的(5、10、25 μM)木犀草素30分鐘，加入 LPS (100 ng/ml)刺激細胞 24 小時後，吸出上清液貯存於 -70℃待測。先於 ELISA 96 孔盤中置入 100 μl/well capture antibody (稀釋於 1:250 in coating buffer)；放置 4℃隔夜。以 wash buffer (0.05% Tween 20 in PBS) 重複沖洗 3 次，加入 200 μl Assay diluent 在室溫下放置 1 小時後，以 washing buffer 沖洗 3 次再加入 200 μl細胞培養液或標準品，在室溫下放置 2 小時。以 washing buffer 沖洗 3 次，加入 working detector (Detection antibody + Avidin-HRP reagent 1:250 in assay diluent) 室溫放置 1 小時。Washing buffer 沖洗 7 次後，加入 100 μl TMB (tetramethylbenzidine)呈色，室溫下避光反應 30 分鐘。加入 50 μl stop solution (2N H_2SO_4)，30 分鐘內以 ELISA reader 於 450 nm 之波長測量該樣品之吸光值，再以標準曲線換算 cytokine 之濃度。

三、一氧化氮 (NO) 含量測定

細胞以 2 X 10^5 cell/well 種於 96 孔盤中隔夜培養，前處理不同濃度的(5、10、25 μM) 木犀草素 30 分鐘，加入 LPS (100 ng/ml) 刺激細胞 16 小時後，取 100 μl 上清液加 100 μl Griess reagent (含 0.1% napth-alethylenediamine dihydrochloride, 1% sulfanilamine , 2.5% phosphoric acid)，室溫下避光作用 10 分鐘， 30 分鐘內以 ELISA reader 於 550 nm 之波長測量該樣品之吸光值，並以 sodium nitrite 為 standard solution 得到的標準曲線換算出原樣本中的 nitrite 濃度。

四、PGE_2 含量測定

PGE_2 之測定是利用競爭型酵素免疫分析(competitive enzyme immunoassay) 之原理。方法為待測樣品的 PGE_2 與已知固定濃度的 PGE_2 alkaline phosphatase tracer 競爭有限的 PGE_2 monoclonal antibody。當三者共同於室溫作用 1 小時之後，此時 PGE_2 monoclonal antibody 會與連結在 plate 底部的 goat anti-mouse IgG antibody 結合。以 washing buffer 沖洗未連結的物質後，加入 substrate (*p*-nitrophenyl phosphate) 於室溫中呈色 60 到 90 分鐘，以波長 420 nm 之 ELISA reader 讀取吸光值。由標準品的吸光值換算待測樣品中 PGE_2 的濃度。

五、細胞免疫螢光染色

細胞以 5 X 10^4 cell/well 種於置有圓蓋玻片之 12 孔盤中，前處理 25 μM 木犀草素 30 分鐘，加入 LPS (100 ng/ml) 刺激細胞 15-60 min，將種有細胞之玻片取出置於新的 12 孔盤中，細胞免疫螢光染色方法如前述。以 mouse anti-p65 antibody (1:500) 4℃作用 24 小時，PBS 清洗 10 分鐘 3 次，接著以 FITC-conjugated goat anti-mouse IgG antibody (1:200) 37 °C用1 小時，細胞核以 propidium iodide 染色 15 min，PBS 清洗 5 分鐘 3 次。待乾後覆蓋於載玻片上以 Vectashield/Tris buffer (pH 8.4) 封片。使用 Leica laser scanning confocal 顯微鏡照相記錄。

六、反轉錄聚合酶鏈反應

以前述方法收集細胞 lysates，室溫下加入 RNABee (約1 ml/dish) 作用 5 分鐘之後，加入 0.2 ml chloroform 室溫下作用 15 分鐘，之後在 4℃下以 12000 rpm 離心 15 分鐘，取約 0.5 至 0.6 ml 的上層液加入含 0.5 ml isopropanol 的 eppendorff 中，沉澱 10 分鐘後，再 4℃ 下以 12000 rpm

離心 10 分鐘，離心後吸去上層液體， 以 75% ethanol 清洗，最後在 4℃下以 12000 rpm 離心 5 分鐘，吸去上層液體後風乾，待 pellet 成透明狀後加入 30-50 μl 的 ddH_2O 置於 4℃冰箱隔夜儲存。反轉錄聚合酶鏈反應方法同前述。Primers 序列示於 Table 1. 。

Table 1. Primers used in RT-PCR analysis

Primer	Sequence
TNF-α	**Forward 5'-ATGAGCACAGAAAGCATGATCCGC-3'**
(502 bp)	**Reverse 5'-CTCAGGCCCGTCCAGATGAAACC-3'**
IL-6	**Forward 5'-ATGAAGTTCCTCTCTGCAAGAGACT-3'**
(247 bp)	**Reverse 5'-CACTAGGTTTGCCGAGTAGATCTC-3'**
iNOS	**Forward 5'-CAACCAGTATTATGGCTCCT-3'**
(835 bp)	**Reverse 5'-GTGACAGCCCGGTCTTTCCA-3'**
COX-2	**Forward 5'-GGAGAGACTATCAAGATAGTGATC-3'**
(860 bp)	**Reverse 5'-ATGGTCAGTAGACTTTTACAGCTC-3'**
IL-10	**Forward 5'-TGA ATT CCC TGG GTG AGA AG-3'**
(136 bp)	**Reverse 5'-ACA CCT TGG TCT TGG AGC TT-3'**
IL-1Ra	**Forward 5'-AAA TCT GCT GGG GAC CCT AC -3'**
(127 bp)	**Reverse 5'-GGT CAA TAG GCA CCA TGT CT-3'**
GAPDH	**Forward 5'-ACCACAGTCCATGCCATCAC-3'**
(451 bp)	**Reverse 5'-TCCACCACCCTGTTGCTGTA-3'**

七、Electrophoretic mobility shift assay (EMSA)

利用細胞核中的轉錄因子與其調控之基因中特定的DNA序列結合的特性，藉著核蛋白與人工合成的寡聚核苷酸探針 (oligonucleotide probe)結合與否，而在電泳膠中所呈現不同的移動相來判定核轉錄因子活化情形。細胞核蛋白的萃取: 細胞離心後取得的pellet加入約其量4倍體積的lysis buffer (50 mM Tris-HCl, 4mM EDTA, 2mM EGTA)，充分混勻後，置於冰上

20分鐘。取少量上述反應液加入等體積trypan blue於顯微鏡下觀察細胞lysis情形，若仍有大部分細胞未反應完成，則再輔以微研磨器研磨數次後，以10000 r.p.m.在4℃下離心20分鐘。收集pellet並與Tortex buffer (20 mM Hepes/ pH 7.9, 350 mM NaCl, 20％ glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA/pH8.0, 0.1 mM EGTA, 0.5 mM DTT, 0.1％ PMSF, 1％ Aporotinin)充分均勻混合，在4℃反應過夜。以12000 r.p.m.在4℃離心15分鐘後，收集上清液進行蛋白質濃度的測定。分裝每管5~15 μg的蛋白，保存於-80℃備用。取 5 μg 細胞核的萃取物加入 20 μl binding reaction mixture 【含2 μL的buffer D (20 mM HEPES/pH7.9, 20％ glycerol, 100 mM KCl, 0.5 mM EDTA/pH8.0, 0.25％ NP-40, 2 mM DTT, 0.1％ PMSF)、4 μL的buffer F (20％ Ficoll 400, 100 mM HEPES/pH7.9, 300 mM KCl, 10 mM DTT, 0.1％ PMSF) 】及5 μL的biotin標定的探針 (NF-κB或AP-1；5 pmole) ，NF-κB(5’-AGTTGAGGGGACTTTCCCAGGC-3’)；AP-1 (5’-AGTTGAGGGGACTTTCCCAGGC-3’) 30℃反應30分鐘，其間每10分鐘將樣品震盪混合一次。反應完成後加入6倍的loading dye 【20％ 5倍 TBE (0.445 M Tris, 0.445 M boric acid, 0.01 M EDTA), 50％ glycerol, 少許 Xylene cyanol】與樣品混合均勻，在4℃於6％ Native acrylamide gel (需先以20 mA先行電泳1小時)以30 mA進行電泳2小時。待電泳完成，將膠片浸潤於0.5倍的TBE buffer 10分鐘，利用濕式轉漬槽在4℃、150 mA轉漬1小時，將蛋白轉漬至NC membrane上。NC membrane經1倍 TBS (2.7 mM KCl, 10 mM Tris-Cl, 137 mM NaCl/pH 7.5)潤洗後，以UV crosslinker照射固定，再以1倍 TBS所泡成的5%脫脂牛奶進行blocking 30分鐘。以1倍TBS清洗NC membrane 4 次，每次5分鐘。依1：20000的體積比配製 Strepavidin-Horseradish Peroxidase (Jackson ImmunoResearch Laboratories, Inc., PA USA)於1倍TBS中，並將NC membrane 靜置於其中，在4℃中反應過夜。隔天再將其移至室溫下反應1

小時後，以1倍TBS清洗NC membrane 4次，每次5分鐘。將NC membrane與ECL (enhanced chemiluminescence) detection kit (PerkinElmer Life Science Inc., MA USA)在室溫下反應5分鐘後，裝於透明塑膠袋內並置於壓片夾(cassette)中，以X-ray film (Fuji Photo Film Co., Ltd., Tokyo Japan) 感光後，復以自動洗片機 (Hope X-ray Products Inc., PA USA)洗片顯影。

八、流式細胞儀分析活性氧化物 (ROS)

ROS 的受質 2', 7'-dichlorofluorescin diacetate (H_2DCFDA) 可直接進入細胞內接著被分解成無螢光親水性物質 dichlorofluorescein (DCFH)。當細胞產生活性氧分子(ROS) 可將 DCFH 氧化形成螢光物質 2', 7'-dichlorofluorescein (DCF)，利用流式細胞儀偵測螢光物質的多寡以代表 H_2O_2 生成量。 MH-S 細胞先以 H_2DCFDA (5 μM, Molecular Probes, Eugene, Oreg) 和 luteolin (5-25 μM) 前處理 30 分鐘，接著以 LPS (100 ng/ml) 於 37^{o}C 培養 0-15 分鐘，收集不同時間及不同處理組別的細胞以預冷的PBS清洗，利用流式細胞分析儀 (FACSCalibur flow cytometer, Becton Dickinson, CA, USA) 及 Cell Quest software 分析各組細胞的螢光變化量。

第三節 統計分析

實驗結果以平均值±標準偏差(mean ± SD.)表示，使用 ANOVA 分析，並以 Bonferroni correction 和 Student's t-test 來評估藥物處理組與對照組之差異。當 p <0.05、p <0.01或 p <0.001 時，顯示統計上具顯著差異。本論文之細胞學實驗，每一處理至少三重複，同樣實驗至少重複執行三次以上。

第四章 結 果

Part A 以活體動物及離體細胞模式評估木犀草素抗肺纖維化作用及分子機轉探討

一、木犀草素減少 bleomycin 引發之急性期肺炎

肺灌洗液中的發炎細胞數量的增加可反應出急性肺損傷之後產生的肺部發炎程度。我們也從從小鼠肺灌洗液中的發炎細胞數量來評估木犀草素對於 bleomycin 引發急性期肺炎之抗發炎效果。在 bleomycin 造模後第3天，肺灌洗液中的發炎細胞總數明顯增加，到了第 7 天發炎細胞總數激增為原來的 5 倍之多(Table 2)，以木犀草素或類固醇藥物治療組的老鼠，在整個期間肺灌洗液中的發炎細胞總數都比 bleomycin 組少，在第 7 天減少程度最為明顯 (2.28 ± 0.17 / 1.35 ± 0.11 $x10^5$)。以細胞分群所佔的比例來看，木犀草素可明顯減少 bleomycin 造成的嗜中性白血球增加 (21.07 ± 2.43 / 14.4 ± 2.99 %)。

急性肺炎的另一項指標為肺灌洗液中早期發炎細胞激素的激增，尤其是 TNF-α 和 IL-6。從 ELISA 分析結果發現，在 bleomycin 造模後第 3 天，IL-6 濃度增加為正常的 3 倍 (Figure 1B)；在 bleomycin 造模後第 7 天，TNF-α 濃度增加為正常的 4 倍多 (Figure 1A)，而木犀草素治療組明顯地降低由 bleomycin 引發的 TNF-α 和 IL-6 釋放，與類固醇藥物治療組相較並無明顯差異。

直接從 H&E 染色的小鼠的肺組織切片也可以觀察肺損傷程度。在 bleomycin 造模後第 7 天，大量的單核球浸潤，佔據了大部分的肺泡與間質，也破壞了正常的肺實質結構，到了第 14 天，單核球浸潤現象略為減緩。木犀草素治療組可減少單核球的聚集，保留肺組織的完整性，類固醇治療組也有接近的保護效果。統計所有切片的肺損傷程度量化值顯示木犀

草素治療組受到的破壞較少，與類固醇藥物治療組相較並無明顯差異(Figure 2)。

二、木犀草素減少 bleomycin 引發之肺纖維化

在對木犀草素的抗肺纖維化作用進行評估之前，首先要瞭解由 bleomycin 造成小鼠肺纖維化的病程進展。 Figure 3A 為 bleomycin 造模後經過不同天數的 Masson's Trichrome 染色病理切片，紅色部分為正常的細胞構成的肺組織結構，藍色部分為膠原蛋白的沉積。正常的肺組織及肺泡結構完整，bleomycin 造模後第 3 天肺泡開始被大量的發炎細胞佔據，第 7 天時，肺泡間隔明顯加寬，可見數量不等的纖維組織增生伴有些微的膠原產生；到了第 10 天，膠原蛋白明顯增加；到了第 14天，肺泡間隔可見大量增生的上皮細胞，並有為數不少的巨噬細胞散布在肺泡中(Figure 3B)，肺泡間質累積大量的膠原蛋白(Figure 3C)；第 21 天，肺泡壁被大量沉積的膠原蛋白瓦解，肺泡塌陷，形成局部的纖維化病變（focal fibrotic lesions)。

接著進行木犀草素的抗肺纖維化作用評估，並參照實驗方法中所述之量化標準進行統計(Figure 4)。實驗分為二部分:(1)早期給藥療程:於造模後隔天每日以胃管餵食藥物；(2)後期給藥療程: 於造模後第 10 天才開始每日以胃管餵食藥物。從第 21 天的組織病理切片圖發現，在早期給予木犀草素治療的小鼠的肺組織纖維化現象明顯比 bleomycin 造模組輕微許多(Figure 5A)，膠原沉積的面積和密度也比較小，統計所有切片的肺纖維化程度量化值顯示，早期給予木犀草素治療可以預防肺纖維化的產生 (Mean fibrosis score, p<0.001 vs. BLM)；早期給予傳統的抗發炎藥物類固醇也有相近的效果 (p<0.01 vs. BLM)。在造模後第 10 天也就是膠原沉積已形成之後才開始給予木犀草素治療，對肺纖維化也有明顯的改善 (Figure 5B)

(Mean fibrosis score, $p<0.01$ vs. BLM)；後期的類固醇治療效果則較不明顯 ($p<0.05$ vs. BLM)。

膠原沉積是肺纖維化的重要指標；促纖維化激素 (profibrotic cytokine) TGF-β1 的高度表現也對纖維化有很大的促進作用。我們也從小鼠肺組織的膠原沉積量和 TGF-β1 基因表現來評估木犀草素對肺纖維化的改善程度。在 bleomycin 造模後 21 天，肺組織的膠原蛋白量明顯增加為正常的 5 倍，早期給予木犀草素治療可以明顯減少肺組織的膠原蛋白量 (Figure 6A) ($p<0.001$ vs. BLM)；早期給予類固醇也有相近的效果 ($p<0.01$ vs. BLM)。在造模後第 10 天才開始給予木犀草素治療，也可以減少肺組織的膠原蛋白量 (Figure 6C) ($p<0.01$ vs. BLM)；後期的類固醇治療則無法減少肺組織的膠原蛋白量。在肺組織的 TGF-β1 基因表現部分，早期給予木犀草素治療可以明顯減少肺組織的 TGF-β1 mRNA 表現量 (Figure 6B) ($p<0.001$ vs. BLM)；在造模後第 10 天才開始給予木犀草素治療，也可以減少肺組織的 TGF-β1 mRNA 表現量 (Figure 6D) ($p<0.01$ vs. BLM)；晚期類固醇治療與早期類固醇治療一樣，對肺組織的 TGF-β1 mRNA 減少程度較不明顯 ($p<0.05$ vs. BLM)。 這些結果提供了更多的證據證明木犀草素不僅在肺損傷的急性期有良好的抗發炎作用，在纖維化已形成的階段也有明顯的改善效果。

三、木犀草素抑制小鼠肺纖維母細胞的生長分化與分泌 ECM

從動物實驗結果得知木犀草素有良好的抗肺纖維化作用，為了能更確認木犀草素抑制肺纖維化的作用機轉，我們對一些參與肺纖維化的細胞進行分析。肺纖維母細胞在纖維化起始及進展過程中扮演相當重要的角色，它會分泌多種 fibrogenic mediator，而且是 ECM 的主要製造者，肺纖維母細胞在 TGF-β1 的刺激下，會增加 α-SMA 及 ECM 蛋白的表現，也會進

一步分化成肌纖維母細胞，分泌更多的 fibrogenic mediator。

我們從小鼠的肺分離出肺纖維母細胞，繼代培養純化後，以不同濃度木犀草素 (10, 25, 50 μM) 單獨處理肺纖維母細胞，從 72 小時的生長曲線圖中可發現，木犀草素對肺纖維母細胞有生長抑制作用，而且有劑量依存效應 (Figure 7)。25 μM 的木犀草素幾乎可完全地抑制細胞的生長；50 μM 的木犀草素並不會對肺纖維母細胞造成傷害。

木犀草素除了能有效抑制肺纖維母細胞的生長，對纖維母細胞分化的影響是進一步探討的重點。α-SMA 的表現增加是纖維母細胞分化的一項指標，分化後的肌纖維母細胞 大量表現 α-SMA 以形成 stress fiber，分泌 ECM 的能力也增加。從細胞免疫螢光染色的照片中發現，肺纖維母細胞單獨處理 TGF-β1 (5 ng/ml) 72小時後，α-SMA 大量表現且呈平行束狀排列；前處理 25 μM 的木犀草素的細胞則可明顯抑制由 TGF-β1 刺激所造成的 α-SMA 大量表現 (Figure 8)。 Western blot 的 α-SMA 蛋白表現量數據也再一次驗證此項觀察結果(Figure 9) (p<0.01 vs. TGF-β1)。

TGF-β1 除了改變纖維母細胞的型態之外，還會促使細胞製造更多的細胞間質蛋白，其中最主要的是 collagen I/III。從 Figure 9 western blot 的結果顯示，單獨處理木犀草素已明顯減少肺纖維母細胞製造 collagen I (p<0.01 vs. Veh)；單獨處理 TGF-β1 時 collagen I 被大量製造；細胞前處理木犀草素則可抑制由 TGF-β1 刺激所造成的 collagen I 大量表現。Vimentin 是另一種肌纖維母細胞 的指標蛋白，木犀草素單獨或與 TGF-β1合併處理細胞都可抑制 vimentin 的表現 (Figure 9)。

Smads 蛋白家族是細胞內負責將 TGF-β1 刺激所產生的訊息傳遞至細胞核的傳遞者，研究也顯示在 BLM-induced 的肺纖維化組織有 Smads protein 的過度表現。因此我們也將探究木犀草素是否影響早期的 TGF-β1/Smads

訊息傳遞。Western blot 的結果顯示 (Figure 10)， TGF-β1 刺激 肺纖維母細胞 20-30 分鐘，出現大量的 Smad3 磷酸化蛋白，細胞前處理 25 μM 的木犀草素則可抑制由 TGF-β1 刺激所造成的 p-Smad3大量表現 (p<0.05)；而細胞前處理 TGF-β1 type 1 receptor kinase inhibitor (SB431542) 則完全地抑制了 Smad3 的磷酸化 (p<0.001)。 活化的 p-Smad3 (R-Smad) 必須與 Smad4 (Co-Smad4) 結合成 heterodimer，才能進核參與基因的調控，因此 Smad4 蛋白的表現也會影響 TGF-β1的訊息傳遞。 Western Blot 的結果顯示(Figure 10)，細胞單獨 25 μM 的木犀草素就會抑制 Smad4 蛋白的表現 (p<0.05 vs. Veh)；合併 TGF-β1時亦可降低由 TGF-β1刺激所造成的 Smad4 過度表現 (p<0.01 vs. TGF-β1)。 SB431542 對 TGF-β1刺激所造成的 Smad4 過度表現沒有影響。 綜合以上結果得知，透過抑制 Smad3 磷酸化和減少 Smad4 的表現而阻斷 TGF-β1 訊息傳遞，可能是木犀草素抑制肺纖維母細胞生長、分化和分泌 ECM 的分子機轉之一。

四、木犀草素抑制肺上皮細胞株 A549 進行 EMT

如前述，肺上皮細胞在肺纖維化的過程中也扮演重要角色，除了會分泌與纖維化有關的物質和 ECM components之外，肺上皮細胞在 TGF-β1 刺激下具有 epithelial-mesenchymal transition (EMT) 的能力，會從上皮細胞轉變為具肌纖維母細胞 phenotype 的細胞。接著我們也將探討木犀草素對 TGF-β1- induced EMT 的作用。如 Figure 11 A 所示，正常的 A549 細胞具有上皮細胞的形狀 (cobblestone epithelial morphology) 及 cell-cell adhesion，免疫螢光染色可發現在細胞膜附近有明顯的 epithelial marker (E-cadherin)表現，在TGF-β1 刺激 48小時後，細胞形狀明顯改變，變成 mesenchymal-like 的梭狀細胞，cell-cell adhesion 消失，E-cadherin 分解到細胞質中，木犀草素對這些改變有明顯的抑制作用。 Western blot 的結果

顯示(Figure 11B)，A549 細胞單獨處理 TGF-β1 48小時後，epithelial marker (E-cadherin) 表現減少；mesenchymal phenotypic markers (fibronectin 和 vimentin) 明顯增加，表示 A549 細胞在 TGF-β1 刺激下進行 epithelial-mesenchymal transition。木犀草素對這些改變有明顯的抑制作用(除了vimentin之外)。綜合以上結果得知，透過抑制 EMT 而減少肌纖維母細胞的分布，可能是木犀草素抗纖維化作用的機轉之一。

Part B 木犀草素對於肺泡巨噬細胞的免疫調控

一、木犀草素對細胞存活率之影響

MH-S 和 RAW 264.7 細胞分別以不同濃度 (5、10、25、50 μM) 的木犀草素處理，經 24 小時後，以 Trypsin 染色及 MTT 法偵測木犀草素對細胞是否有毒性，結果發現 50 μM 以下的藥物濃度均不會造成細胞毒性(Figure 12)。

二、木犀草素對 LPS 刺激巨噬細胞產生促發炎細胞激素及發炎物質的影響

TNF-α 和 IL-6 等促發炎細胞激素 (Proinflammatory cytokines) 在發炎過程中扮演重要角色，因此我們首先探討木犀草素是否對 LPS 刺激巨噬細胞產生 TNF-α 和 IL-6 有抑制效果。如 Figure 13 所示，RAW 264.7 和 MH-S 細胞單獨處理 LPS (100 ng/ml) 造成細胞激素大量釋出，而細胞前處理木犀草素 (5-25 μM) 以濃度依存性的方式抑制 LPS 刺激細胞生成 TNF-α (Figure 13A) 和 IL-6 (Figure 13B)。 在 NO 和 PGE_2 的實驗結果也有相同的趨勢(Figure 14)，但因細胞種類不同略有差異，在 RAW 264.7 細胞觀察到木犀草素 5 μM 對 NO 即有明顯的抑制 (50%)(Figure 14A)；而在 MH-S 細胞，木犀草素 5 μM 對 PGE_2 的抑制已達到 80% (Figure 14B)。

三、木犀草素對 iNOS 和 COX-2 的蛋白質的調控

為了探究木犀草素對 LPS 刺激巨噬細胞產生 NO 和 PGE_2 是否與 iNOS 和 COX-2 二種催化酵素有關，接著以 western blot 來分析木犀草素對 iNOS 和 COX-2 蛋白的表現。在未加 LPS 刺激及單獨處理木犀草素的細胞中偵測不到 iNOS 和 COX-2 蛋白的表現。LPS (100 ng/ml) 刺激 8 小時後，iNOS 和 COX-2 蛋白開始有表現，到 14 小時達高峰 (Figure 15)。木犀草素 (25 μM) 在這二株巨噬細胞皆可顯著地抑制由 LPS 刺激所增加 iNOS 和 COX-2 的蛋白表現。

四、木犀草素對相關分子的基因調控

為了探討木犀草素抑制發炎物質的產生是否因為參與相關分子的基因調控之故，接著以反轉錄聚合酶鏈反應 (RT-PCR) 來分析相關分子的 mRNA 表現。MH-S 和 RAW264.7 細胞前處理木犀草素再給予 LPS 刺激，在幾個不同的時間點收集細胞的 RNA 分析 iNOS、COX-2、TNF-α 和 IL-6 mRNA。如 Figure 16 所示，木犀草素 (25 μM) 顯著地抑制由 LPS 刺激所引起的 iNOS、COX-2、TNF-α 和 IL-6 mRNA level。此現象亦符合於蛋白層次的結果。比較 MH-S 和 RAW264.7 二株細胞之反應時間，MH-S 在處理 LPS 2 小時而 RAW264.7 在處理 LPS 4 小時後相關分子開始進行轉錄。綜合上述結果顯示，木犀草素是在基因轉錄層次抑制細胞激素 (TNF-α, IL-6) 及催化酵素 (iNOS, COX-2) 的基因表現，導致下游的 TNF-α, IL-6, NO 和 PGE_2 產量減少。

巨噬細胞分泌的 Interleukin-1 Receptor antagonist (IL-1Ra) 和 Interleukin-10 (IL-10) 有抑制 TNF-α、IL-1、IL-6 和 IL-8 的產生，調節發炎反應之功能。因此本論文也探討木犀草素對這些抗發炎細胞激素有何影響。如 Figure 17 之 RT-PCR 結果所示，MH-S 細胞單獨處理 LPS 或

木犀草素 8 小時後， IL-1Ra mRNA 表現增加 (分別為 $p< 0.05$ 和 $p< 0.01$)；同時處理 LPS 和木犀草素組，木犀草素對 LPS 刺激 IL-1Ra mRNA 有加成的效果 ($p< 0.01$)。MH-S 細胞單獨處理 LPS 會增加 IL-10 mRNA 表現，而木犀草素不會對 IL-10 mRNA 的表現造成任何影響。

五、木犀草素對 LPS 刺激 NF-κB 活化的影響

NF-κB 是一參與發炎物質基因轉錄重要的調節分子 [189]，因此我們推測木犀草素抑制發炎物質的基因表現可能與抑制 NF-κB 的活化有關。本實驗利用 EMSA 實驗來偵測木犀草素對 NF-κB 與 DNA 結合能力的影響。如 Figure 18A 所示，LPS 處理 MH-S 細胞於 60 分鐘時 NF-κB p65 次單元的核轉移現象達到最高峰，此活化作用可因前處理木犀草素而被抑制 (Figure 18B)；細胞如以 PDTC (NF-κB 抑制劑) 前處理亦得到相同的抑制效果。另外我們也利用細胞免疫螢光染色法觀察活化態 NF-κB p65 次單元的核轉移現象。如 Fig 19 所示木犀草素明顯地抑制由 LPS 刺激所造成的 NF-κB p65 nuclear translocation。

NF-κB p65 的核轉移歸因於細胞質內與 NF-κB 結合的抑制性蛋白 IκB-α 被磷酸化 (phosphorylation)、泛素化 (ubiquitination; ubiquitin 是用來接在欲降解蛋白質上的標籤蛋白質)，最後被送到 proteosome 分解[190, 191]，因此利用西方點墨法 (western blot analysis) 來觀察木犀草素對由 LPS 刺激所造成 IκB-α 分解的影響。如 Figure 20A 所示，以 100 ng/ml LPS 處理 MH-S 或 RAW 264.7，引發 IκB-α 快速被分解（10-20 分鐘)，在 60 分鐘時可恢復為原始狀態。細胞若以木犀草素 (25 μM) 或 PDTC (200 μM) 前處理 30 分鐘則可有效抑制由 LPS 刺激所造成 IκB-α 分解 (Fig 20B)。 綜合以上結果得知，木犀草素抑制 NF-κB 的活化，包括 IκB-α 分解、NF-κB p65 次單元的核轉移和 NF-κB 與 DNA 的結合能力。

六、木犀草素對 LPS 刺激 AP-1 活化的影響

文獻報導 activating protein-1 (AP-1) 是另一個轉錄調控因子，細胞在被 LPS 刺激後活化 AP-1 也會增加促發炎激素的製造[192, 193]，因此我們也進一步探討木犀草素對 AP-1 活化的影響。首先利用 EMSA 實驗來偵測木犀草素對 AP-1 與 DNA 結合能力的影響。如 Figure 21 EMSA 實驗所示，LPS 處理 MH-S 細胞於 60 分鐘時 AP-1-DNA complex 結合量達到最高峰 (Figure 21A)，此活化作用可因前處理木犀草素或 curcumin (AP-1 抑制劑) 而被抑制 (Figure 21B)。 從 RT-PCR 的結果顯示，細胞以 10 μM curcumin 前處理，可抑制由 LPS 刺激所造成的 TNF-α、IL-6、iNOS 和 COX-2 mRNA 過度表現 (Figure 22A)。 細胞以 100 μM PDTC (NF-κB 抑制劑)，也可抑制由 LPS 刺激所造成的 IL-6、iNOS 和 COX-2 mRNA 過度表現 (Figure 22B)。木犀草素(25 μM) 對 LPS 刺激所造成的 TNF-α、IL-6 和 iNOS mRNA 抑制效果優於同時使用 curcumin 和 PDTC (Figure 22C)。綜合以上結果得知，LPS 活化了 NF-κB 和 AP-1 二個轉錄分子，導致下游發炎相關分子 (TNF-α、 IL-6、iNOS 和 COX-2) 表現量增加。 木犀草素則抑制由 LPS 刺激所造成 NF-κB 和 AP-1 活化，阻斷了發炎物質的產生。 PDTC 一般被認為可以抑制 NF-κB 的活化，但在我們的幾次重複實驗中發現它並不能使 NF-κB 下游 TNF-α mRNA 的表現減少 (Figure 22B)。

七、木犀草素對對 LPS 刺激 ROS 產生的影響

文獻報導 LPS 會刺激巨噬細胞在很短的時間內產生 ROS [194, 195]。我們利用流式細胞儀偵測 DCF 螢光量表示細胞所產生的 H_2O_2 和 •OH。如 Figure 23A 所示，LPS 處理 MH-S 細胞，5 分鐘時細胞內的 ROS 急速升高。細胞前處理木犀草素 (5-25 μM)可阻止 ROS 的產生，並有劑量依存性 (Figure 23B)。

八、木犀草素對 Akt 與 Ikk 磷酸化的影響

根據前人研究得知 NF-κB 的轉錄活化與上游 IKK 或 Akt 等激酶有關。因此我們也進一步探討木犀草素對 NF-κB 上游的訊息傳遞路徑的參與。從 Figure 24 Western blot 的結果顯示，MH-S 細胞前處理木犀草素 (25 μM) 可抑制由 LPS 刺激在 10 分鐘時所引起的 Akt (Thr308/Ser473) 和 IKKα/β (Ser176/180) 磷酸化。當細胞以 10 μM LY294002 (PI_3K selective inhibitor) 前處理，也有類似的抑制作用。綜合以上結果顯示，木犀草素阻斷了 NF-κB 上游的訊息傳遞路徑，導致 NF-κB 的轉錄無法進行。

第五章　討 論

肺纖維化是一種進行性細胞外間質蛋白過度沉積導致缺氧甚至死亡的呼吸系統疾病，至今還沒有一種藥物能有效地治癒肺纖維化。肺纖維化最主要的病理特徵是肺泡上皮細胞無法再上皮化(re-epithelialization) 和纖維母細胞/ 肌纖維母細胞持續地活化，釋放出大量的胞外間質蛋白破壞了肺組織的結構。儘管近幾年來對肺纖維化的病理變化較為明瞭，但對其病因學及所有參與的細胞及分子機轉仍所知有限。肺纖維化的發展過程通常是不活躍但也不易改變的，因此在治療上有很大的限制，臨床上以類固醇及免疫抑制劑為建議使用的治療藥物，但對多數肺纖維化的病人效果是有限的。長久以來的學說認為急性或慢性肺部發炎是導致肺纖維化的主因，但從許多的臨床及動物實驗結果發現，抗發炎藥物(包括類固醇)對肺纖維化的改善有限；也不是所有原因引起的肺部發炎都會造成肺纖維化的後果，所以近年來的研究傾向於將發炎與纖維化分開來討論。從大多數特異性肺纖維化病人 (IPF) 的病理分析發現，肺部發炎並不是最主要組織病理特徵，反而有明顯的 epithelial–mesenchymal interaction 和不正常的傷口癒合過程，也有越來越多的研究認為纖維細胞的增生引起一連串的肺組織破壞是造成肺纖維化最主要的原因。總之，肺纖維化過度的膠原沉積是在免疫學或遺傳因素影響下，引發各種不同類型的細胞之間複雜的相互作用，造成膠原形成與降解調控失衡的結果。從疾病的相關分子機轉研究中找到新的治療標靶是目前抗肺纖維化藥物研發的方向也是迫不及待的工作。

木犀草素是一種植物黃酮，也是傳統中醫常用來治療呼吸系統疾病的中藥金銀花的主要成分之一，有學者研究顯示木犀草素具有明顯的抗氧化及抗發炎作用，在本研究的預試驗中篩選了幾種植物成分，也發現木犀草素的抗發炎效果優於槲皮素、黃芩素和小檗鹼。因此本論針對木犀草素抗

肺纖維化作用進行療效評估及相關細胞分子機轉的探討。 C57BL/6J 品系小鼠以 bleomycin 氣管投藥會造成持續性的肺部纖維化[196]，因此這種疾病動物模式普遍地被應用於肺纖維化的研究。在熟練的實驗技巧及適當的劑量控制下，我們成功地從氣管注入 bleomycin 誘發小鼠的肺纖維化，其肺纖維化現象可維持到第 4 週，因此我們可以明確地判斷肺纖維化形成的時間及評估藥物的療效。在誘發小鼠肺纖維化的時程中發現在造模後的第 7 天觀察到膠原蛋白已開始形成；在造模後的第 10 天可觀察到明顯的膠原蛋白沉積 (Figure 3A)，推測此時肺部已從發炎期近入纖維化期，結果與 Izbicki 等人的研究相近[197]。Chaudhary 等人認為有許多治療肺纖維化的試驗性藥物在動物實驗時有很好的抗纖維化效果，多無法應用在臨床上，可能是因為大部分的試驗性藥物都具有抗發炎作用，而且在 bleomycin 造模之前或造模同時給藥，能有效阻止或減緩實驗動物的早期肺損傷，因此減少後續的肺纖維化病變，但對臨床上正在進行中或已形成的纖維化病變改善效果則有限[198]。為了區別木犀草素的抗發炎及抗纖維化作用，我們設計了早期與後期治療二個不同給藥時間的動物實驗。從實驗結果發現，bleomycin 造模之後早期給與木犀草素治療的實驗小鼠，肺纖維化明顯減輕，此結果與 bleomycin 造模初期木犀草素的抗發炎作用有關 (Figure 5, 6)。木犀草素透過抑制免疫細胞及纖維細胞的趨化及活化，同時抑制 profibrotic cytokines 與 growth factors 釋放，以減少對肺泡上皮細胞的傷害及胞外膠原的產生，木犀草素在此階段對肺組織纖維化的形成有明顯的預防作用。當 bleomycin 造模之後第 10 天發炎現象已趨緩，膠原沉積已產生時再給與木犀草素治療，肺纖維化也有改善，顯示木犀草素對進行中或已形成的肺組織纖維化有治療的效果，是透過抑制纖維細胞的增生及 TGF-β1 的釋放 (Figure 5, 6)。不論是早期或後期的治療，木犀草素都可以減

少肺組織 TGF-β1 mRNA 的表現與 collagen 含量，也證明木犀草素是透過抑制 TGF-β1 的活性而減少 collagen 的製造與沉積。

Selman 等人認為，纖維化病變除了發炎因素之外還有更多值得研究的主題[85]，例如內因性的異常傷口癒合 (intrinsic aberrant wound healing)，在此種調控機制下，受損的肺泡上皮細胞及實質纖維母細胞之間的相互作用促使細胞轉型成為 mesenchymal cell phenotype，並往纖維化發展(fibroblast proliferation, myofibroblast differentiation and matrix synthesis)。 TGF-β1 是調控上皮細胞與 mesenchymal 細胞的交互作用最重要的分子，與傷口癒合及纖維化的關係密不可分。Munger 也發現過度的 integrin αvβ6 表現可促進 latent TGF-β 活化，製造有利於纖維化發展的條件，這個現象與發炎反應不一定有關[81]，雖然發炎反應最後也會造成 TGF-β 的活化。或許將肺纖維化定義為由包括發炎在內的多種病因造成的“併發症”，而不是一種“特殊的疾病”比較不會造成模糊的印象。

從動物實驗的結果與相關的文獻探討印證後我們進一步的推測，木犀草素也會作用於除了免疫細胞之外參與纖維化形成的其它細胞，例如肺纖維母細胞和肺泡上皮細胞，因此我們利用細胞學實驗來探討木犀草素對這些細胞的作用。實驗結果發現，木犀草素可以抑制肺纖維母細胞的生長，對 TGF-β1 刺激所產生的 肌纖維母細胞 differentiation 及 collagen I production 都有抑制作用 (Figure 7, 8, 9)，驗證了動物實驗的結果。此外，肺纖維母細胞單獨處理木犀草素時可減少 mesenchymal related marker (α-SMA, Vimentin and collagen production) 的表現，顯示木犀草素可能具有促進 MET (mesenchymal epithelial transition) 的能力，有助於肺泡上皮細胞的再上皮化(re-epithialization) 與正常肺結構的重建。 TGF-β1與纖維化的關係密不可分，TGF-β1在細胞內的訊息傳遞過程及如何調控相關分子的表現也變得極為重要。許多的研究都發現 TGF-β1下游的 Smad3 的活化對纖

維化有重要的影響[199-201]。Zhao 等人指出 SARS-CoV nucleocapsid (N) protein 促進上皮細胞 TGF-β1與 Smad3 的活化使下游 PAI-1 表現增加，與肺纖維化的形成有重大關聯[202]，這也可以解釋為何 SARS 病人發病早期就可觀察到肺纖維化的病灶，除了發炎損傷後的修復動作之外，直接活化 Smad3 也是因素之一。我們也證明了在短時間內木犀草素會透過抑制 Smad3 的磷酸化與 Smad4 的表現，阻斷 TGF-β1的訊息傳遞，導致下游與組織癒合及纖維化相關分子的表現減少 (Figure 10)，為日後研究金銀花及木犀草素在 SARS 病毒感染造成呼吸系統病變的應用方面提供參考數據。此外我們也首次發現木犀草素在短時間內會減少 Smad4 (Co-Smad) 的表現而影響 TGF-β1的訊息傳遞 (Figure 10)，推測木犀草素會影響 Smad4 蛋白的穩定性，至於木犀草素是透過何種方式(例如 proteosome 的分解) 影響 Smad4 蛋白的穩定性，是值得繼續探討的題目。

肌纖維母細胞在肺纖維化發展過程中是最主要的胞外基質分泌細胞，從 IPF 切片觀察中發現肌纖維母細胞數量增加，形成越多的 fibroblastic foci，對肺纖維化的癒後越不利[203]。這些被活化的肌纖維母細胞會大量表現出 mesenchymal immunocytochemical markers、有收縮能力的 intracytoplasmic stress fibers (例如 α-SMA) 並製造大量的膠原蛋白。因此，探究損傷後的肌纖維母細胞如何增生及其來源細胞的種類是很重要的，因為調控 fibroblast phenotype 細胞的分化和增生是完全不同的機制。從 IPF 病人的肺分離出的纖維母細胞具有與正常人的肺纖維母細胞不同的 heterogenous phenotypes 和特性，因此推測這些 heterogenous phenotypes 的細胞可能是由許多不同種類細胞分化而來[204]。越來越多的研究發現肺泡上皮細胞 (AECs) 經過 epithelial–mesenchymal transition 可轉變成具有 mesenchymal phenotype 的細胞並作為纖維母細胞和肌纖維母細胞的來源細胞[75, 76, 205]。從這個層面來說，肺泡上皮細胞在參與肺纖維化的過程中扮

演關鍵性角色，它是一種“multipotent progenitor”，受不同的因素刺激就可能走向不同的命運，包括 re-epithelialization 結構重建、apoptosis 或經由 EMT 分化成 fibrogenesis 的細胞等，對研究肺纖維化的發展來說，釐清在何種調控因素下使 AECs走向不同的命運有助於對疾病的控制。Kevin K. 曾指出 TGF-β1 的活化和某些特殊的 ECM 蛋白過多是促使 AECs 走向 EMT 及發展為 fibrotic lung 的重要影響因素[206]。從我們的實驗結果也印證了這個現象，TGF-β1 不但改變了 A549 細胞原來上皮細胞的形態，原本聚集在細胞膜周圍作為 cell-junction 的 E-cadherin 崩解 (Figure 11A)，隨著 epithelial marker 的減少，取而代之的是 mesenchymal marker (fibronectin 和 vimentin) 的增加，木犀草素對 TGF-β1-induced EMT 則有抑制的作用(Figure 11B)，推測應該也是與阻斷 TGF-β1 訊息的傳遞有關。

黃酮類化合物為普遍存在於植物中的二次代謝產物，一般認為其具有廣泛的生物活性，如抗氧化、抗菌、抗發炎、抑制腫瘤生長及血管新生等作用[207]。 在哺乳動物的細胞學研究顯示，某些植物黃酮類化合物可抑制發炎物質 (pro-inflammatory cytokines and mediators) 的產生而具有抗發炎作用[208]。 發炎期過多的細胞激素 (如TNF-α 和 IL-6) 會造成器官組織的傷害，如風溼性關節炎、血管硬化，甚或導致敗血性休克。可抑制發炎物質的產生或可調控發炎物質基因表現的化合物都具有成為抗發炎藥物的潛力，而天然植物黃酮類化合物具有這些生理活性，現今已成為熱門的抗發炎藥物研發標的。木犀草素屬植物黃酮類化合物，之前的研究顯示木犀草素對 C57BL/6J老鼠的細菌性肺炎具有保護作用，可降低死亡率及肺組織發炎程度[40]。木犀草素能有效地減緩實驗小鼠由 bleomycin 氣管投藥所造成的急性肺炎(Table 2, Figure 1, 2)，其抗發炎效果與傳統抗發炎藥物類固醇相近。木犀草素的抗發炎機轉為減少發炎細胞尤其是嗜中性白血球的趨化聚集及抑制發炎激素的釋放。此項動物實驗的結果也驗證了木犀草素在細

胞免疫方面的調節作用。肺泡巨噬細胞為分布於肺泡周圍對病原體最先起反應的免疫細胞，在本研究中發現，木犀草素不管是在肺泡巨噬細胞 MH-S 或是周邊巨噬細胞 RAW 264.7，都明顯地抑制由 LPS 引發 TNF-α、IL-6、COX-2 和 iNOS 的基因表現。我們的結果與前人在不同的巨噬細胞之研究結果一致[31, 33, 209]。

單核球/巨噬細胞包括骨髓中的前單核球、周邊血中的單核球及遷移到組織內的巨噬細胞，組織內的巨噬細胞來自於血液中的單核球。組織內特化的巨噬細胞除負責一般的免疫功能之外，還有維持生理恆定及組織修復等功能。分布到不同組織的巨噬細胞因組織環境的刺激，在型態上及功能上有不同的分化特性(包括cytotoxicity, migration, oxygen radical production, pinocytosis 和 tissue factor production)。 分布到肺泡的巨噬細胞會受表面張力劑或其它肺組織液成分所影響[210]。 肺泡巨噬細胞是呼吸系統中重要的防禦細胞，但與血液中的單核球和腹腔巨噬細胞相較之下，為較差的 antigen-presenting cell。也有研究指出不同組織特化的巨噬細胞，對 LPS 刺激的基因活化表現有不同的調控方式[211]。在此，我們比較了木犀草素對 LPS 刺激分布於不同組織的巨噬細胞發炎相關基因表現的異同處，從 Figure 16 發現分布於不同組織的巨噬細胞對 LPS 刺激產生的基因活化的反應時程不同，肺泡巨噬細胞 MH-S 在 LPS 刺激 2 小時即啟動發炎相關物質基因的轉錄；RAW 264.7 則要在 LPS 刺激 4 小時後，發炎相關物質基因始進行轉錄。此外，木犀草素在 MH-S 與 RAW 264.7 的抑制現象有相同的趨勢，但是在 MH-S 的抑制程度比在 RAW 264.7 觀察到的結果來的明顯。由以上結果可歸納出肺泡巨噬細胞對於刺激與保護作用 (LPS 和木犀草素) 都比周邊巨噬細胞敏感。

我們的研究更發現木犀草素除了明顯地抑制巨噬細胞產生發炎相關物質，同時對於巨噬細胞細胞本身產生的抗發炎分子也有促進活化的效果。

MH-S 細胞單獨處理木犀草素會增加 IL-1Ra 的基因表現；木犀草素與 LPS 同時處理細胞時，會加強 IL-1Ra 的基因表現，顯示木犀草素有"抑制發炎"和 "促進抗發炎" 分子表現的雙重作用。

腫瘤壞死因子 (TNF-α) 在發炎反應中扮演重要角色，在 LPS 刺激下的巨噬細胞可大量產生 TNF-α，並促使其它的細胞激素分泌。 *In vivo* 及 *in vitro* [212, 213] 的研究都認為 PDTC (NF-κB 抑制劑)可以抑制 NF-κB 的活化而減少由TNF-α 及 LPS 引起的發炎物質之基因表現。因此， PDTC 常被用來作為 NF-κB signaling pathway 的阻斷劑[37]。 然而，在我們的實驗中確發現 MH-S 細胞前處理 PDTC 的確可抑制由 LPS 刺激引起的 IκB degradation (Figure 20B)及繼發的 NF-κB-DNA binding (Figure 18B)，但確無法使 NF-κB 下游 TNF-α mRNA 的表現減少 (Figure 22B)。 推測可能原因有二個: (1) MH-S 細胞在 LPS 刺激下導致發炎物質的產生，除了 NF-κB 之外可能還有其它訊息傳路徑的參與；而木犀草素對 TNF-α mRNA 的抑制應該還有其它轉錄分子的調控，(2) PDTC 一般被認為可以抑制 NF-κB 的活化，但是有可能在某些細胞此藥物對 NF-κB 的抑制效果專一性不夠，無法完全阻斷 NF-κB 下游的基因表現。

曾有學者研究指出 TNF-α gene 的 promoter region 前端除了 NF-κB 之外還有 AP-1 的 binding sequence [214]。接著也證實 LPS 的刺激使 AP-1 活化並結合至 TNF-α gene binding site，並啟動 TNF-α 的基因轉錄，使 TNF-α mRNA 表現增加[215]。在我們的研究中也發現，MH-S 細胞前處處理 curcumin (AP-1 抑制劑) 顯著地抑制由 LPS 刺激導致 TNF-α mRNA 的表現 (Figure 22A, C)；再者，木犀草素和 curcumin 皆明顯地抑制由 LPS 刺激導致 AP-1-DNA binding (Figure 21B)。綜合上述結果推測木犀草素抑制 LPS 刺激導致 TNF-α mRNA 的表現主要是透過阻斷 AP-1 的活化。

IL-6 是一種多功能的 (pleiotropic) 細胞激素，其功能包括調節人體的免疫反應、造血作用及骨質代謝，也與神經系統及內分泌系統的生理功能有關[216]。因感染、外傷或其它壓力造成的急性發炎期 IL-6 快速的被製造，過多的 IL-6 造成嚴重發炎及組織傷害。 我們的研究結果顯示，木犀草素不止抑制了 MH-S 和 RAW 264.7 細胞由 LPS 刺激產生的 IL-6，也減少由 LPS 刺激產生的 IL-6 mRNA 的表現，證明木犀草素是在基因轉錄層次調控 IL-6 的表現。我們的結果顯示， 雖然木犀草素都會抑制 NF-κB 和 AP-1 的活化 (Figure 18B, 21B)，但從 PDTC 和 curcumin 抑制 IL-6 基因表現的結果顯示 (Figure 22)，LPS 刺激產生的 IL-6 基因表現最主要還是透過 NF-κB 的活化，此結果與前人的研究有一致性[217]。

木犀草素在不同的細胞均可抑制由 LPS 刺激產生 NO 及 iNOS 的表現[33, 43, 44]。我們的實驗結果也顯示在 MH-S 和 RAW 264.7 巨噬細胞，木犀草素抑制由 LPS 刺激產生 NO 、iNOS 的蛋白及 iNOS 基因表現為一致性 (Figure 14, 15, 16)。細胞受到 LPS 刺激誘發 iNOS 基因表現，其中 NF-κB 與 AP-1的活化都有可能是調控因素[218, 219]， 從我們的實驗結果發現木犀草素抑制細胞由 LPS 刺激 iNOS 基因表現最主要是透過抑制 NF-κB 的活化。

ROS 在發炎過程中扮演著訊息傳遞的角色，促使細胞產生更多的發炎物質，同時也對宿主的組織器官造成傷害。許多的研究指出，ROS 透過活化包括 NF-κB 和 AP-1 在內的轉錄分子而增加下游與發炎相關基因的表現[220]。ROS 形成細胞內的氧化壓力造成 IKK 或 Akt kinase 活化，再將下游的 IκB 磷酸化後分解[221]；加入抗氧化劑可抑制 NF-κB 和 AP-1 的活化並減少發炎激素產生，可能與抗氧化劑減少 ROS 有關[33, 222]。 木犀草素為黃酮類分子，具有抗氧化及清除自由基的作用[209]。在本研究中，以 LPS 刺激細胞後 5 分鐘就有 ROS 產生；接著在 10-20 分鐘 IκBα 磷酸

化分解；NF-κB p65 約在 30 分鐘進入細胞核與 DNA binding。 綜合這些結果推測木犀草素抑制肺泡巨噬細胞發炎物質的產生可能是因為它的抗氧化作用而阻斷 NF-κB 與 AP-1 的訊息傳遞。

當巨噬細胞上的 receptor 受到 LPS 的刺激後，會活化細胞內的訊息傳遞，過程中牽涉到一連串的激酶磷酸化反應。有研究指出 LPS 刺激會先將 IKK 或 Akt kinase 磷酸化，再將 IκB 磷酸化而活化 NF-κB[223]。Xagorari 等人指出，木犀草素可抑制 LPS 刺激 RAW 264.7 巨噬細胞 MAPKKS family 的活化，包括 MEK1/2、MKK3/6、ERK 和 p38 的磷酸化[31]。 Ozes 和 Madrid 的研究指出，NF-κB 的活化必須靠 Akt 將 IKKα/β 磷酸化[224, 225]。我們的實驗結果也顯示 MH-S 細胞在前處理木犀草素時會抑制由 LPS 刺激所引發的 Akt 磷酸化 (Ser473 和 Thr308) 及 IKKα/β 磷酸化 (Ser176/180)，其抑制效果與前處理 Akt 抑制劑 LY294002 接近 (Figure 24)，此抑制作用造成 NF-κB 無法活化。由此可推測木犀草素會抑制由 LPS 刺激所引發的 Akt 及 IKKα/β 磷酸化也可能是其抗發炎作用的路徑之一。

黃酮類分子大都具有抗氧化的作用，但結構上的差異會產生不同的生物活性。 從 structure-activity relationship (SAR) 的分析來看，有些結構是抗發炎活性所必備[33]。如結構上同時具備下列幾項:在 5, 7, 3'和4' 位置的碳上接有-OH 基；C 環的 C2-C3 為雙鍵； C 環以 C2 位置與 B 環連接，其抗發炎作用最佳。在我們的預試驗中，曾經比較過幾個黃酮類分子 (槲皮素、黃芩素和木犀草素) 在巨噬細胞的抗發炎效果及相關分子的作用，其中以 luteolin 的抗發炎效果是最好的，符合 SAR 的分析結果。其它黃酮類分子的抗發炎作用是否透過其它作用機轉或者會在不同的分析系統得到不同的結果，這些都需要更多的研究來釐清。

中草藥的使用有悠久的歷史，近幾年也開始被西方醫學所重視。 陸續增加的現代藥理研究也使中藥的藥理機轉日益明確。一般認為中草藥在抗發炎的應用上有很大的開發潛力，不論在動物體內或體外的研究都有不錯的成效。植物黃酮類化合物是抗發炎中草藥中已知的活性成分之一，但這些研究結果是否與臨床的實際結果相符，則需更進一步的探討這些活性成分進入人體後的動力學來驗證。本論文所探討的木犀草素對肺部發炎有良好的治療效果，將其製成噴霧劑型直接作用於呼吸系統或許是可以考慮的投藥方式。

第六章　結 論

從我們的研究結果也發現木犀草素具有多靶點的藥理特性，在不同的疾病進展階段對不同的標靶分子產生特定的作用。總結如下：

一、 在 bleomycin 引發小鼠肺纖維化的早期，木犀草素有抗發炎的作用；而其抗發炎的機轉與其抗氧化特性及抑制 NF-κB 和 AP-1 等轉錄分子的訊息傳遞有關。

二、 早期給與木犀草素對 bleomycin 引發肺纖維化的保護作用，其機轉可能與減少發炎的傷害或直接抑制 profibrotic cytokine (TGF-β1) 的產生有關。

三、 後期給與木犀草素對 bleomycin 引發肺纖維化的抑制作用，其機轉與抑制 TGF-β1/Smads 的訊息傳遞，導致相關分子的表現減少有關。

此結果可說明傳統抗發炎中藥金銀花在生物體內的藥效展現與其成分木犀草素有關，日後仍需對金銀花其它成分之作用加以分析研究。此外，也應以中醫分期論治及扶正祛邪的理論為基礎，透過科學化的研究，確立傳統治療肺纖維化的中藥，如丹參、三七、黃耆等之作用機轉。

Table 2 Effects of luteolin on total and differential cell counts in BALF.

Group	Total cells (x 10^5 /ml)	Cell Differentiation (%)		
		Neutrophils	Macrophages	Lymphocytes
Day 3				
Control	0.38 ± 0.04	3.08 ± 0.65	94.36 ± 6.41	3.35 ± 0.56
BLM	1.49 ± 0.12	29.48 ± 3.27	66.27 ± 6.96	3.58 ± 0.81
BLM+Lut	1.16 ± 0.11*	27.32 ± 2.42	67.42 ± 7.39	3.94 ± 0.57
BLM+Pred	1.01 ± 0.09*	25.16 ± 3.02	69.97 ± 6.44	4.41 ± 0.73
Day 7				
Control	0.41 ± 0.06	3.38 ± 0.85	92.54 ± 5.15	3.81 ± 0.47
BLM	2.28 ± 0.17	21.07 ± 2.43	69.79 ± 6.64	8.75 ± 1.17
BLM+Lut	1.35 ± 0.11***	14.04 ± 2.99**	75.89 ± 6.77	8.44 ± 0.79
BLM+Pred	1.43 ± 0.18***	12.36 ± 2.54**	76.92 ± 10.37	8.76 ± 1.12
Day 14				
Control	0.36 ± 0.07	2.84 ± 0.54	95.49 ± 5.15	3.53 ± 0.32
BLM	1.45 ± 0.15	16.88 ± 2.43	74.72 ± 3.48	7.96 ± 1.36
BLM+Lut	1.01 ± 0.09	10.48 ± 2.13	79.89 ± 6.77	8.23 ± 1.13
BLM+Pred	0.96 ± 0.11	11.68 ± 1.65	77.72 ± 9.66	8.05 ± 1.33

Total and differential white blood cell counts in bronchoalveolar lavage fluid (BALF) measured at 3, 7 and 14 days after intratracheal instillation of bleomycin or saline as indicated. Data are presented as mean ± SD. in the group of ten mice. * $p < 0.05$,** $p < 0.01$, *** $p < 0.001$ as compared with the BLM treatment. BLM, bleomycin; Lut, luteolin; Pred, prednisolone.

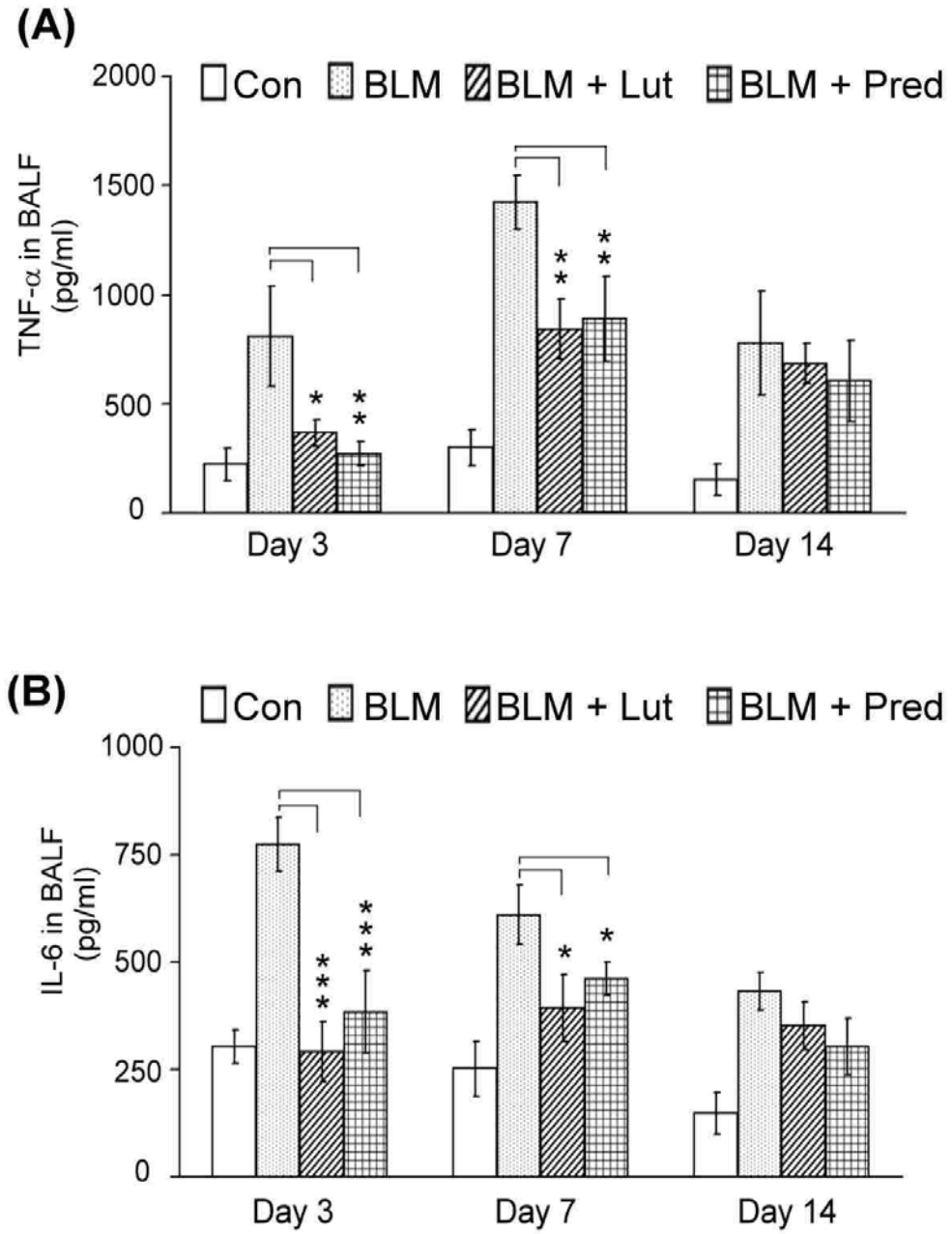


Figure 1. Time-course changes in the levels of TNF-α and IL-6 level in BALF.

Time-course changes of TNF-α and IL-6 level in bronchoalveolar lavage fluid (BALF) at 3, 7 and 14 days postbleomycin or saline in experimental groups as indicated. Mice were daily and orally treated with vehicle, luteolin (10 mg/kg) or prednisolone (5 mg/kg). Bronchoalveolar lavage was performed as described in *Materials and Methods*. The levels of (A) TNF-α and (B) IL-6 in BAL fluid were measured. Data are presented as mean ± SD. in the group of ten mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared with the BLM treatment. BLM, bleomycin; Lut, luteolin; Pred, prednisolone.

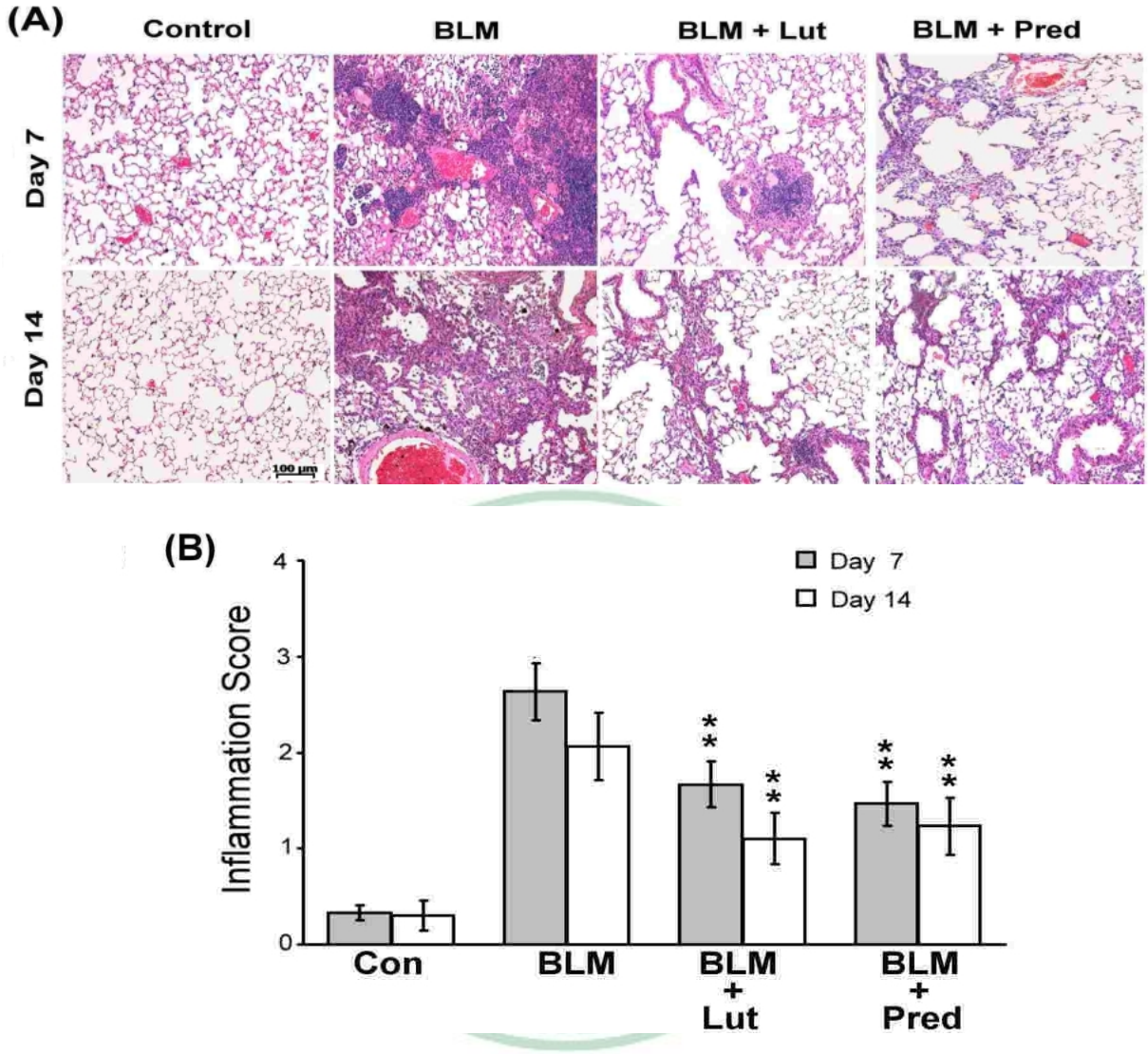


Figure 2. Attenuation of bleomycin-induced lung inflammation by luteolin.

Mice were treated as described in Fig 1, lung tissues were collected and stained with H&E. Images were selected according to the alveolitis scores. Bleomycin-treated mice appeared inflammation in the lung interstitium (A). Magnification x10. Scale bars, 100 μm. Histological lesions scores of lung tissues from experimental groups (B). Data are presented as mean ± S.D. in the group of ten mice. ** $p< 0.01$ as compared with the BLM treatment. BLM, bleomycin; Lut, luteolin; Pred, prednisolone.

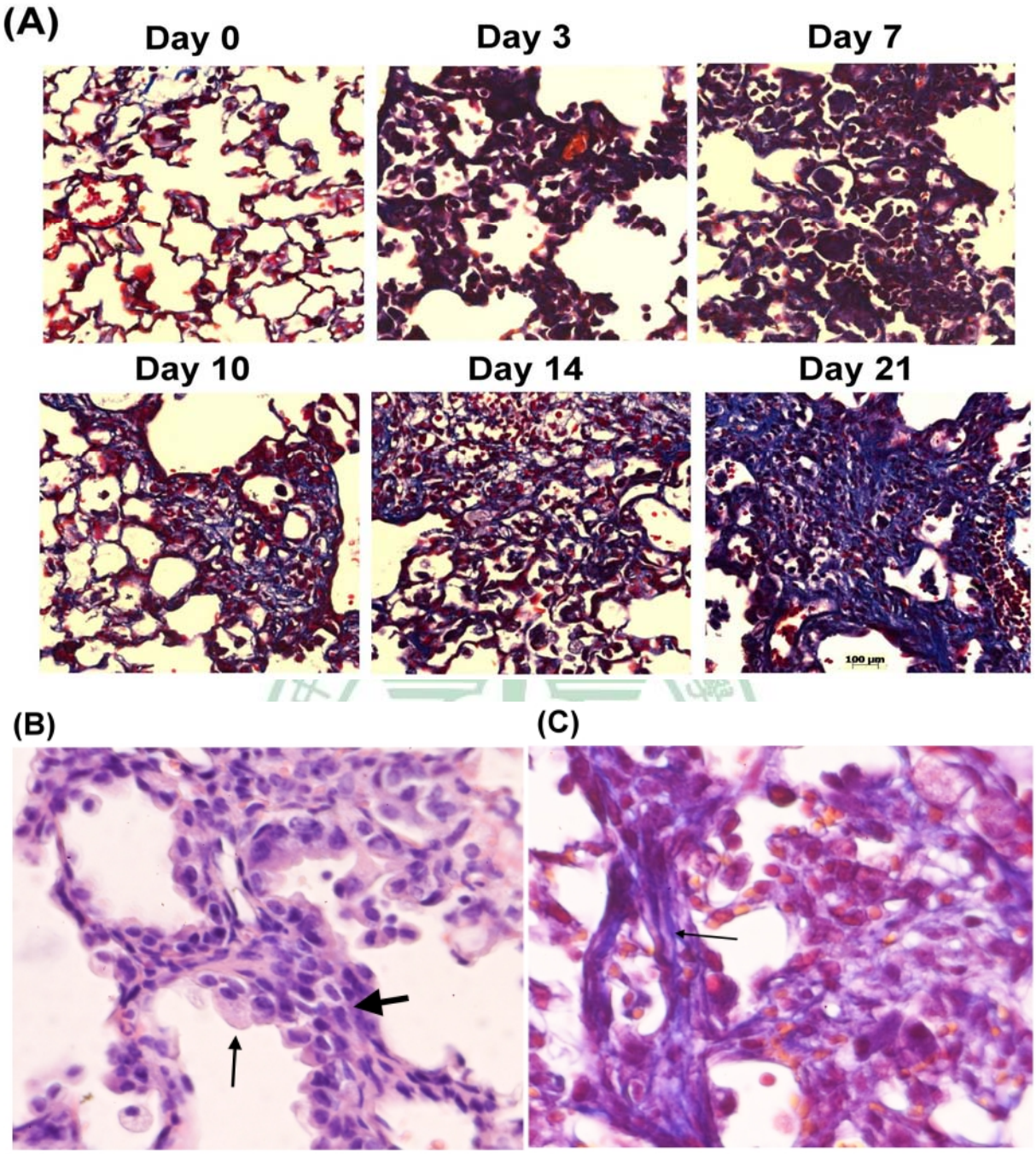


Figure 3. Time course of BLM-induced lung fibrosis.

Histological sections of mice lung demostrating the course of lung fibrosis. Masson's Trichrome stain (A). 14 days after intratracheal bleomycin, a marked peribronchial interstitial infiltration with inflammatory cells, oedema and fibrosis (B). Thick arrow in panel B indicates thickening of interalveolar septum; thin arrow in panel B indicates intra-alveolar macrophage; arrow in panel C identifies interstitial collagen. Original magnification of x40 for A, and x60 for B and C.

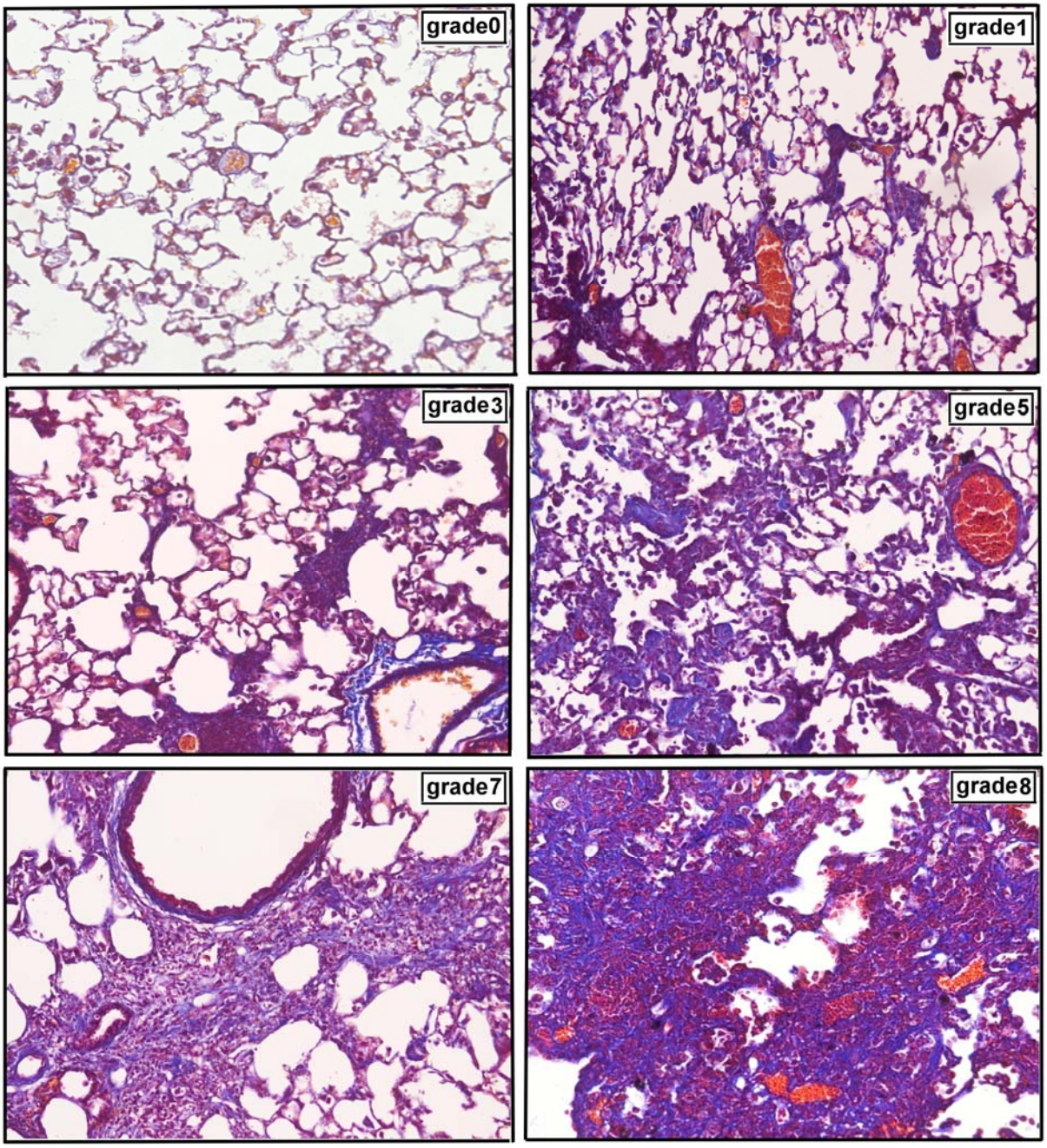


Figure 4. Criteria for grading lung fibrosis. H&E stain.Magnification x20.

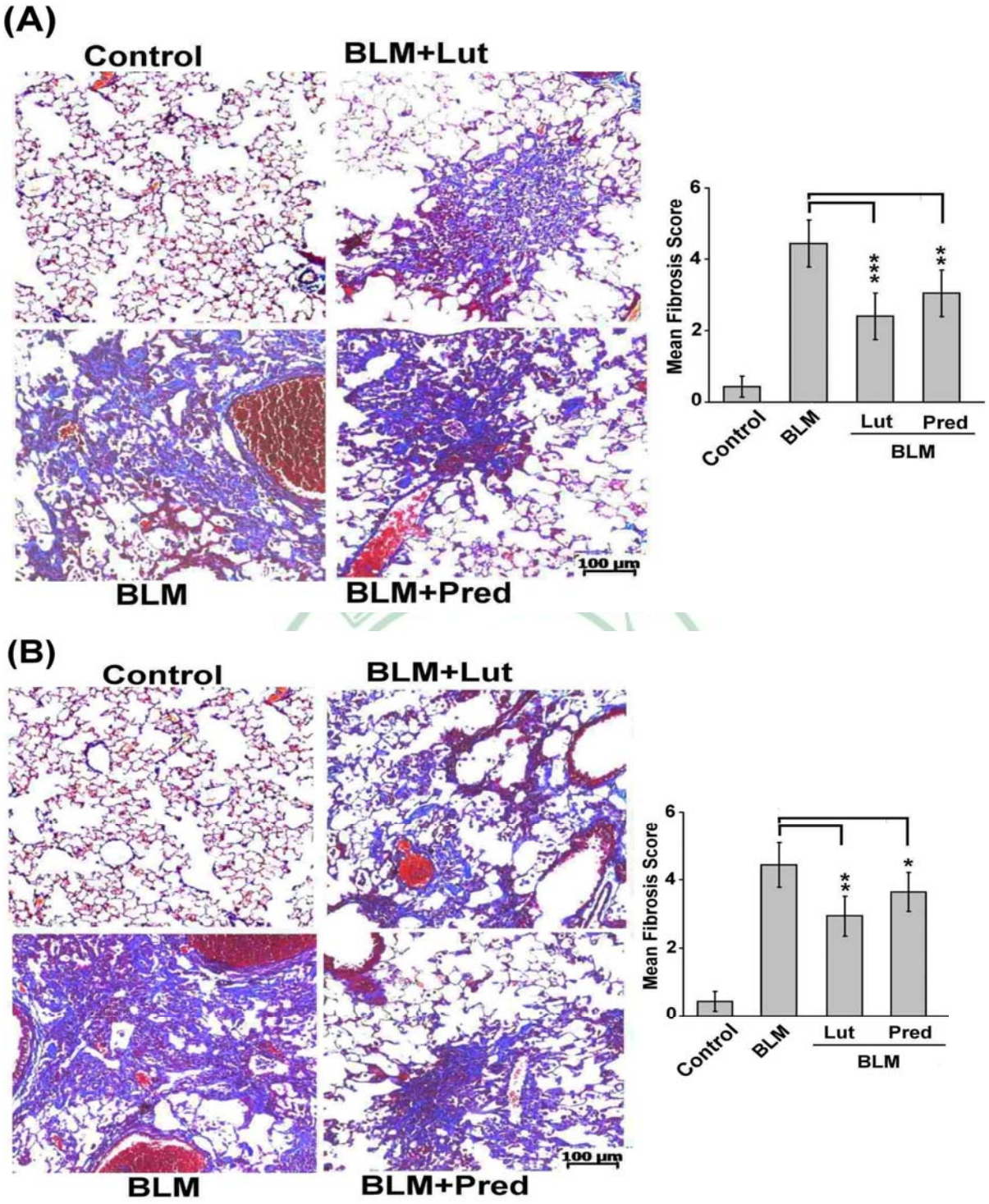


Figure 5. Luteolin ameliorate bleomycin-induced lung fibrosis in mice.

Mice were administered bleomycin (5 mg/kg) at day 0 and drug treatments (luteolin or prednisolone) commenced from day 1 to day 21 (A) or from day 10 to day 21 (B). Evaluation of fibrotic change in the lung from experimental groups using fibrotic scoring system. Magnification x100. Scale bars, 100 μm. * $p < 0.05$, ** $p < 0.01$ vs. BLM.

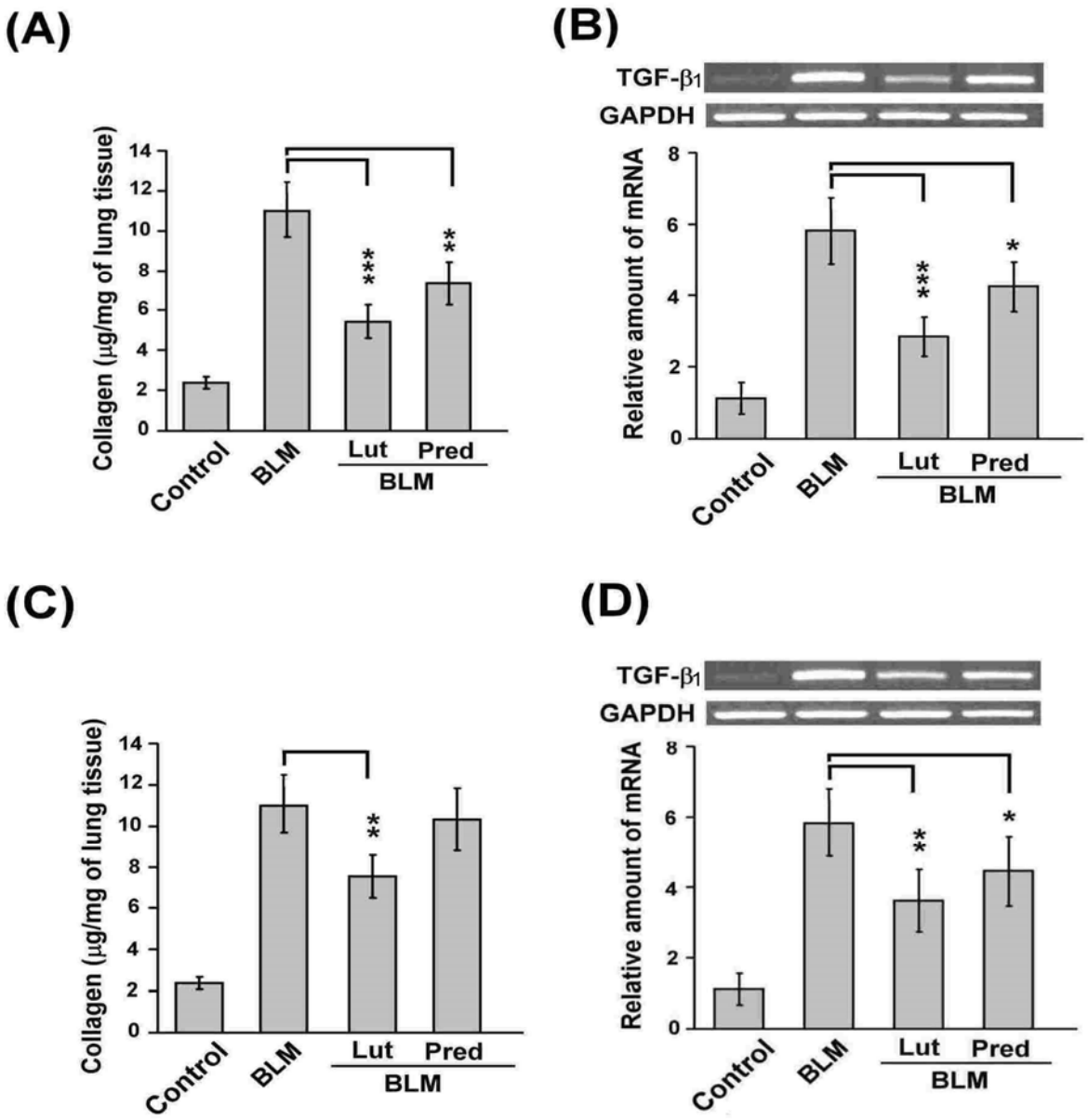


Figure 6. Down-regulation of bleomycin-stimlated collagen and TGF-β1 gene expression by luteolin.

Mice were administered bleomycin (5 mg/kg) at day 0 and drug treatments (luteolin or prednisolone) commenced from day 1 to day 21 (A-B)；drug treatments (luteolin or prednisolone) commenced from day 10 to day 21 (C-D). Collagen content in the lung from experimental groups on day 21 was measured using a Sircol collagen kit. TGF-β1 mRNA expression in the lung from experimental groups examined by RT-PCR analysis. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$ vs. BLM.

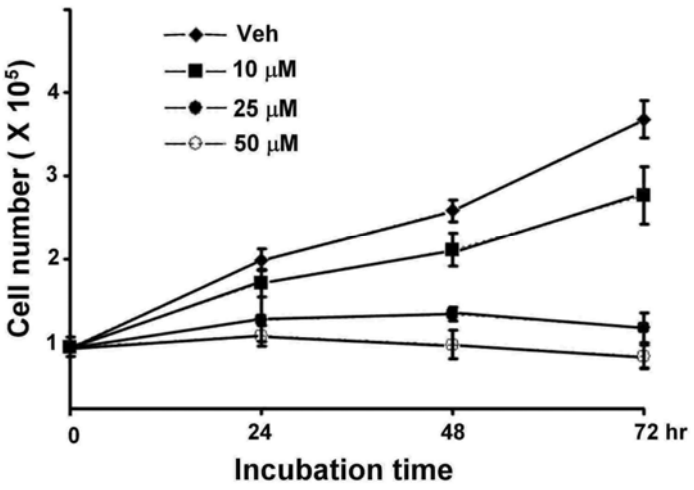


Figure 7. Luteolin inhibited cell proliferation in a dose-dependent manner.

Lung fibroblasts were treated with various concentrations of luteolin for 72 hours, cell number was evaluated by trypan blue dye exclusion method.

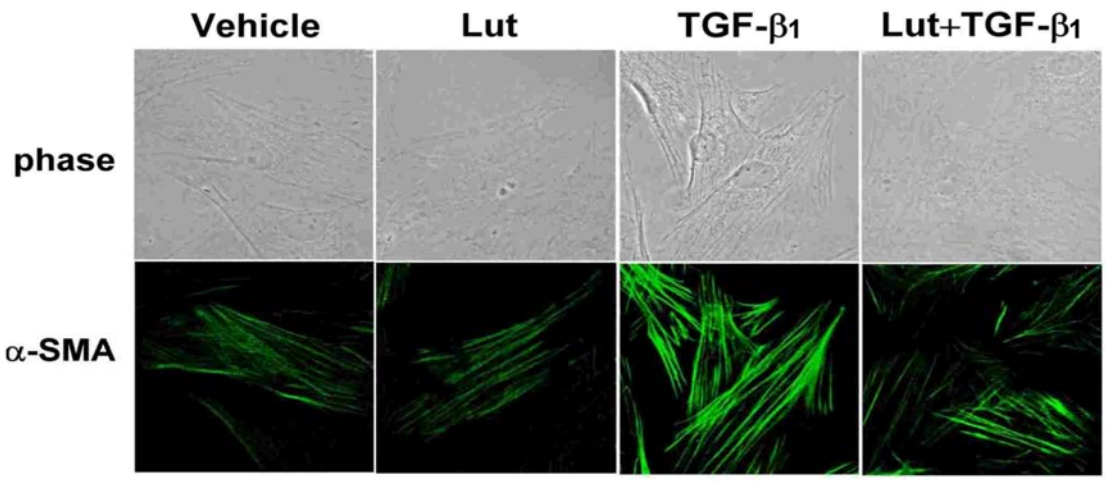


Figure 8. Luteolin inhibited TGF-β1-induced myofibroblastic differentiation.

Serum-starved lung fibroblasts were induced with TGF-β1 (5 ng/ml) in the absence or presence of luteolin (25 μM) for 72 hours. α-SMA was immunocytochemically determined with monoclonal antibody. Magnification x630.

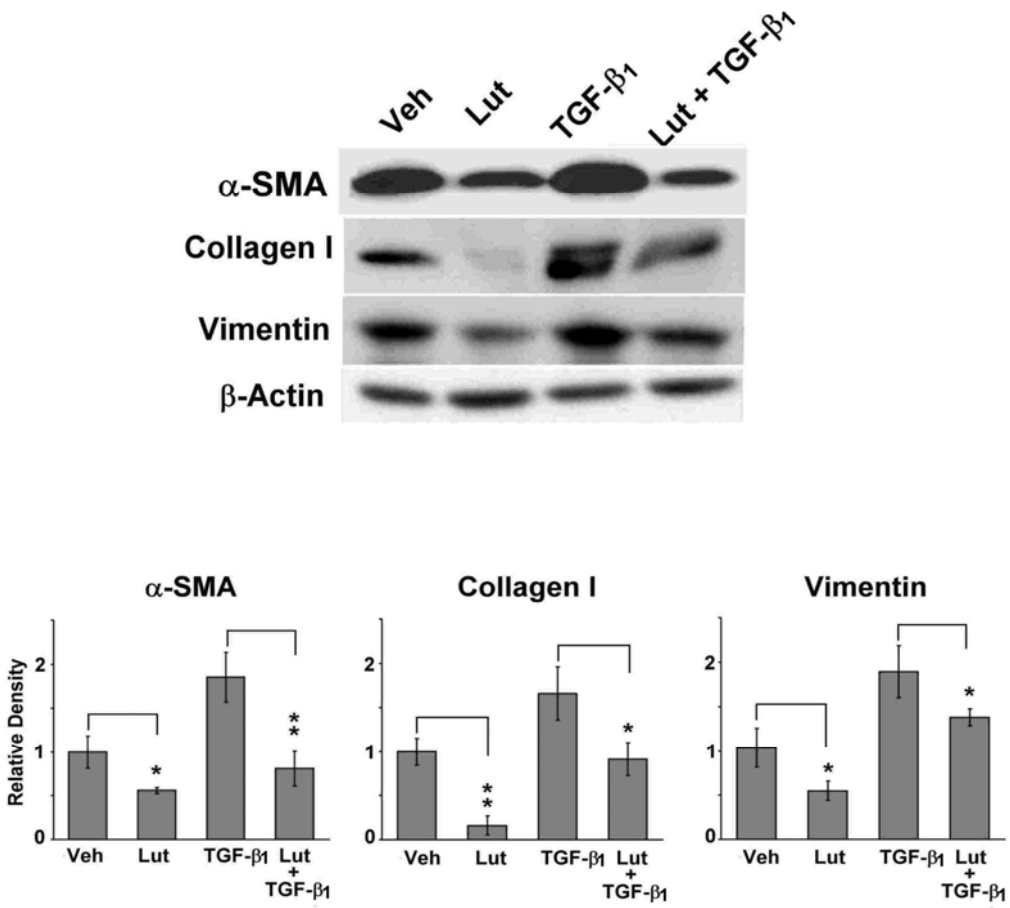


Figure 9. Luteolin decreased α-SMA, collagen 1 and vimentin production induced by TGF-β1 in lung fibroblasts.

Serum-starved lung fibroblasts were induced with TGF-β1 (5 ng/ml) in the absence or presence of luteolin (25 μM) for 72 hours. Western blot analysis was performed to detect α-SMA, vimentin and collagen protein expression in lung fibroblasts. The blots were analyzed by densitometry and the results expressed as relative integration units. Data from triplicate experiments are shown as mean ± SD. * $p< 0.05$, ** $p< 0.01$ vs. Veh.

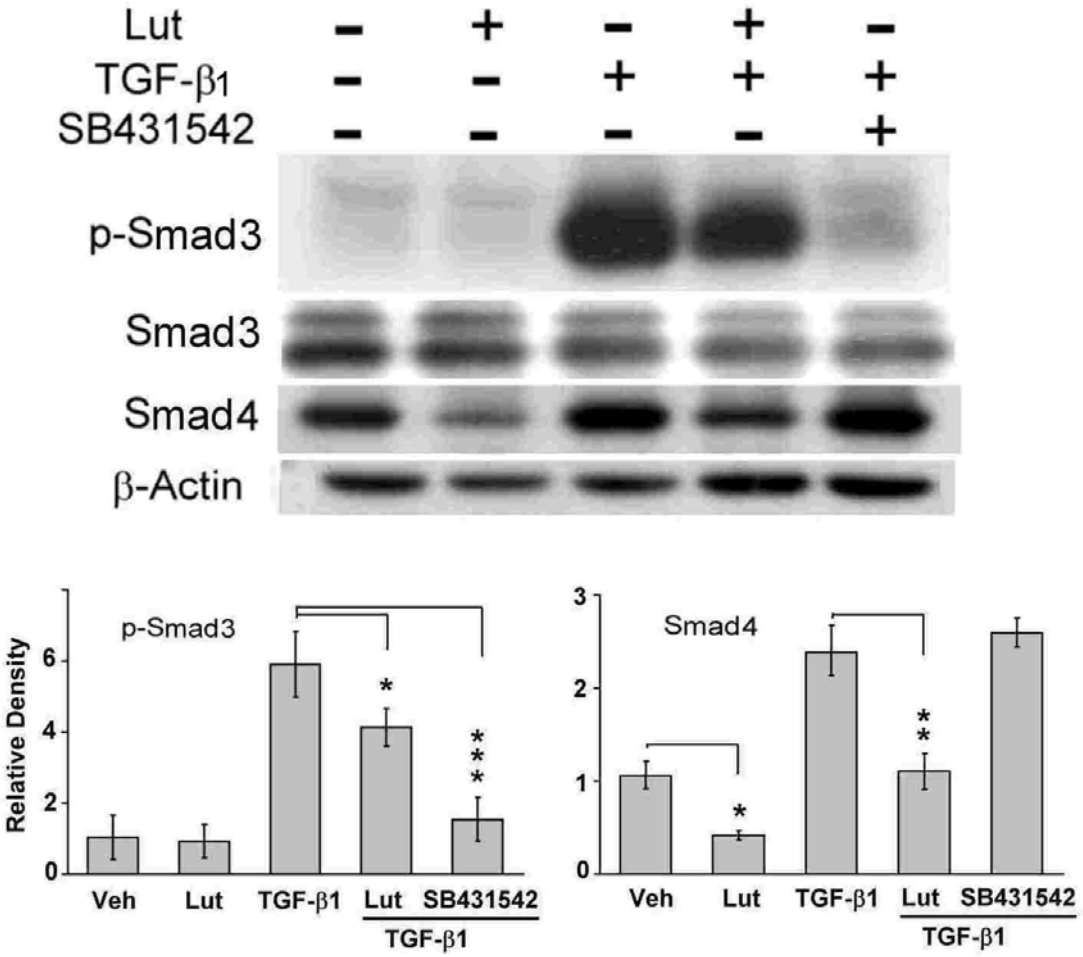


Figure 10. Decrease of TGF-β1-induced Smad3 phosphorylation and Smad4 expression by luteolin in primary lung fibroblasts.

Cells were pretreated with luteolin (25 μM) or TGF-β1 inhibitor (SB431542, 10 μM) for 30 min then treated with TGF-β1 (5 ng/ml) for 30 min. Smad3 phosphorylation and total Smad3 were measured with Western Blot analysis using antibodies for p-Smad3 and Smad3. The blots were analyzed by densitometry and the results expressed as relative integration units. Data from triplicate experiments are shown as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. TGF-β1.

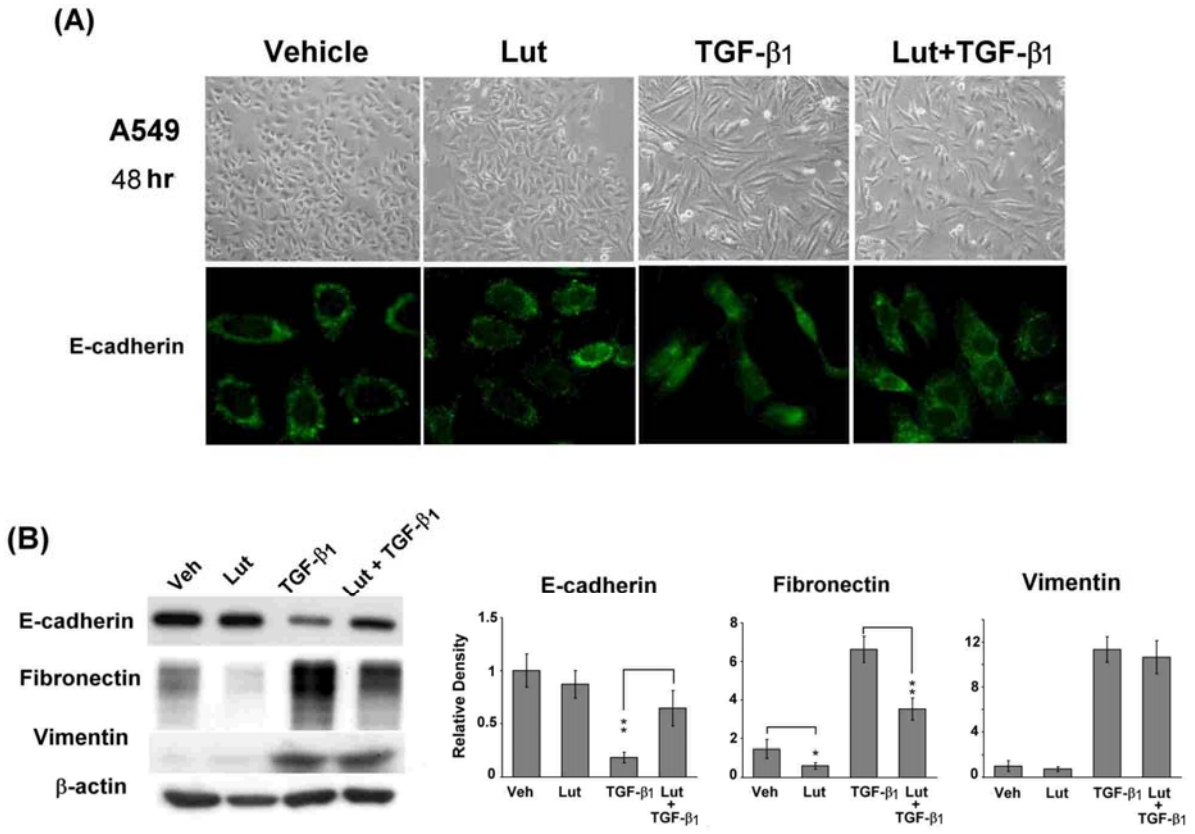


Figure 11. Prevention of TGF-β1-induced EMT by luteolin in A549.

(A) Effects of luteolin on TGF-β1-induced morphological changes in A549 cells. Serum-starved A549 cells were induced with TGF-β1 (5 ng/ml) in the absence or presence of luteolin (25 μM) for 48 hours (200×), E-cadherin was immunocytochemically determined with monoclonal antibody (630×). (B) Expression changes of EMT-related markers in A549 cells. Epithelial marker (E-cadherin) is down-regulated by TGF-β1 and expression of fibronectin and vimentin, the mesenchymal markers, are up-regulated by TGF-β1 in parallel with the down regulation in the epithelial marker. These phenomenons are all inhibited by luteolin. The blots were analyzed by densitometry and the results expressed as relative integration units. Data from triplicate experiments are shown as mean ± SD. * $p < 0.05$, ** $p < 0.01$ vs. TGF-β1.

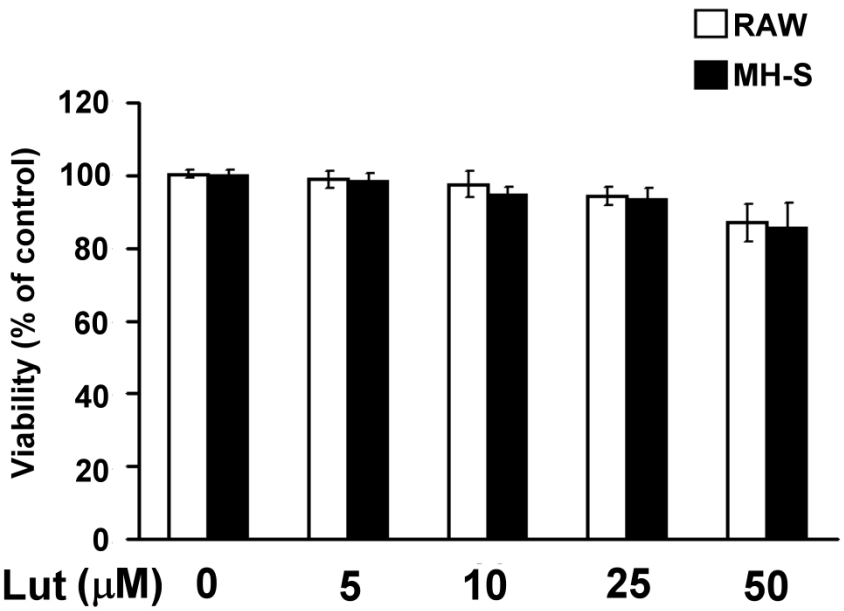


Figure 12. Effect of luteolin on cell viability.

MH-S and RAW 264.7 cells were incubated with 5-50 μM luteolin for 24 h. The results were displayed in percentage of control samples. Data are presented as the mean ± SD. (n=3) for three independent experiments; Lut, luteolin.

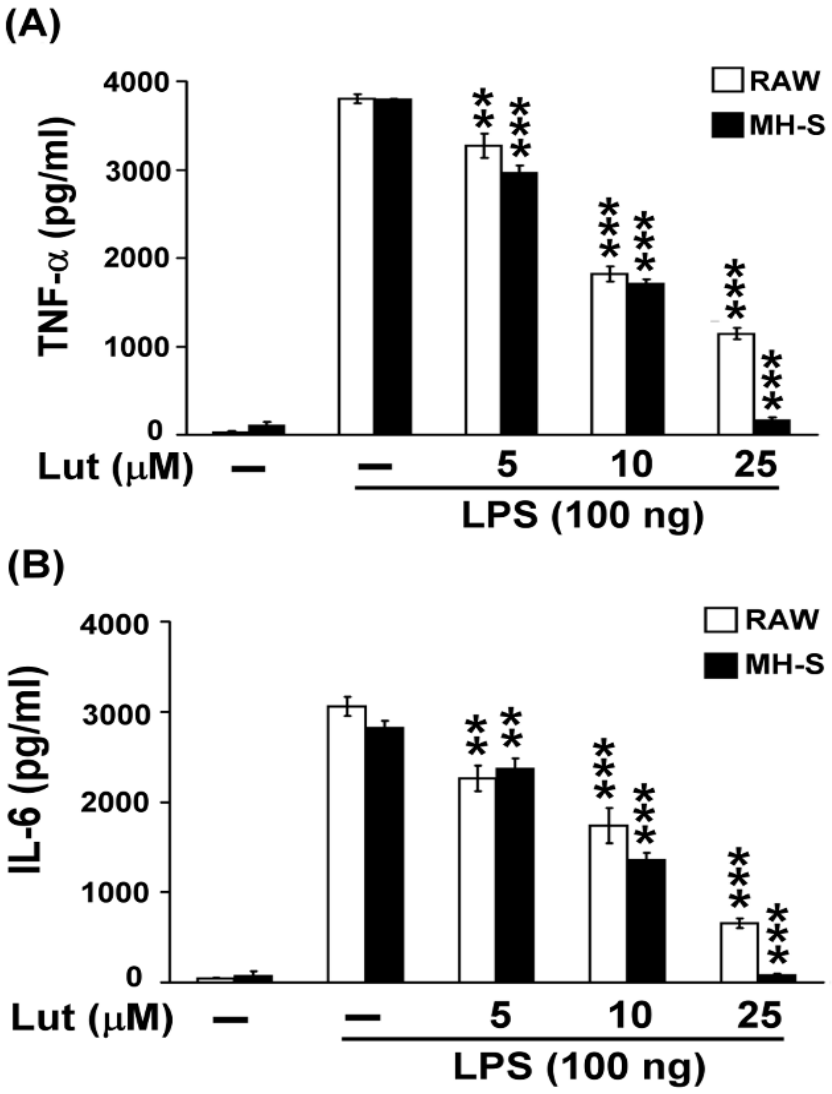


Figure 13. Luteolin inhibited LPS-induced TNF-α and IL-6 production.

MH-S and RAW 264.7 cells were pretreated with luteolin (5-25 μM) for 30 min, and then stimulated with LPS (100 ng/ml). Culture media were collected at 8 hr for TNF-α (A) and IL-6 (B) analysis. Data are presented as the mean ± SD. (n=3) for three independent experiments; ** $p < 0.01$, *** $p < 0.001$ vs. LPS treatment. Lut, luteolin.

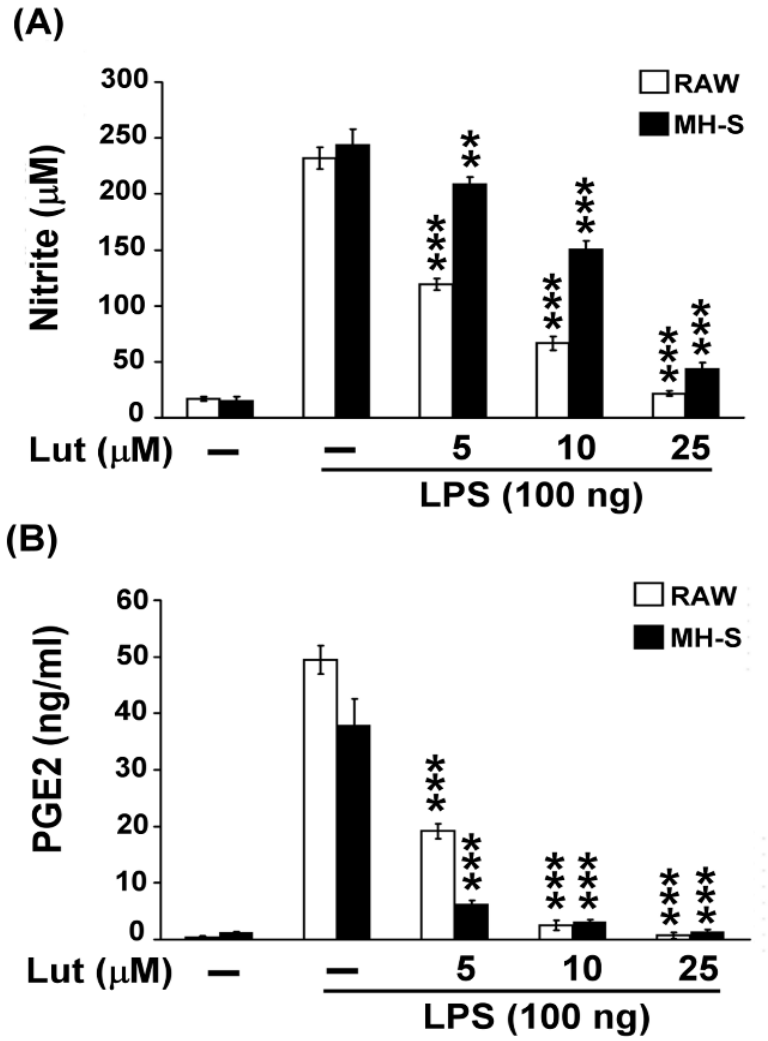


Figure 14. Luteolin inhibited LPS-induced NO and PGE_2 production.

MH-S and RAW 264.7 cells were pretreated with luteolin (5-25 μM) for 30 min, and then stimulated with LPS (100 ng/ml). Culture media were collected at 16 h for NO (A) and PGE_2 (B) analysis. Data are presented as the mean ± SD. (n=3) for three independent experiments; ** $p < 0.01$, *** $p < 0.001$ vs. LPS treatment. Lut, luteolin.

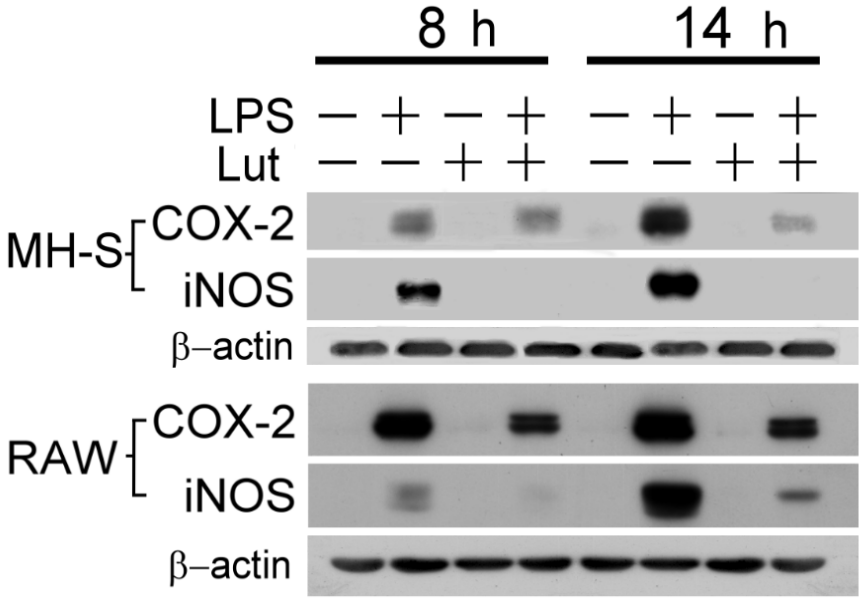


Figure 15. Luteolin decreased the protein levels of COX-2 and iNOS in LPS-stimulated macrophages.

MH-S and RAW 264.7 cells were pretreated with luteolin (25 μM) for 30 min, and then stimulated with LPS (100 ng/ml) for 8-14 h. Total protein extract was separated by SDS-PAGE, and then Western blot analysis with specific antibodies against COX-2 and iNOS. β-actin was used as an internal loading control. The figures were obtained from three independent experiments with similar patterns.

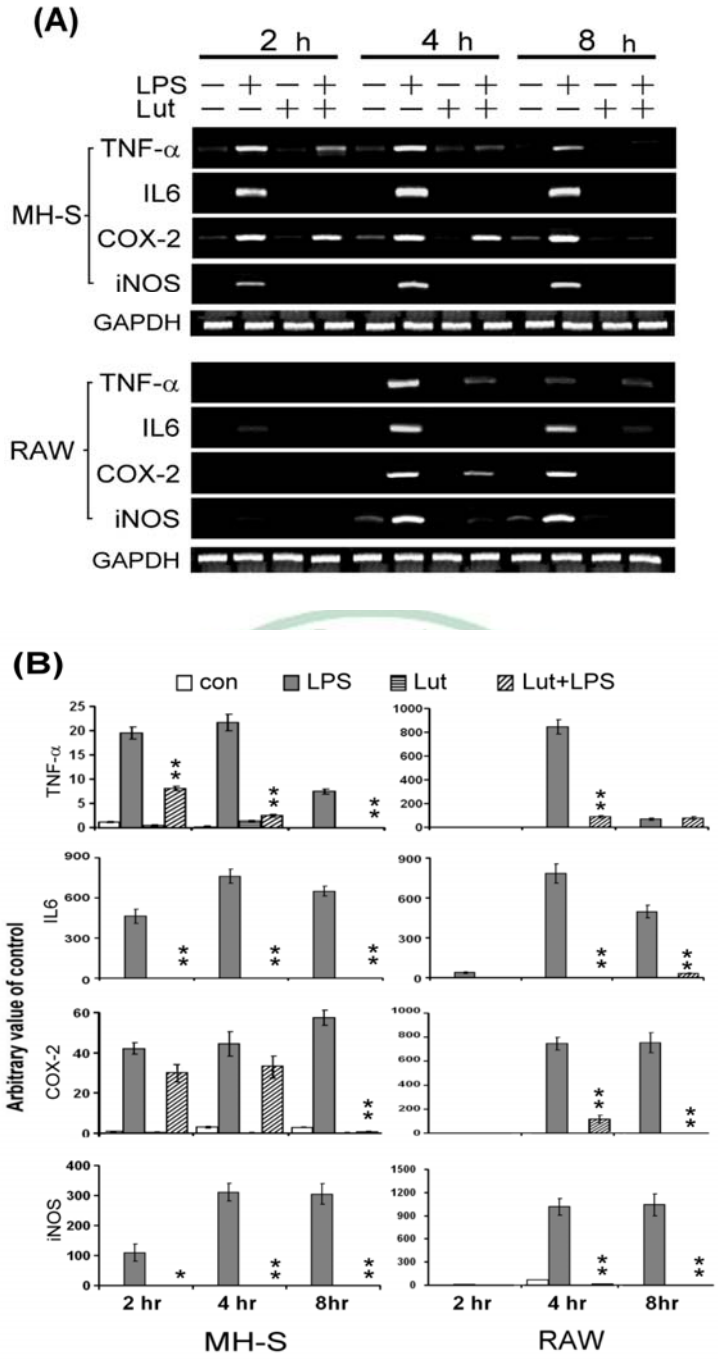


Figure 16. Luteolin decreased the mRNA levels of inflammation -associated genes in LPS-stimulated macrophages.

Cells were pretreated with luteolin (25 μM) for 30 min, and then stimulated with LPS (100 ng/ml) for 2-8 h. TNF-α, IL-6, COX-2 and iNOS mRNA expression were analyzed by RT-PCR as described under *Materials and Methods*. Data are presented as the mean ± SD. (n=3) for three independent experiments; ** $p < 0.01$, vs. LPS treatment. Lut, luteolin.

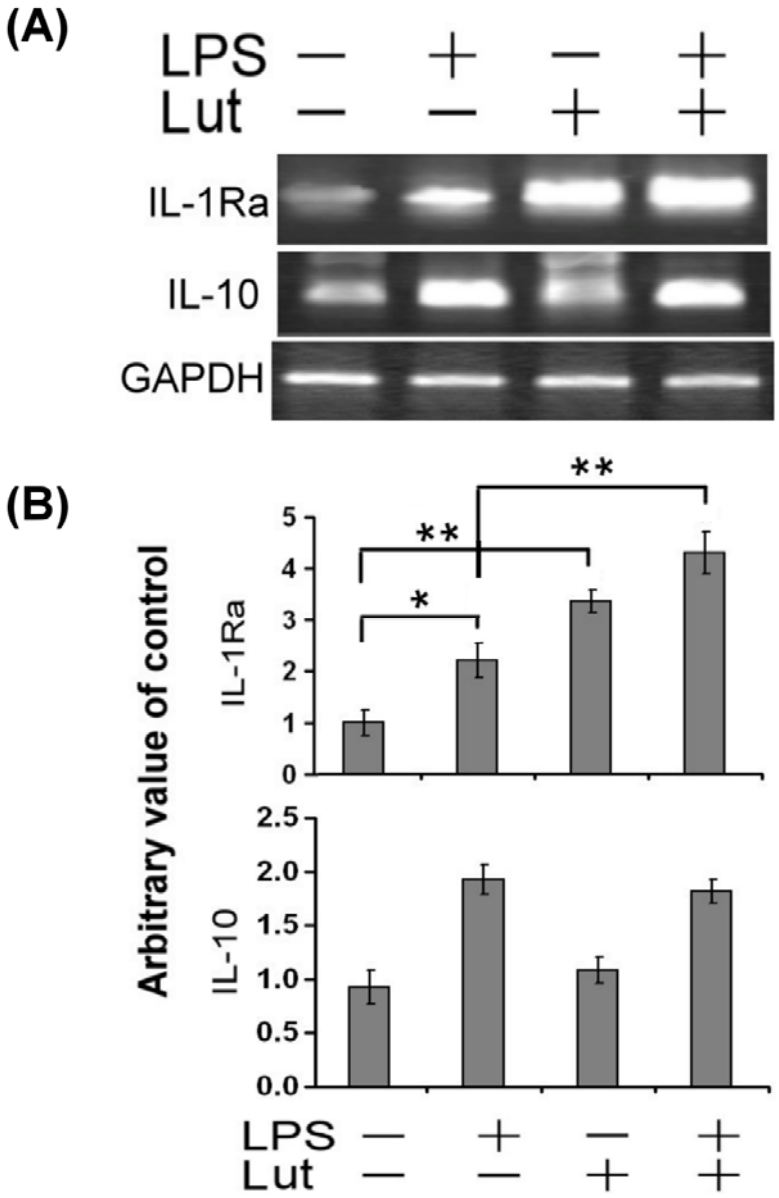


Figure 17. RT-PCR analysis of IL-1Ra and IL-10 mRNA expression.

MH-S cells were pretreated with luteolin (25 μM) for 30 min, and then stimulated with LPS (100 ng/ml) for 8 h. Total RNAwas analyzed by RT-PCR. The figures were obtained from three independent experiments with similar patterns. Data are presented as the mean±SD. (n=3) for three independent experiments; * $p< 0.05$, ** $p< 0.01$. Lut, luteolin.

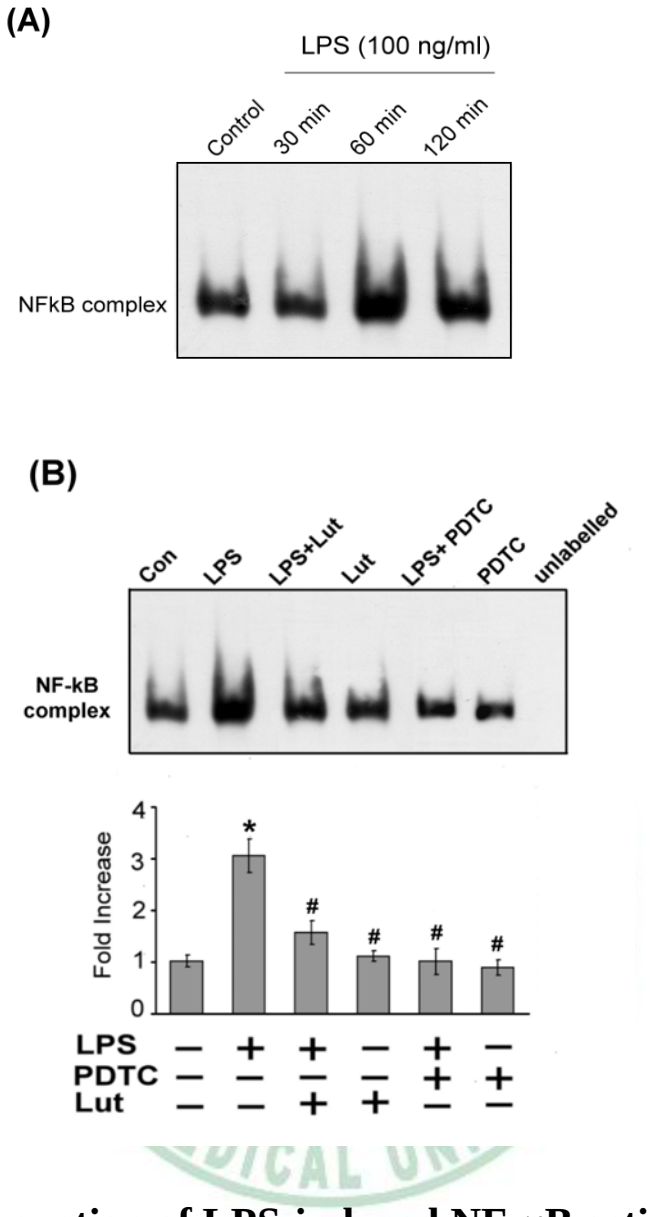


Figure 18. Attenuation of LPS-induced NF-κB activation by luteolin.

MH-S cells were pretreated with luteolin (25 μM) or PDTC (200 μM) for 30 min, and then stimulated with or without LPS (100 ng/ml) for 60 min. Nuclear extracts were prepared and electrophoretic mobility shift assay was performed using biotin-labeled NF-κB consensus binding sequence. Competitive EMSA using an unlabeled NF-κB consensus sequence at 100-fold excess confirmed the specificity of NF-κB protein binding. Values were expressed as mean ± SD. (n=3). *p< 0.05 as compared with the corresponding vehicle control; # p< 0.05 as compared with the LPS treatment.

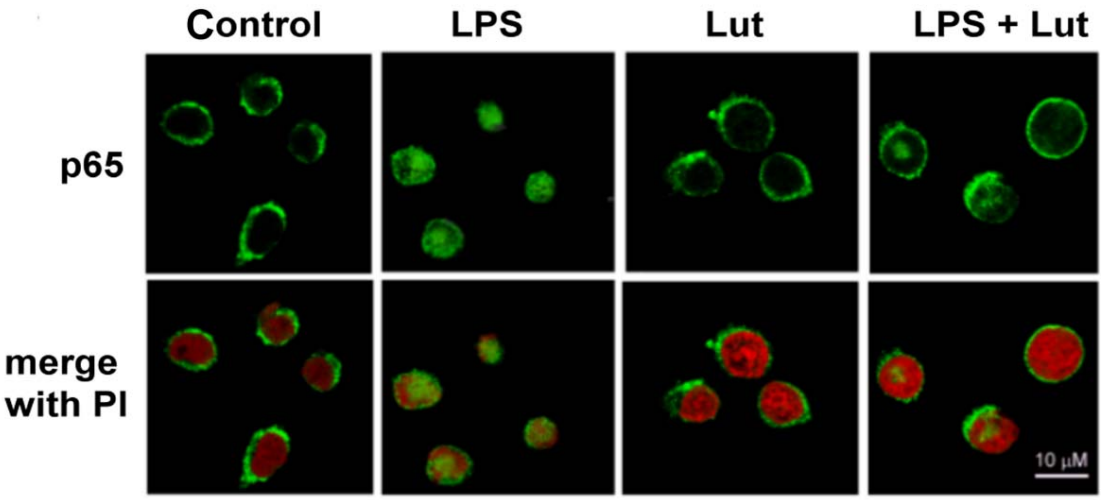


Figure 19.Effects of luteolin on LPS-induced nuclear translocation of p65 subunit of NF-κB.

MH-S cells were preincubated with luteolin (25 μM) for 30 min, and then treated with LPS (100 ng/ml) for 60 min. The subcellular localization of NF-κB p65 subunit was detected by immunofluorescence with antibody against p65 as described in *Materials and Methods*. The same fields were counter stained with PI for location of nuclei.

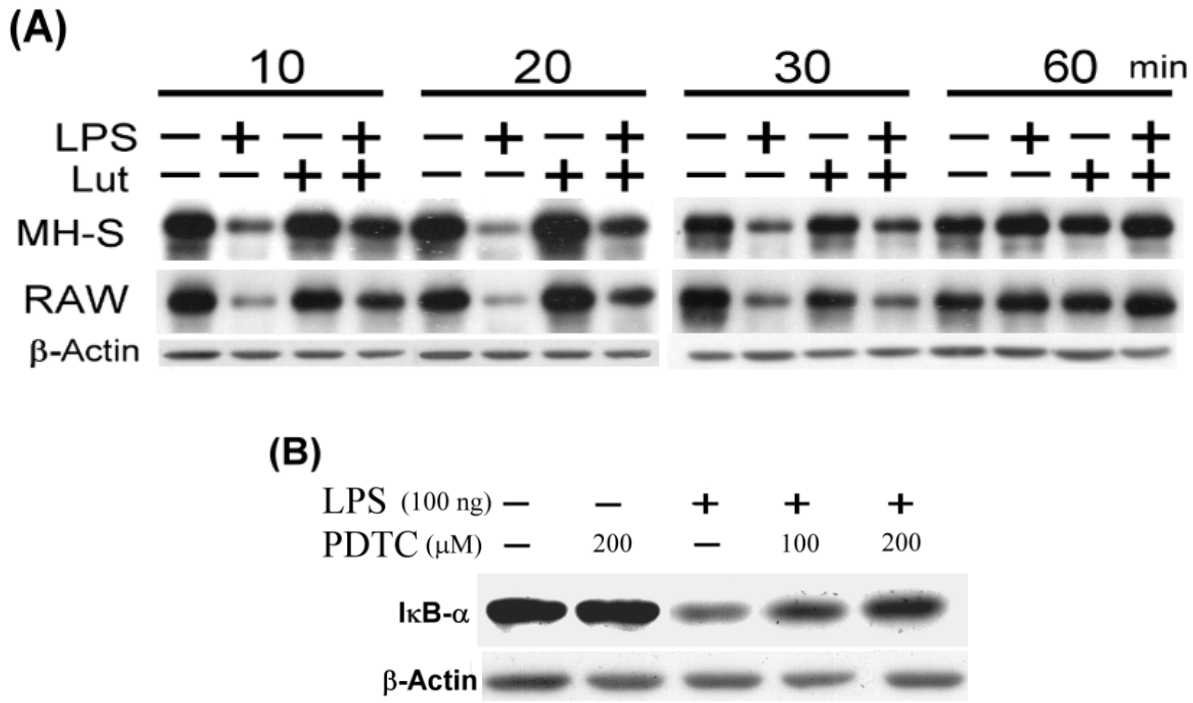


Figure 20. Luteolin blocked LPS-induced IκBα degradation.

MH-S and RAW 264.7 cells were pretreated with 25 μM luteolin for 30 min, and then stimulated with LPS (100 ng/ml) for 10 and 20 min. Protein extract was separated by SDS-PAGE followed Western blot analysis was performed with specific antibodies against IκBα. Equal loading of proteins was verified by actin immunoblotting. The figures were obtained from three independent experiments with similar patterns.

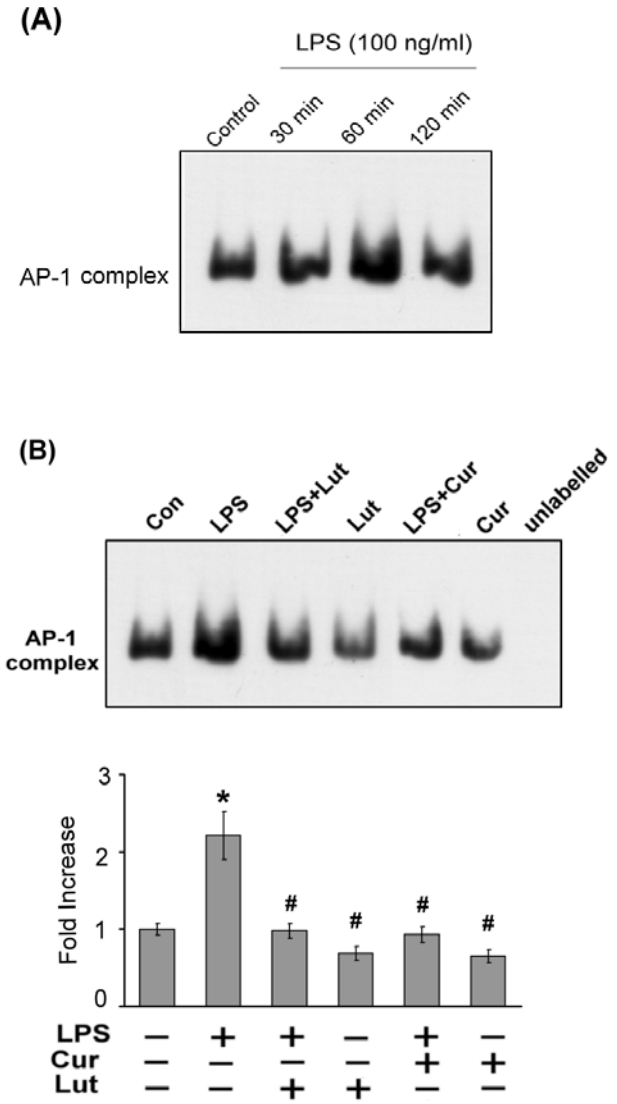


Figure 21.Suppression of LPS-induced AP-1 activation by luteolin.

MH-S cells were pretreated with luteolin (25 μM) or curcumin (10 μM) for 30 min, and then stimulated with or without LPS (100 ng/ml) for 60 min. Nuclear extracts were prepared and EMSA was performed using biotin-labeled AP-1 consensus binding sequence. Competitive EMSA using an unlabeled AP-1 consensus sequence at 100-fold excess confirmed the specificity of AP-1 protein binding. Values were expressed as mean ± SD. (n=3). * $p< 0.05$ as compared with the corresponding vehicle control; # $p< 0.05$ as compared with the LPS treatment. The figures were obtained from three independent experiments with similar patterns. Lut, luteolin; Cur, curcumin.

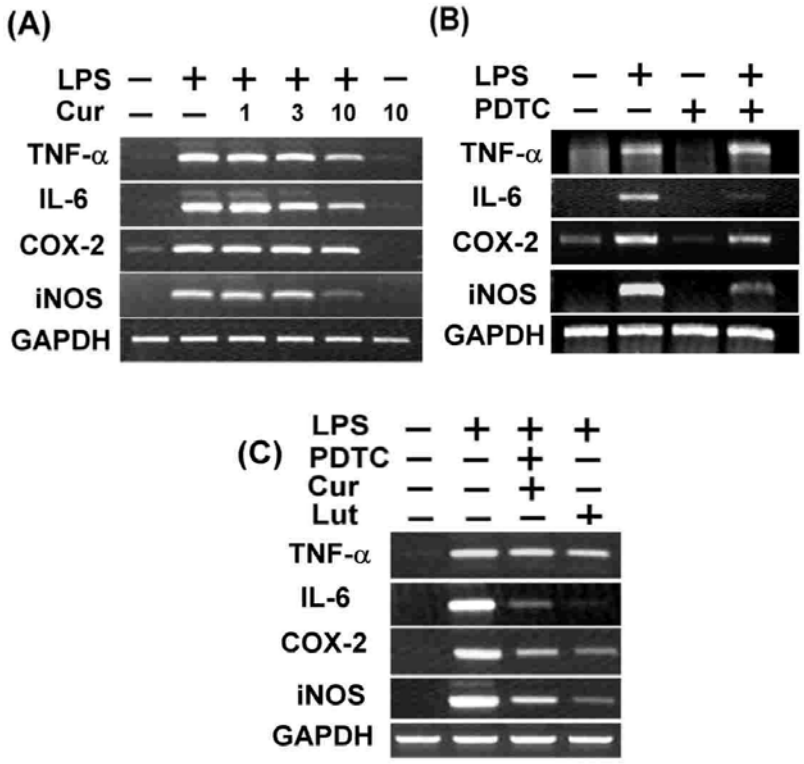


Figure 22. Effects of PDTC and curcumin on LPS-induced inflammation-associated genes expression.

(A) MH-S cells were pretreated with curcumin, (B) 200 μM PDTC or (C) combined treatment with PDTC and curcumin for 30 min, and then stimulated with LPS (100 ng/ml) for 4 h. TNF-α, IL-6, COX-2 and iNOS mRNA expression were analyzed by RT-PCR as described under *Materials and Methods.* The figures were obtained from three independent experiments with similar patterns. Lut, luteolin; Cur, curcumin.

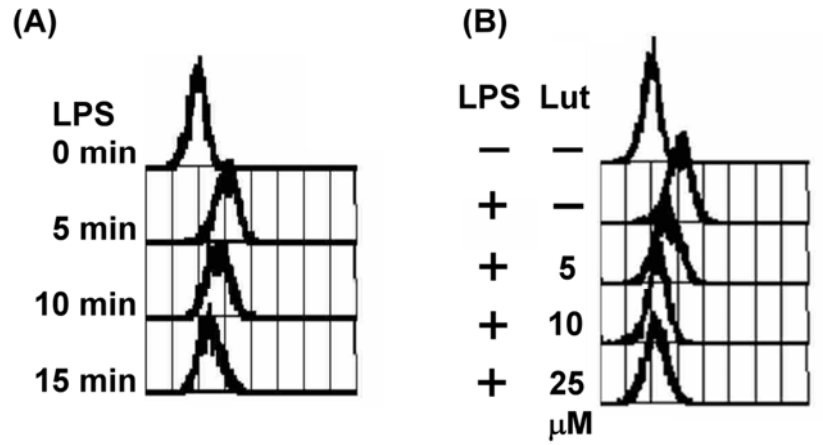


Figure 23. Effect of luteolin on LPS-induced ROS generation.

(A) MH-S macrophages were pretreated with H_2DCFDA (5 μM) for 30 min in serum-free medium and then exposed to LPS (100 ng/ml) at 37°C for 0, 5, 10, 20 min. (B) MH-S cells were pretreated with H_2DCFDA (5 μM) and luteolin (5, 10, and 25 μM) for 30 min, and then exposed to LPS (100 ng/ml) at 37°C for another 5 min. Cells were collected and fluorescence was measured by a flow cytometer using Cell Quest software. The figure was obtained from three independent experiments with similar patterns.

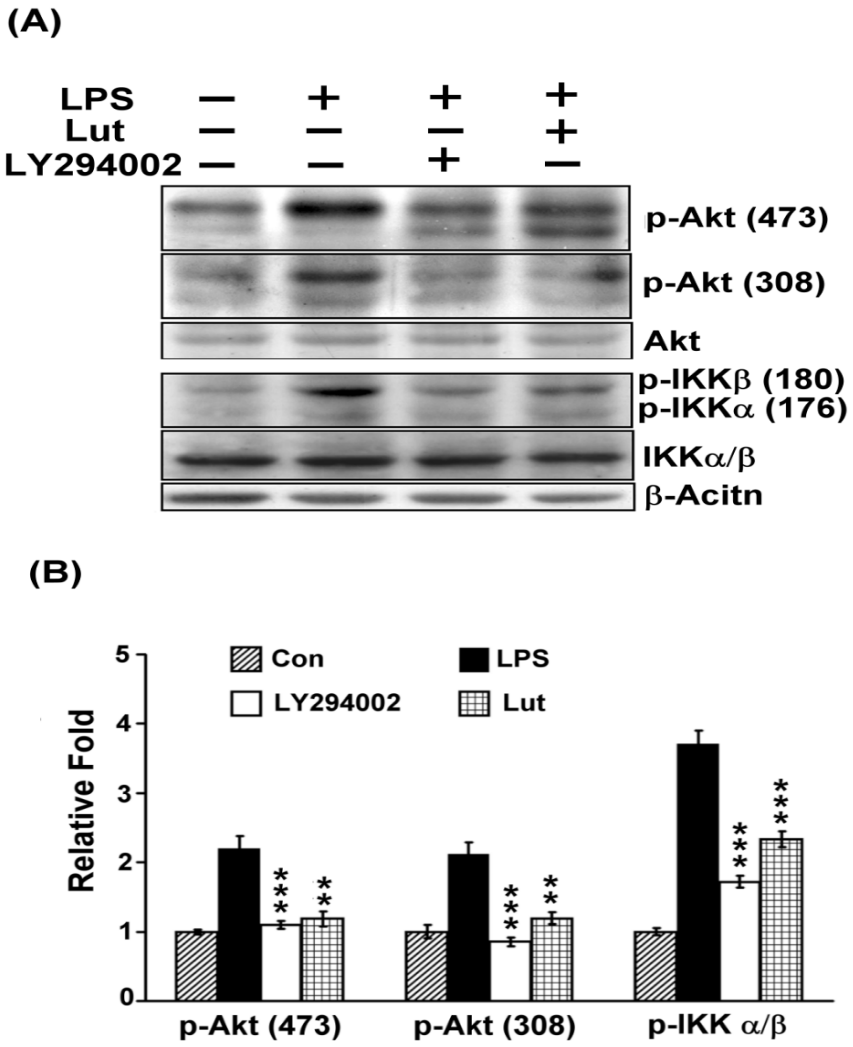


Figure 24. Luteolin decreased the LPS-induced Akt and IKKα/β phosphorylation.

MH-S cells were pretreated with luteolin (25 μM) or LY294002 (10 μM) for 30 min, and then stimulated with LPS (100 ng/ml) for 10 min. 50 μg of each protein was separated by SDS-PAGE, and then Western blot analysis with specific antibodies against phospho-Akt (Thr308), phospho-Akt (Ser473) and phosphor-IKKα/β (Ser176/180) were performed. β-actin was used as a control. Results are expressed as the mean ± SD. for three independent experiments; ** $p < 0.01$, *** $p < 0.001$ vs. LPS treatment. Lut, luteolin.

參考文獻

1. Downey GP, Granton JT. Mechanisms of acute lung injury. *Current Opinion in Pulmonary Medicine* 1997;3(3):234-241.

2. Ashbaugh DG, Bigelow DB, Petty TL, Levine BE. Acute respiratory distress in adults. *Critical Care and Resuscitation* 2005;7(1):60-61.

3. Wang CH, Liu CY, Wan YL, Chou CL, Huang KH, Lin HC, Lin SM, Lin TY, Chung KF, Kuo HP. Persistence of lung inflammation and lung cytokines with high-resolution CT abnormalities during recovery from SARS. *Respiratory Research* 2005;6:42.

4. Nicholls JM, Poon LL, Lee KC, Ng WF, Lai ST, Leung CY, Chu CM, Hui PK, Mak KL, Lim W, Yan KW, Chan KH, Tsang NC, Guan Y, Yuen KY, Peiris JS. Lung pathology of fatal severe acute respiratory syndrome. *Lancet* 2003;361(9371):1773-1778.

5. 單愛蓮, 權菊香, 錢麗旗, 肖永紅. 對預防與治療非典型肺炎的中藥處方情況調查分析. *中國臨床藥理學雜誌* 2003;19(4):289.

6. Ware LB, Matthay MA. The acute respiratory distress syndrome. *The New England Journal of Medicine* 2000;342(18):1334-1349.

7. 中華中藥典. 行政院衛生署編印 2004:102-104.

8. 梁曉天. 常用中藥基礎研究. *中國醫學雜誌* 2004;9(6):535.

9. 何晶. 金銀花的化學成分及藥理作用 *天津藥學* 2007;19(5):67.

10. 張玲, 彭廣芳, 林慧彬. 山東金銀花揮發油化學成分研究. *中國藥學雜誌* 1995;30(11):651.

11. 高玉敏. 金銀花化學成分的研究. *中草藥* 1995;26 (11):568.

12. 李永梅, 王天志, 王志霄. 細氈毛忍冬花蕾化學成分研究[*中國中藥雜誌* 2001;26(1):45－47.

13. 陳敏, 吳威巍, 沈國強. 灰氈毛忍冬化學成分研究
灰氈毛忍冬素 F 和 G 的結構測定. *藥學學報* 1994;29(8):617-620.

14. 茅青, 曹東. 灰氈毛忍冬化學成分的研究. *藥學學報* 1993;28(4):273-281.

15. 婁紅祥, 郎偉君, 呂木堅. 金銀花中水溶性化合物的分離與結構確定. *中草藥* 1996;27(4):195-199.

16. 邢俊波, 李會軍, 李萍. 忍冬花蕾化學成分研究. *中國新藥雜誌* 2002;11(11):856-859.

17. 趙良忠, 蔣賢育, 段林東. 金銀花水溶性抗菌物質的提取及其抑菌效果研究. *中國生物製品學雜誌* 2006;19(2):201.

18. 孫延波, 王雲, 關顯智. 金銀花對口腔病原性微生物體外抑菌試驗的研究. *中國中藥雜誌* 1996;21(4):242.

19. 馬雙成, 劉燕, 畢培曦. 金銀花藥材中抗呼吸道病毒感染的黃酮類成分的定量研究. *藥物分析雜誌* 2006;26 (4):426.

20. 李永梅, 李莉, 柏川. 金銀花的抗腺病毒作用研究. *華西藥學雜志*, 2001;16(5):327.

21. 李斐, 黎海祇. 金銀花水提物對卵清蛋白致敏小鼠的抗過敏作用研究. *重慶醫科大學學報* 2004;29(3):288.

22. 劉傑, 夏利, 陳秀芬. . 黃褐毛忍冬總皂苷的抗炎作用. *中國藥理學報* 1988;9(5):395.

23. 時京珍, 劉耕陶. 黃褐毛忍冬皂甙對乙醯氨基酚致小鼠肝臟毒性的保護作用. *藥學學報* 1995;30(4):311.

24. 潘競鏘. 金銀花能降低小鼠血糖血脂水準. *廣州醫藥* 1998;29(3):59.

25. 何顯忠, 蘭榮德. 金銀花的藥理作用與臨床應用. *時珍國醫國藥* 2004;15(12):865.

26. 葛冰, 盧向陽, 易克. 金銀花活性成分,藥理作用及其應用. *中國野生植物資源* 2004;23(5):13.

27. The Merck Index.

28. 王文信. 金銀花及其成分木犀草素改善藥物誘發大鼠記憶障礙作用之研究. *中國醫藥大學中國藥學研究所碩士論文* 2004

29. 王麗婷, 楊敏麗. 金銀花不同品種及不同部位木犀草素含量的比較. *中華中醫藥雜誌* 2007;22 (10):666-668.

30. 王立升,白海強, 劉力恒, 王天文. 木犀草素研究進展. *大眾科技* 2007;9:132-133.

31. Xagorari A, Papapetropoulos A, Mauromatis A, Economou M, Fotsis T, Roussos C. Luteolin inhibits an endotoxin-stimulated phosphorylation cascade and proinflammatory cytokine production in macrophages. *The Journal of Pharmacology and Experimental Therapeutics* 2001; 296(1):181-187.

32. Chen CC, Chow MP, Huang WC, Lin YC, Chang YJ. Flavonoids inhibit tumor necrosis factor-alpha-induced up-regulation of intercellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells through activator protein-1 and nuclear factor-kappaB: structure-activity relationships. *Mol Pharmacol* 2004;66(3):683-693.

33. Comalada M, Ballester I, Bailon E, Sierra S, Xaus J, Galvez J, de Medina FS, Zarzuelo A. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: analysis of the structure-activity relationship. *Biochemical Pharmacology* 2006; 72(8):1010-1021.

34. Kanazawa K, Uehara M, Yanagitani H, Hashimoto T. Bioavailable flavonoids to suppress the formation of 8-OHdG in HepG2 cells. *Archives of Biochemistry and Biophysics* 2006;455(2):197-203.

35. Tsai FS, Peng WH, Wang WH, Wu CR, Hsieh CC, Lin YT, Feng IC, Hsieh MT. Effects of luteolin on learning acquisition in rats: involvement of the central cholinergic system. *Life Sciences* 2007;80(18):1692-1698.

36. Mastuda H, Morikawa T, Ueda K, Managi H, Yoshikawa M. Structural requirements of flavonoids for inhibition of antigen-Induced degranulation, TNF-alpha and IL-4 production from RBL-2H3 cells. *Bioorganic & Medicinal Chemistry* 2002;10(10):3123-3128.

37. Bessho R, Matsubara K, Kubota M, Kuwakado K, Hirota H, Wakazono Y, Lin YW, Okuda A, Kawai M, Nishikomori R. Pyrrolidine dithiocarbamate, a potent inhibitor of NF-kB activation, prevents apoptosis in human promyelocytic leukemia HL-60 cells and thymocytes. *Biochemical Pharmacology* 1994; 48(10):1883-1889.

38. Kotanidou A, Xagorari A, Bagli E, Kitsanta P, Fotsis T, Papapetropoulos A, Roussos C. Luteolin reduces lipopolysaccharide-induced lethal toxicity and expression of proinflammatory molecules in mice. *American Journal of Respiratory and Critical Care Medicine* 2002; 165(6):818-823.

39. Das M, Ram A, Ghosh B. Luteolin alleviates bronchoconstriction and airway hyperreactivity in ovalbumin sensitized mice. *Inflammation Research* 2003; 52(3):101-106.

40. Tormakangas L, Vuorela P, Saario E, Leinonen M, Saikku P, Vuorela H. In vivo treatment of acute *Chlamydia pneumoniae* infection with the flavonoids quercetin and luteolin and an alkyl gallate, octyl gallate, in a mouse model. *Biochemical Pharmacology* 2005; 70(8):1222-1230.

41. Xagorari A, Roussos C, Papapetropoulos A. Inhibition of LPS-stimulated pathways in macrophages by the flavonoid luteolin. *British Journal of Pharmacology* 2002; 136(7):1058-1064.

42. Kim SH, Shin KJ, Kim D, Kim YH, Han MS, Lee TG, Kim E, Ryu SH, Suh PG. Luteolin inhibits the nuclear factor-kappa B transcriptional activity in Rat-1 fibroblasts. *Biochemical Pharmacology* 2003;66(6):955-963.

43. Gutierrez-Venegas G, Kawasaki-Cardenas P, Arroyo-Cruz SR, Maldonado-Frias S. Luteolin inhibits lipopolysaccharide actions on human gingival fibroblasts. *European Journal of Pharmacology* 2006; 541(1-2):95-105.

44. Kim JS, Lee HJ, Lee MH, Kim J, Jin C, Ryu JH. Luteolin inhibits LPS-stimulated inducible nitric oxide synthase expression in BV-2 microglial cells. *Planta Medica* 2006; 72(1):65-68.

45. Kimata M, Shichijo M, Miura T, Serizawa I, Inagaki N, Nagai H. Effects of luteolin, quercetin and baicalein on immunoglobulin E-mediated mediator release from human cultured mast cells. *Clinical and Experimental Allergy* 2000;30(4):501-508.

46. Dempsey OJ. Clinical review: idiopathic pulmonary fibrosis--past, present and future. *Respiratory Medicine* 2006;100(11):1871-1885.

47. American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and t reatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). *American Journal of Respiratory and Critical Care Medicine* 2000;161(2 Pt 1):646-664.

48. Hiwatari N, Shimura S, Sasaki T, Aikawa T, Ando Y, Ishihara H, Sekizawa K, Sasaki H, Takishima T. Prognosis of idiopathic pulmonary fibrosis in patients with mucous hypersecretion. *The American Review of Respiratory Disease* 1991;143(1):182-185.

49. Mannino DM, Etzel RA, Parrish RG. Pulmonary fibrosis deaths in the United States, 1979-1991. An analysis of multiple-cause mortality data. *American Journal of Respiratory and Critical Care Medicine* 1996;153(5):1548-1552.

50. Coker RK, Laurent GJ. Pulmonary fibrosis: cytokines in the balance. *The European Respiratory Journal* 1998;11(6):1218-1221.

51. Gauldie J, Kolb M, Sime PJ. A new direction in the pathogenesis of idiopathic pulmonary fibrosis? *Respiratory Research* 2002;3:1.

52. Gross TJ, Hunninghake GW. Idiopathic pulmonary fibrosis. *The New England Journal of Medicine* 2001;345(7):517-525.

53. Gharaee-Kermani M, Phan SH. Role of cytokines and cytokine therapy in wound healing and fibrotic diseases. *Current Pharmaceutical Design* 2001;7(11):1083-1103.

54. Mason RJ, Schwarz MI, Hunninghake GW, Musson RA. NHLBI Workshop Summary. Pharmacological therapy for idiopathic pulmonary fibrosis. Past, present, and future. *American Journal of Respiratory and Critical Care Medicine* 1999;160(5 Pt 1):1771-1777.

55. Vourlekis JS, Brown KK, Cool CD, Young DA, Cherniack RM, King TE, Schwarz MI. Acute interstitial pneumonitis. Case series and review of the literature. *Medicine (Baltimore)* 2000;79(6):369-378.

56. Demedts M, Costabel U. ATS/ERS international multidisciplinary consensus classification of the idiopathic interstitial pneumonias. *The European Respiratory Journal* 2002;19(5):794-796.

57. Shimabukuro DW, Sawa T, Gropper MA. Injury and repair in lung and airways. *Critical Care Medicine* 2003;31(8 Suppl):S524-531.

58. Pugin J, Verghese G, Widmer MC, Matthay MA. The alveolar space is the site of intense inflammatory and profibrotic reactions in the early phase of acute respiratory distress syndrome. *Critical Care Medicine* 1999;27(2):304-312.

59. Gharaee-Kermani M, Gyetko MR, Hu B, Phan SH. New insights into the pathogenesis and treatment of idiopathic pulmonary fibrosis: a potential role for stem cells in the lung parenchyma and implications for therapy. *Pharmaceutical Research* 2007;24(5):819-841.

60. Engelhardt E, Toksoy A, Goebeler M, Debus S, Brocker EB, Gillitzer R. Chemokines IL-8, GROalpha, MCP-1, IP-10, and Mig are sequentially and differentially expressed during phase-specific infiltration of leukocyte subsets in human wound healing. *The American Journal of Pathology* 1998;153(6):1849-1860.

61. Carre PC, Mortenson RL, King TE, Jr., Noble PW, Sable CL, Riches DW. Increased expression of the interleukin-8 gene by alveolar macrophages in idiopathic pulmonary fibrosis. A potential mechanism for the recruitment and activation of neutrophils in lung fibrosis. *The Journal of Clinical Investigation* 1991;88(6):1802-1810.

62. Steppling H, Nagel M, Strausz J, Muller-Quernheim J. Increased oxidative metabolism of alveolar macrophages in idiopathic fibrosing alveolitis. *Pneumologie* 1990;44 Suppl 1:303-305.

63. Obayashi Y, Yamadori I, Fujita J, Yoshinouchi T, Ueda N, Takahara J. The role of neutrophils in the pathogenesis of idiopathic pulmonary fibrosis. *Chest* 1997;112(5):1338-1343.

64. Franke-Ullmann G, Pfortner C, Walter P, Steinmuller C, Lohmann-Matthes ML, Kobzik L. Characterization of murine lung interstitial macrophages in comparison with alveolar macrophages in vitro. *The Journal of Immunology* 1996;157(7):3097-3104.

65. Wang L, Antonini JM, Rojanasakul Y, Castranova V, Scabilloni JF, Mercer RR. Potential role of apoptotic macrophages in pulmonary inflammation and fibrosis. *Journal of Cellular Physiology* 2003;194(2):215-224.

66. Lohmann-Matthes ML, Steinmuller C, Franke-Ullmann G. Pulmonary macrophages. *European Respiratory Journal* 1994;7(9):1678-1689.

67. Campbell EJ, Cury JD, Lazarus CJ, Welgus HG. Monocyte procollagenase and tissue inhibitor of metalloproteinases. Identification, characterization, and regulation of secretion. *The Journal of Biological Chemistry* 1987;262(33):15862-15868.

68. Martin EL, Sheikh TA, Leco KJ, Lewis JF, Veldhuizen RA. Contribution of alveolar macrophages to the response of the TIMP-3 null lung during a septic insult. *American Journal of Physiology* 2007;293(3):L779-789.

69. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. . *The Journal of Immunology* 2005;175(1):5-14.

70. Cher DJ, Mosmann TR. Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by TH1 clones. *The Journal of Immunology* 1987;138(11):3688-3694.

71. Infante-Duarte C, Kamradt T. Th1/Th2 balance in infection. *Springer Seminars in Immunopathology* 1999;21(3):317-338.

72. Adamson IY, Young L, Bowden DH. Relationship of alveolar epithelial injury and repair to the induction of pulmonary fibrosis. *The American Journal of Pathology* 1988;130(2):377-383.

73. Shoji S, Ertl RF, Linder J, Romberger DJ, Rennard SI. Bronchial epithelial cells produce chemotactic activity for bronchial epithelial cells. Possible role for fibronectin in airway repair. *The American Review of Respiratory Disease* 1990;141(1):218-225.

74. Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM, Borok Z. Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. *The American Journal of Pathology* 2005;166(5):1321-1332.

75. Kasai H, Allen JT, Mason RM, Kamimura T, Zhang Z. TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respiratory Research* 2005;6:56.

76. Yao HW, Xie QM, Chen JQ, Deng YM, Tang HF. TGF-beta1 induces alveolar epithelial to mesenchymal transition in vitro. *Life Sciences* 2004;76(1):29-37.

77. Shook D, Keller R. Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. *Mechanisms of Development* 2003;120(11):1351-1383.

78. Tosh D, Slack JM. How cells change their phenotype? *Nature Reviews Molecular Cell Biology* 2002;3(3):187-194.

79. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *The Journal of Clinical Investigation* 2002;110(3):341-350.

80. Li Y, Yang J, Dai C, Wu C, Liu Y. Role for integrin-linked kinase in mediating tubular epithelial to mesenchymal transition and renal interstitial fibrogenesis. *The Journal of Clinical Investigation* 2003;112(4):503-516.

81. Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, Pittet JF, Kaminski N, Garat C, Matthay MA, Rifkin DB, Sheppard D. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 1999;96(3):319-328.

82. Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *The Journal of Clinical Investigation* 1997;100(4):768-776.

83. Roberts AB, Tian F, Byfield SD, Stuelten C, Ooshima A, Saika S, Flanders KC. Smad3 is key to TGF-beta-mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis. *Cytokine and Growth Factor Reviews* 2006;17(1-2):19-27.

84. Zavadil J, Bottinger EP. TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene* 2005;24(37):5764-5774.

85. Selman M, Pardo A. The epithelial/fibroblastic pathway in the pathogenesis of idiopathic pulmonary fibrosis. *American Journal of Respiratory Cell and Molecular Biology* 2003;29(3 Suppl):S93-97.

86. Kuhn C, McDonald JA. The roles of the myofibroblast in idiopathic pulmonary fibrosis. Ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. *The American Journal of Pathology* 1991;138(5):1257-1265.

87. Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, Belperio JA, Keane MP, Strieter RM. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *The Journal of Clinical Investigation* 2004;114(3):438-446.

88. Desmouliere A, Redard M, Darby I, Gabbiani G. Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *The American journal of pathology* 1995;146(1):56-66.

89. Zhang HY, Gharaee-Kermani M, Phan SH. Regulation of lung fibroblast alpha-smooth muscle actin expression, contractile phenotype, and apoptosis by IL-1beta. *The Journal of Immunology* 1997;158(3):1392-1399.

90. Zhang HY, Phan SH. Inhibition of myofibroblast apoptosis by transforming growth factor beta-1. *American Journal of Respiratory Cellular and Molecular Biology* 1999;21(6):658-665.

91. Mattey DL, Dawes PT, Nixon NB, Slater H. Transforming growth factor myofibroblast-like differentiation in human synovial fibroblasts in vitro: modulation by basic fibroblast growth factor. *Annals of The Rheumatic Diseases* 1997;56(7):426-431.

92. Hashimoto S, Gon Y, Takeshita I, Matsumoto K, Maruoka S, Horie T. Transforming growth Factor-beta1 induces phenotypic modulation of human lung fibroblasts to myofibroblast through a c-Jun-NH2-terminal kinase-dependent pathway. *American Journal of Respiratory and Critical Care Medicine* 2001;163(1):152-157.

93. Desmouliere A, Rubbia-Brandt L, Abdiu A, Walz T, Macieira-Coelho A, Gabbiani G. Alpha-smooth muscle actin is expressed in a subpopulation of cultured and cloned fibroblasts and is modulated by gamma-interferon. *Experimental Cell Research* 1992;201(1):64-73.

94. Desmouliere A, Chaponnier C, Gabbiani G. Tissue repair, contraction, and the myofibroblast. *Wound Repair Regen* 2005;13(1):7-12.

95. Ramos C, Montano M, Garcia-Alvarez J, Ruiz V, Uhal BD, Selman M, Pardo A. Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis, and tissue inhibitor of metalloproteinases expression. *American Journal of Respiratory Cell and Molecular Biology* 2001;24(5):591-598.

96. Winkler MK, Fowlkes JL. Metalloproteinase and growth factor interactions: do they play a role in pulmonary fibrosis? *American Journal of Physiology-Lung Cell Molecular Physiology* 2002;283(1):L1-11.

97. Shapiro SD, Senior RM. Matrix metalloproteinases. Matrix degradation and more. *American Journal of Respiratory Cell and Molecular Biology* 1999;20(6):1100-1102.

98. Swiderski RE, Dencoff JE, Floerchinger CS, Shapiro SD, Hunninghake GW. Differential expression of extracellular matrix remodeling genes in a murine model of bleomycin-induced pulmonary fibrosis. *The American Journal of Pathology* 1998;152(3):821-828.

99. Massague J. The transforming growth factor-beta family. *Annual Review of Cell Biology* 1990;6:597-641.

100. Massague J, Chen YG. Controlling TGF-beta signaling. *Genes & Development* 2000;14(6):627-644.

101. Wakefield LM, Smith DM, Broz S, Jackson M, Levinson AD, Sporn MB. Recombinant TGF-beta 1 is synthesized as a two-component latent complex that shares some structural features with the native platelet latent TGF-beta 1 complex. *Growth Factors* 1989;1(3):203-218.

102. Taipale J, Miyazono K, Heldin CH, Keski-Oja J. Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein. *The Journal of Cell Biology* 1994;124(1-2):171-181.

103. Saharinen J, Hyytiainen M, Taipale J, Keski-Oja J. Latent transforming growth factor-beta binding proteins (LTBPs)--structural extracellular matrix proteins for targeting TGF-beta action. *Cytokine and Growth Factor Reviews* 1999;10(2):99-117.

104. Fortunel NO, Hatzfeld A, Hatzfeld JA. Transforming growth factor-beta: pleiotropic role in the regulation of hematopoiesis. *Blood* 2000;96(6):2022-2036.

105. Kaminska B, Wesolowska A, Danilkiewicz M. TGF beta signaling and its role in tumour pathogenesis. *Acta Biochimica Polonica* 2005;52(2):329-337.

106. Souchelnytskyi S, Tamaki K, Engstrom U, Wernstedt C, ten Dijke P, Heldin CH. Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-beta signaling. *The Journal of Biological Chemistry* 1997;272(44):28107-28115.

107. Massague J, Gomis RR. The logic of TGFbeta signaling. *FEBS Letters* 2006;580(12):2811-2820.

108. Ten Dijke P, Hill CS. New insights into TGF-beta-Smad signaling. *Trends in Biochemical Sciences* 2004;29(5):265-273.

109. Elliott RL, Blobe GC. Role of transforming growth factor Beta in human cancer. *Journal of Clinical Oncology* 2005;23(9):2078-2093.

110. Akhurst RJ, Derynck R. TGF-beta signaling in cancer--a double-edged sword. *Trends in Cell Biology* 2001;11(11):S44-51.

111. Johansson N, Ala-aho R, Uitto V, Grenman R, Fusenig NE, Lopez-Otin C, Kahari VM. Expression of collagenase-3 (MMP-13) and collagenase-1 (MMP-1) by transformed keratinocytes is dependent on the activity of p38 mitogen-activated protein kinase. *Journal of Cell Science* 2000;113 Pt 2:227-235.

112. Tanaka Y, Kobayashi H, Suzuki M, Kanayama N, Terao T. Transforming growth factor-beta1-dependent urokinase up-regulation and promotion of invasion are involved in Src-MAPK-dependent signaling in human ovarian cancer cells. *The Journal of Biological Chemistry* 2004;279(10):8567-8576.

113. Wakahara K, Kobayashi H, Yagyu T, Matsuzaki H, Kondo T, Kurita N, Sekino H, Inagaki K, Suzuki M, Kanayama N, Terao T. Transforming growth factor-beta1-dependent activation of Smad2/3 and up-regulation of PAI-1 expression is negatively regulated by Src in SKOV-3 human ovarian cancer cells. *Journal of Cellular Biochemistry* 2004;93(3):437-453.

114. Leask A, Abraham DJ. TGF-beta signaling and the fibrotic response. *The FASEB Journal* 2004;18(7):816-827.

115. Fukuda Y, Ishizaki M, Kudoh S, Kitaichi M, Yamanaka N. Localization of matrix metalloproteinases-1, -2, and -9 and tissue inhibitor of metalloproteinase-2 in interstitial lung diseases. *Laboratory Investigation* 1998;78(6):687-698.

116. Gurujeyalakshmi G, Hollinger MA, Giri SN. Regulation of transforming growth factor-beta1 mRNA expression by taurine and niacin in the bleomycin hamster model of lung fibrosis. *American Journal of Respiratory Cell and Molecular Biology* 1998;18(3):334-342.

117. Liu JY, Sime PJ, Wu T, Warshamana GS, Pociask D, Tsai SY, Brody AR. Transforming growth factor-beta(1) overexpression in tumor necrosis factor-alpha receptor knockout mice induces fibroproliferative lung disease. *American Journal of Respiratory Cell and Molecular Biology* 2001;25(1):3-7.

118. Yang YA, Dukhanina O, Tang B, Mamura M, Letterio JJ, MacGregor J, Patel SC, Khozin S, Liu ZY, Green J, Anver MR, Merlino G, Wakefield LM. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. *The Journal of Clinical Investigation* 2002;109(12):1607-1615.

119. Noble PW. Idiopathic pulmonary fibrosis. New insights into classification and pathogenesis usher in a new era therapeutic approaches. *American Journal of Respiratory Cell and Molecular Biology* 2003;29(3 Suppl):S27-31.

120. Chapman HA. Disorders of lung matrix remodeling. *The Journal of Clinical Investigation* 2004;113(2):148-157.

121. Moustakas A, Souchelnytskyi S, Heldin CH. Smad regulation in TGF-beta signal transduction. *Journal of Cell Science* 2001;114(Pt 24):4359-4369.

122. Dhainaut JF, Charpentier J, Chiche JD. Transforming growth factor-beta: a mediator of cell regulation in acute respiratory distress syndrome. *Critical Care Medicine* 2003;31(4 Suppl):S258-264.

123. 劉曉濱, 姜曉妹, 周亞濱. 活血化瘀、益氣養陰法對實驗性肺纖維化 LPO 和 SOD 的影響. *中國中西結合雜誌* 1994;14(12):733-751.

124. 張紓難, 王辛秋, 李蘭群. 益肺化纖方治療肺纖維化的實驗研究. *中國醫藥學報* 1999;14(6):422-425.

125. 戴令娟, 侯傑, 蔡後榮. 川芎嗪當歸治療肺間質纖維化的實驗研究. *中華結核和呼吸雜誌* 1996;19(1):26-28.

126. 朱建偉, 李貴海. 丹參、當歸對小鼠肺纖維化的抑制作用. *山東中醫學院學報* 1995;19(4):267.

127. Lynch JP, 3rd, White E, Flaherty K. Corticosteroids in idiopathic pulmonary fibrosis. *Current Opinion in Pulmonary Medicine* 2001;7(5):298-308.

128. Zhang K, Gharaee-Kermani M, McGarry B, Remick D, Phan SH. TNF-alpha-mediated lung cytokine networking and eosinophil recruitment in pulmonary fibrosis. *Journal of Immunology* 1997;158(2):954-959.

129. Okada T, Sugie I, Aisaka K. Effects of gamma-interferon on collagen and histamine content in bleomycin-induced lung fibrosis in rats. *Lymphokine and Cytokine Research* 1993;12(2):87-91.

130. Card JW, Racz WJ, Brien JF, Margolin SB, Massey TE. Differential effects of pirfenidone on acute pulmonary injury and ensuing fibrosis in the hamster model of amiodarone-induced pulmonary toxicity. *Toxicological Sciences* 2003;75(1):169-180.

131. Nakayama S, Mukae H, Sakamoto N, Kakugawa T, Yoshioka S, Soda H, Oku H, Urata Y, Kondo T, Kubota H, Nagata K, Kohno S. Pirfenidone inhibits the expression of HSP47 in TGF-beta1-stimulated human lung fibroblasts. *Life Sciences* 2008;82(3-4):210-217.

132. Krishna G, Liu K, Shigemitsu H, Gao M, Raffin TA, Rosen GD. PG490-88, a derivative of triptolide, blocks bleomycin-induced lung fibrosis. *The American Journal of Pathology* 2001;158(3):997-1004.

133. Kobayashi H, Kobayashi M, Heming TA, Bidani A, Pollard RB, Suzuki F. Cytokine production by rabbit alveolar macrophages: differences between activated and suppressor cell phenotypes. *Immunology Letters* 1999;69(3):339-346.

134. Gordon S. Alternative activation of macrophages. *Nature Reviews Immunology* 2003;3(1):23-35.

135. Nicod LP. Pulmonary defence mechanisms. *Respiration* 1999;66(1):2-11.

136. Chaudhuri N, Dower SK, Whyte MK, Sabroe I. Toll-like receptors and chronic lung disease. *Clinical Science* 2005;109(2):125-133.

137. Guha M, Mackman N. LPS induction of gene expression in human monocytes. *Cellular Signaling* 2001;13(2):85-94.

138. Akira S, Yamamoto M, Takeda K. Role of adapters in Toll-like receptor signaling. *Biochemical Society Transactions* 2003;31(Pt 3):637-642.

139. Wu Q, Lu Z, Verghese MW, Randell SH. Airway epithelial cell tolerance to Pseudomonas aeruginosa. *Respiratory Research* 2005;6:26.

140. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proceedings of the National Academy of Sciences of the United States of America* 1975;72(9):3666-3670.

141. Vassalli P, Grau GE, Piguet PF. TNF in autoimmune diseases, graft-versus-host reactions, and pulmonary fibrosis. *Immunology Series* 1992;56:409-430.

142. Olivenstein R, Du T, Xu LJ, Martin JG. Microvascular leakage in the airway wall and lumen during allergen induced early and late responses in rats. *Pulmonary Pharmacology &Therapeutics* 1997;10(4):223-230.

143. Ward PA, Hunninghake GW. Lung inflammation and fibrosis. *American Journal of Respiratory and Critical Care Medicine* 1998;157(4 Pt 2):S123-129.

144. Ishihara K, Hirano T. IL-6 in autoimmune disease and chronic inflammatory proliferative disease. *Cytokine and Growth Factor Reviews* 2002;13(4-5):357-368.

145. Akira S, Kishimoto T. IL-6 and NF-IL6 in acute-phase response and viral infection. *Immunological Reviews* 1992;127:25-50.

146. Elias JA, Lentz V. IL-1 and tumor necrosis factor synergistically stimulate fibroblast IL-6 production and stabilize IL-6 messenger RNA. *The Journal of Immunology* 1990;145(1):161-166.

147. Roth M, Nauck M, Yousefi S, Tamm M, Blaser K, Perruchoud AP, Simon HU. Platelet-activating factor exerts mitogenic activity and stimulates expression of interleukin 6 and interleukin 8 in human lung fibroblasts via binding to its functional receptor. *The Journal of Experimental Medicine* 1996;184(1):191-201.

148. Desgeorges A, Gabay C, Silacci P, Novick D, Roux-Lombard P, Grau G, Dayer JM, Vischer T, Guerne PA. Concentrations and origins of soluble interleukin 6 receptor-alpha in serum and synovial fluid. *The Journal of Rheumatology* 1997;24(8):1510-1516.

149. Mosmann TR, Coffman RL. Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Advances in Immunology* 1989;46:111-147.

150. Asadullah K, Sterry W, Volk HD. Interleukin-10 therapy--review of a new approach. *Pharmacological Reviews* 2003;55(2):241-269.

151. Rennick DM, Fort MM. Lessons from genetically engineered animal models. XII. IL-10-deficient (IL-10(-/-) mice and intestinal inflammation. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 2000;278(6):G829-833.

152. Nakagome K, Dohi M, Okunishi K, Tanaka R, Miyazaki J, Yamamoto K. In vivo IL-10 gene delivery attenuates bleomycin induced pulmonary fibrosis by inhibiting the production and activation of TGF-beta in the lung. *Thorax* 2006;61(10):886-894.

153. Carl VS, Gautam JK, Comeau LD, Smith MF, Jr. Role of endogenous IL-10 in LPS-induced STAT3 activation and IL-1 receptor antagonist gene expression. *Journal of Leukocyte Biology* 2004;76(3):735-742.

154. Arend WP. The balance between IL-1 and IL-1Ra in disease. *Cytokine and Growth Factor Reviews* 2002;13(4-5):323-340.

155. Andersson J, Bjork L, Dinarello CA, Towbin H, Andersson U. Lipopolysaccharide induces human interleukin-1 receptor antagonist and interleukin-1 production in the same cell. *European Journal of Immunology* 1992;22(10):2617-2623.

156. Schonbohn H, Schuler M, Kolbe K, Peschel C, Huber C, Bemb W, Aulitzky WE. Plasma levels of IL-1, TNF alpha, IL-6, IL-8, G-CSF, and IL1-RA during febrile neutropenia: results of a prospective study in patients undergoing chemotherapy for acute myelogenous leukemia. *Annals of Hematology* 1995;71(4):161-168.

157. MacDermot J, Kelsey CR, Waddell KA, Richmond R, Knight RK, Cole PJ, Dollery CT, Landon DN, Blair IA. Synthesis of leukotriene B4, and prostanoids by human alveolar macrophages: analysis by gas chromatography/mass spectrometry. *Prostaglandins* 1984;27(2):163-179.

158. Takahashi H, Abe M, Hashimoto S, Takayama K, Miyazaki M. In vivo effect of lipopolysaccharide on alveolar and peritoneal macrophages of rats: superoxide anion generation and 5-lipoxygenase metabolism of arachidonic acid. *American Journal of Respiratory Cell and Molecular Biology* 1993;8(3):291-298.

159. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews* 1991;43(2):109-142.

160. Nathan C. Nitric oxide as a secretory product of mammalian cells. *The FASEB Journal* 1992;6(12):3051-3064.

161. Marletta MA. Nitric oxide synthase structure and mechanism. *The Journal of Biological Chemistry* 1993;268(17):12231-12234.

162. Fang FC. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *The Journal of Clinical Investigation* 1997;99(12):2818-2825.

163. Hamid Q, Springall DR, Riveros-Moreno V, Chanez P, Howarth P, Redington A, Bousquet J, Godard P, Holgate S, Polak JM. Induction of nitric oxide synthase in asthma. *Lancet* 1993;342(8886-8887):1510-1513.

164. Kharitonov SA, Yates D, Robbins RA, Logan-Sinclair R, Shinebourne EA, Barnes PJ. Increased nitric oxide in exhaled air of asthmatic patients. *Lancet* 1994;343(8890):133-135.

165. Beutler B, Rietschel ET. Innate immune sensing and its roots: the story of endotoxin. *Nature Reviews Immunology* 2003;3(2):169-176.

166. Nathan C. Inducible nitric oxide synthase: what difference does it make? *The Journal of Clinical Investigation* 1997;100(10):2417-2423.

167. Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annual Review of Immunology* 1998;16:225-260.

168. Zhang G, Ghosh S. Toll-like receptor-mediated NF-kB activation: a phylogenetically conserved paradigm in innate immunity. *The Journal of Clinical Investigation* 2001;107(1):13-19.

169. Orian A, Whiteside S, Israel A, Stancovski I, Schwartz AL, Ciechanover A. Ubiquitin-mediated processing of NF-kB transcriptional activator precursor p105. Reconstitution of a cell-free system and identification of the ubiquitin-carrier protein, E2, and a novel ubiquitin-protein ligase, E3, involved in conjugation. *The Journal of Biological Chemistry* 1995;270(37):21707-21714.

170. Malek S, Chen Y, Huxford T, Ghosh G. IkappaBbeta, but not IkappaBalpha, functions as a classical cytoplasmic inhibitor of NF-kappaB dimers by masking both NF-kappaB nuclear localization sequences in resting cells. *The Journal of Biological Chemistry* 2001;276(48):45225-45235.

171. Tam WF, Sen R. IkappaB family members function by different mechanisms. *The Journal of Biological Chemistry* 2001;276(11):7701-7704.

172. Arenzana-Seisdedos F, Turpin P, Rodriguez M, Thomas D, Hay RT, Virelizier JL, Dargemont C. Nuclear localization of IkB alpha promotes active transport of NF-kB from the nucleus to the cytoplasm. *Journal of Cell Science* 1997;110 (Pt 3):369-378.

173. DiDonato J, Mercurio F, Rosette C, Wu-Li J, Suyang H, Ghosh S, Karin M. Mapping of the inducible IkB phosphorylation sites that signal its ubiquitination and degradation. *Molecular and Cellular Biology* 1996;16(4):1295-1304.

174. Miller BS, Zandi E. Complete reconstitution of human IkappaB kinase (IKK) complex in yeast. Assessment of its stoichiometry and the role of IKKγ on the complex activity in the absence of stimulation. *The Journal of Biological Chemistry* 2001;276(39):36320-36326.

175. Karin M. How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. *Oncogene* 1999;18(49):6867-6874.

176. Li ZW, Chu W, Hu Y, Delhase M, Deerinck T, Ellisman M, Johnson R, Karin M. The IKKbeta subunit of IkB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *The Journal of Experimental Medicine* 1999;189(11):1839-1845.

177. Hu Y, Baud V, Delhase M, Zhang P, Deerinck T, Ellisman M, Johnson R, Karin M. Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. *Science* 1999;284(5412):316-320.

178. Malinin NL, Boldin MP, Kovalenko AV, Wallach D. MAP3K-related kinase involved in NF-kB induction by TNF, CD95 and IL-1. *Nature* 1997;385(6616):540-544.

179. Gabrielsen OS. The NF-kB/Rel family. *http://www.uio.no/studier/emner/matnat/molbio*.

180. Abate C, Patel L, Rauscher FJ, 3rd, Curran T. Redox regulation of fos and jun DNA-binding activity in vitro. *Science* 1990;249(4973):1157-1161.

181. Karin M, Liu Z, Zandi E. AP-1 function and regulation. *Current Opinion in Cell Biology* 1997;9(2):240-246.

182. Shaulian E, Karin M. AP-1 in cell proliferation and survival. *Oncogene* 2001;20(19):2390-2400.

183. Whitmarsh AJ, Davis RJ. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *Journal of Molecular Medicine* 1996;74(10):589-607.

184. Gwinn MR, Vallyathan V. Respiratory burst: role in signal transduction in alveolar macrophages. *Journal of Toxicology and Environmental Health* 2006;9(1):27-39.

185. Martin LD, Krunkosky TM, Voynow JA, Adler KB. The role of reactive oxygen and nitrogen species in airway epithelial gene expression. *Environmental Health Perspectives* 1998;106 Suppl 5:1197-1203.

186. http://www.egms.de/figures/journals/cto/2004-3/cto000002.f12.png.

187. Tanino Y, Makita H, Miyamoto K, Betsuyaku T, Ohtsuka Y, Nishihira J, Nishimura M. Role of macrophage migration inhibitory factor in bleomycin-induced lung injury and fibrosis in mice. *American Journal of Physiology,Lung Cellular and Molecular Physiology* 2002;283(1):L156-162.

188. Ashcroft T, Simpson JM, Timbrell V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *Journal of Clinical Pathology* 1988;41(4):467-470.

189. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature* 2000; 406(6797): 782-787.

190. Hayden MS, Ghosh S. Signaling to NF-kappaB. *Genes and Developement* 2004; 18(18):2195-2224.

191. Ghosh S, Baltimore D. Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B. *Nature* 1990; 344(6267):678-682.

192. Granger RL, Hughes TR, Ramji DP. Gene, stimulus and cell-type specific regulation of activator protein-1 in mesangial cells by lipopolysaccharide and cytokines. *Biochimica et Biophysica Acta* 2000; 1492(1):100-107.

193. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. *Nature Cell Biology* 2002; 4(5):E131-136.

194. Hsu HY, Wen MH. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *The Journal of Biological Chemistry* 2002; 277(25):22131-22139.

195. Lu Y, Wahl LM. Oxidative stress augments the production of matrix metalloproteinase-1, cyclooxygenase-2, and prostaglandin E2 through enhancement of NF-kB activity in lipopolysaccharide-activated human primary monocytes. *Journal of Immunology* 2005; 175(8):5423-5429.

196. Schrier DJ, Kunkel RG, Phan SH. The role of strain variation in murine bleomycin-induced pulmonary fibrosis. *The American Review of Respiratory Disease* 1983;127(1):63-66.

197. Izbicki G, Segel MJ, Christensen TG, Conner MW, Breuer R. Time course of bleomycin-induced lung fibrosis. *International Journal of Experimental Pathology* 2002;83(3):111-119.

198. Chaudhary NI, Schnapp A, Park JE. Pharmacologic differentiation of inflammation and fibrosis in the rat bleomycin model. *American Journal of Respiratory and Critical Care Medicine* 2006;173(7):769-776.

199. Gauldie J, Kolb M, Ask K, Martin G, Bonniaud P, Warburton D. Smad3 signaling involved in pulmonary fibrosis and emphysema. *Proceedings of the American Thoracic Society* 2006;3(8):696-702.

200. Verrecchia F, Mauviel A, Farge D. Transforming growth factor-beta signaling through the Smad proteins: role in systemic sclerosis. *Autoimmunity Reviews* 2006;5(8):563-569.

201. Bonniaud P, Margetts PJ, Ask K, Flanders K, Gauldie J, Kolb M. TGF-beta and Smad3 signaling link inflammation to chronic fibrogenesis. *The Journal of Immunology* 2005;175(8):5390-5395.

202. Zhao X, Nicholls JM, Chen YG. Severe acute respiratory syndrome-associated coronavirus nucleocapsid protein interacts with Smad3 and modulates transforming growth factor-beta signaling. *The Journal of Biological Chemistry* 2008;283(6):3272-3280.

203. King TE, Jr., Schwarz MI, Brown K, Tooze JA, Colby TV, Waldron JA, Jr., Flint A, Thurlbeck W, Cherniack RM. Idiopathic pulmonary fibrosis: relationship between histopathologic features and mortality. *American Journal of Respiratory and Critical Care Medicine* 2001;164(6):1025-1032.

204. Phan SH. The myofibroblast in pulmonary fibrosis. *Chest* 2002;122(6 Suppl):286S-289S.

205. Chen SP, Zhou B, Willis BC, Sandoval AJ, Liebler JM, Kim KJ, Ann DK, Crandall ED, Borok Z. Effects of transdifferentiation and EGF on claudin isoform expression in alveolar epithelial cells. *Journal of Applied Physiology* 2005;98(1):322-328.

206. Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, Sheppard D, Chapman HA. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. *Proceedings of the National Academy of Sciences of the United States of America* 2006;103(35):13180-13185.

207. Shimada H, Miura K, Imamura Y. Characteristics and inhibition by flavonoids of 20alpha-hydroxysteroid dehydrogenase activity in mouse tissues. *Life Sciences* 2006; 78(25):2931-2936.

208. Yuan G, Wahlqvist ML, He G, Yang M, Li D. Natural products and anti-inflammatory activity. *Asia Pacific Journal of Clinical Nutrition* 2006; 15(2):143-152.

209. Harris GK, Qian Y, Leonard SS, Sbarra DC, Shi X. Luteolin and chrysin differentially inhibit cyclooxygenase-2 expression and scavenge reactive oxygen species but similarly inhibit prostaglandin-E2 formation in RAW 264.7 cells. *The Journal of Nutrition* 2006; 136(6):1517-1521.

210. Lambrecht BN. Alveolar macrophage in the driver's seat. *Immunity* 2006;24(4):366-368.

211. Feng GJ, Goodridge HS, Harnett MM, Wei XQ, Nikolaev AV, Higson AP, Liew FY. Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: Leishmania phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. *The Journal of Immunology* 1999;163(12):6403-6412.

212. Nemeth ZH, Hasko G, Vizi ES. Pyrrolidine dithiocarbamate augments IL-10, inhibits TNF-alpha, MIP-1alpha, IL-12, and nitric oxide production and protects from the lethal effect of endotoxin. *Shock* 1998; 10(1):49-53.

213. Ziegler-Heitbrock HW, Sternsdorf T, Liese J, Belohradsky B, Weber C, Wedel A, Schreck R, Bauerle P, Strobel M. Pyrrolidine dithiocarbamate inhibits NF-kB mobilization and TNF production in human monocytes. *Journal of Immunology* 1993;151(12):6986-6993.

214. Lee AK, Sung SH, Kim YC, Kim SG. Inhibition of lipopolysaccharide-inducible nitric oxide synthase, TNF-alpha and COX-2 expression by sauchinone effects on I-kappaBalpha phosphorylation, C/EBP and AP-1 activation. *British Journal of Pharmacology* 2003; 139(1):11-20.

215. Moon SK, Cho GO, Jung SY, Gal SW, Kwon TK, Lee YC, Madamanchi NR, Kim CH. Quercetin exerts multiple inhibitory effects on vascular smooth muscle cells: role of ERK1/2, cell-cycle regulation, and matrix metalloproteinase-9. *Biochemical and Biophysical Research Communications* 2003; 301(4):1069-1078.

216. Kamimura D, Ishihara K, Hirano T. IL-6 signal transduction and its physiological roles: the signal orchestration model. *Reviews of Physiology, Biochemistry and Pharmacology* 2003; 149: 1-38.

217. Guha M, Mackman N. LPS induction of gene expression in human monocytes. *Cellular Signaling* 2001; 13(2):85-94.

218. Aktan F. iNOS-mediated nitric oxide production and its regulation. *Life Sciences* 2004; 75(6):639-653.

219. Cho MK, Suh SH, Kim SG. JunB/AP-1 and NF-kB-mediated induction of nitric oxide synthase by bovine type I collagen in serum-stimulated murine macrophages. *Nitric Oxide* 2002; 6(3):319-332.

220. Pham CG, Bubici C, Zazzeroni F, Papa S, Jones J, Alvarez K, Jayawardena S, De Smaele E, Cong R, Beaumont C, Torti FM, Torti SV, Franzoso G. Ferritin heavy chain upregulation by NF-kB inhibits TNFalpha-induced apoptosis by suppressing reactive oxygen species. *Cell* 2004; 119(4):529-542.

221. Gloire G, Legrand-Poels S, Piette J. NF-kappaB activation by reactive oxygen species: Fifteen years later. *Biochemical Pharmacology* 2006; 72(11):1493-1505.

222. Ma Q, Kinneer K, Ye J, Chen BJ. Inhibition of nuclear factor kappaB by phenolic antioxidants: interplay between antioxidant signaling and inflammatory cytokine expression. *Molecular Pharmacology* 2003; 64(2):211-219.

223. Ardeshna KM, Pizzey AR, Devereux S, Khwaja A. The PI3 kinase, p38 SAP kinase, and NF-kB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. *Blood* 2000; 96(3):1039-1046.

224. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF-kB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* 1999; 401(6748):82-85.

225. Madrid LV, Mayo MW, Reuther JY, Baldwin AS, Jr. Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kB through utilization of the IkB kinase and activation of the mitogen-activated protein kinase p38. *The Journal of Biological Chemistry* 2001;276(22):18934-18940.

Available online at www.sciencedirect.com
ScienceDirect
Life Sciences 81 (2007) 1602–1614
Life Sciences
www.elsevier.com/locate/lifescie

Luteolin suppresses inflammation-associated gene expression by blocking NF-κB and AP-1 activation pathway in mouse alveolar macrophages

Chiu-Yuan Chen [a], Wen-Huang Peng [a], Kuen-Daw Tsai [b], Shih-Lan Hsu [a,c,*]

[a] Graduate Institute of Chinese Pharmaceutical Sciences, China Medical University, Taichung 404, Taiwan, ROC
[b] Department of Internal Medicine, China Medical University Hospital at Beigang, Yunlin 651, Taiwan, ROC
[c] Department of Education & Research, Taichung Veterans General Hospital, Taichung 407, Taiwan, ROC

Received 23 March 2007; accepted 26 September 2007

Abstract

Luteolin, a plant flavonoid, has potent anti-inflammatory properties both in vitro and in vivo. However, the molecular mechanism of luteolin-mediated immune modulation has not been fully understood. In this study, we examined the effects of luteolin on the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2), as well as the expression of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) in mouse alveolar macrophage MH-S and peripheral macrophage RAW 264.7 cells. Luteolin dose-dependently inhibited the expression and production of these inflammatory genes and mediators in macrophages stimulated with lipopolysaccharide (LPS). Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) assay further confirmed the suppression of LPS-induced TNF- α, IL-6, iNOS and COX-2 gene expression by luteolin at a transcriptional level. Luteolin also reduced the DNA binding activity of nuclear factor-kappa B (NF-κB) in LPS-activated macrophages. Moreover, luteolin blocked the degradation of IκB-α and nuclear translocation of NF-κB p65 subunit. In addition, luteolin significantly inhibited the LPS-induced DNA binding activity of activating protein-1 (AP-1). We also found that luteolin attenuated the LPS-mediated protein kinase B (Akt) and IKK phosphorylation, as well as reactive oxygen species (ROS) production. In sum, these data suggest that, by blocking NF-κB and AP-1 activation, luteolin acts to suppress the LPS-elicited inflammatory events in mouse alveolar macrophages, and this effect was mediated, at least in part, by inhibiting the generation of reactive oxygen species. Our observations suggest a possible therapeutic application of this agent for treating inflammatory disorders in lung.

Keywords: Luteolin; LPS; NF-κB; AP-1; Alveolar macrophage; Anti-inflammation

Introduction

It is well documented that pulmonary oxidant stress and inflammatory reaction play important pathological roles in disease conditions, including acute lung injury/adult respiratory distress syndrome (ALI/ARDS), hyperoxia, ischemia–reperfusion, sepsis, radiation injury, lung transplantation, and chronic obstructive pulmonary disease (COPD). Reactive oxygen species (ROS), released from activated leukocytes and macrophage, damage the lungs and initiate cascades of pro-inflammatory reactions multiplying pulmonary and systemic stress (Christofidou-Solomidou and Muzykantov, 2006). Severe acute respiratory syndrome (SARS) is a novel global infectious disease induced by Corona virus. A clinical investigation has shown that pathological changes in SARS patients are similar to those of acute lung injury, as revealed by alveolar cell collapse, severe exudation, acute inflammatory reaction and hyaline membrane formation (Lew et al., 2003). Currently, no effective therapy is available for the management of ALI/ARDS/SARS and only supportive therapies exist. This has prompted us to search for novel compounds with therapeutic effect to reduce lung injuries and relieve symptoms (File and Tsang, 2005; Lai, 2005).

It is noteworthy that the use of medicinal plants or their crude extracts is increasingly becoming an attractive approach as a complement and an alternative for treating various inflammatory

* Corresponding author. Department of Education & Research, Taichung Veterans General Hospital, Taichung 407, Taiwan, ROC. Tel.: +886 4 23592525x4037; fax: +886 4 23592705.
E-mail address: h2326@mail.vghtc.gov.tw (S.-L. Hsu).

0024-3205/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2007.09.028

disorders. *Lonicera japonica* (Caprifoliaceae) has been known as an anti-inflammatory herb in traditional Chinese medicine for thousands of years and has been widely used for upper respiratory tract infections, diabetes mellitus and rheumatoid arthritis (Lee et al., 2001). This herbal medicine has also been employed for treatment of SARS in mainland China (Liu et al., 2006; Wu et al., 2004). Luteolin (3′,4′,5,7-tetra-hydroxyl-flavone), a flavonoid isolated from *L. japonica*, exhibits a strongly anti-inflammatory activity, can effectively inhibit the lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and inducible nitric oxide (NO) production in vitro (Park et al., 2005; Xagorari et al., 2001), and also protect against LPS-induced lethal toxicity by inhibiting pro-inflammatory molecule expression in vivo and reducing leukocyte infiltration in tissues (Kotanidou et al., 2002). LPS from Gram-negative bacteria is well known to cause bacterial sepsis mediated through activation of monocytes, neutrophils and macrophages (Aderem and Ulevitch, 2000). Sometimes activation of these cells may induce hyper-secretion of various pro-inflammatory and toxicity mediating molecules such as TNF-α and IL-6, eicosonoids and nitric oxide (NO) (Nathan, 1987). The excessive inflammatory responses, in turn, may manifest respiratory failure and multiple organ dysfunction syndrome (MODS) i.e. loss of capillary integrity, distributive and septic shock, multiple organ dysfunction and mortality. Exposure of laboratory animals or cultured monocytes to bacterial LPS triggers the generation of ROS and gene induction; the inducible genes encode pro-inflammatory cytokines and enzymes such as TNF-α, IL-6, COX-2, and iNOS, which up-regulate the host defense systems but unfortunately also contribute to pathological conditions such as bacterial sepsis, ischemia/reperfusion injuries, chronic inflammatory disease, and the down-regulation of hepatic drug-metabolizing enzymes (Guha and Mackman, 2001; Guha et al., 2001). Recently, luteolin has been found to be able to inhibit the LPS-induced TNF-α release by inactivating the extracellular-regulated kinases (ERK) and p38 MAPK and blocking the transcriptional activation of nuclear factor-kappa B (NF-κB) (Xagorari et al., 2001, 2002). An in vivo study indicated that feeding of luteolin protects mice against LPS-induced toxicity (Kotanidou et al., 2002). Also, luteolin alleviates bronchoconstriction and airway hyperreactivity in ovalbumin-sensitized mice (Das et al., 2003) and decreases the acute *Chlamydia pneumoniae* infection load and inflammatory reactions in vivo (Tormakangas et al., 2005).

Alveolar macrophages play a vital role in the defense of the lung through their ability to scavenge inhaled particles, ingest and kill microorganisms, recruit and activate other inflammatory cells and elicit the immune response. They perform major roles in antigen presentation, production of a variety of biologically active substances (e.g. ROS and cytokines), and modulation and regulation of immune effector cells (Kobayashi et al., 1999; Matsunaga et al., 2001). Due to the lack of scientific evidence to address the effect of luteolin on the alveolar macrophages, in the present study, we examined the potential anti-inflammatory properties of luteolin in LPS-stimulated mouse alveolar macrophage MH-S and peripheral macrophage RAW 264.7 cell lines. In addition, we also investigate the molecular mechanism responsible for the inhibitory effect of luteolin. In this study, we demonstrate that luteolin inhibits LPS-induced inflammatory reactions in MH-S cells, apparently through blocking the NF-κB and activating the protein (AP-1) transcription activation pathways.

Materials and methods

Reagents and cell culture

Luteolin was obtained from Extrasynthese (Genay, France) and dissolved in EtOH:DMSO (1:1,v/v). Lipopolysaccharide (*Escherichia coli* 055:B5), 1, 7-bis (4-hydroxy-3-methoxy-phenyl)-1, 6-heptadiene-3, 5-dinone (curcumin), ammonium pyrryolidinedithio-carbamate (PDTC) and LY294002 were purchased from Sigma (St. Louis, MO, USA). Two different types of macrophages were used in this study. MH-S and RAW 264.7 cells were obtained from Culture Collection and Research Center (Hsinchu, Taiwan). MH-S were cultured in RPMI 1640 medium containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), penicillin (100 units/ml), and streptomycin (100 μg/ml), 10 mM HEPES, 0.05 mM 2-mercaptoethanol. RAW 264.7 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). These cells were maintained at subconfluence in a 95% air, 5% CO_2 humidified atmosphere at 37 °C.

Cell viability

Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to purple formazan. Cells were incubated with MTT (0.5%) for 4 h at 37 °C. The medium was removed by aspiration, and formazan crystals were dissolved in DMSO. The extent of the reduction of MTT was quantified by measurement of A550.

Table 1
Primers used in RT-PCR analysis

Primer	Sequence
TNF-α	Forward 5′-ATGAGCACAGAAAGCATGATCCGC-3′
(502 bp)	Reverse 5′-CTCAGGCCCGTCCAGATGAAACC-3′
IL-6	Forward 5′-ATGAAGTTCCTCTCTGCAAGAGACT-3′
(247 bp)	Reverse 5′-CACTAGGTTTGCCGAGTAGATCTC-3′
iNOS	Forward 5′-CAACCAGTATTATGGCTCCT-3′
(835 bp)	Reverse 5′-GTGACAGCCCGGTCTTTCCA-3′
COX-2	Forward 5′-GGAGAGACTATCAAGATAGTGATC-3′
(860 bp)	Reverse 5′-ATGGTCAGTAGACTTTTACAGCTC-3′
IL-10	Forward 5′-TGA ATT CCC TGG GTG AGA AG-3′
(136 bp)	Reverse 5′-ACA CCT TGG TCT TGG AGC TT-3′
IL-1Ra	Forward 5′-AAA TCT GCT GGG GAC CCT AC -3′
(127 bp)	Reverse 5′-GGT CAA TAG GCA CCA TGT CT-3′
GAPDH	Forward 5′-ACCACAGTCCATGCCATCAC-3′
(451 bp)	Reverse 5′-TCCACCACCCTGTTGCTGTA-3′

Cytokine measurement

Cytokine concentrations in the supernatants were determined by ELISA kits that were specific against mouse cytokines. Levels of TNF-α and IL-6 were measured by using OptEIA Set from BD Biosciences. Assays were performed according to the manufacturer's instructions.

Nitrite measurement

The nitrite concentration in the medium was measured according to the Griess reaction, and the calculated concentration was taken as an indicator of NO production. The supernatant of cell cultures was mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). The optical density at 550 nm (A550) was measured and calculated against a sodium nitrite standard curve.

PGE_2 measurements

PGE_2 concentration in the supernatant of the culture medium was determined by using an EIA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Western blotting analysis

Confluent cells (1×10^6) in a 3.5-cm petri dish were pretreated without or with luteolin (25 μM, Extrasynthese, Genay, France) for 30 min and then treated with LPS (100 ng/

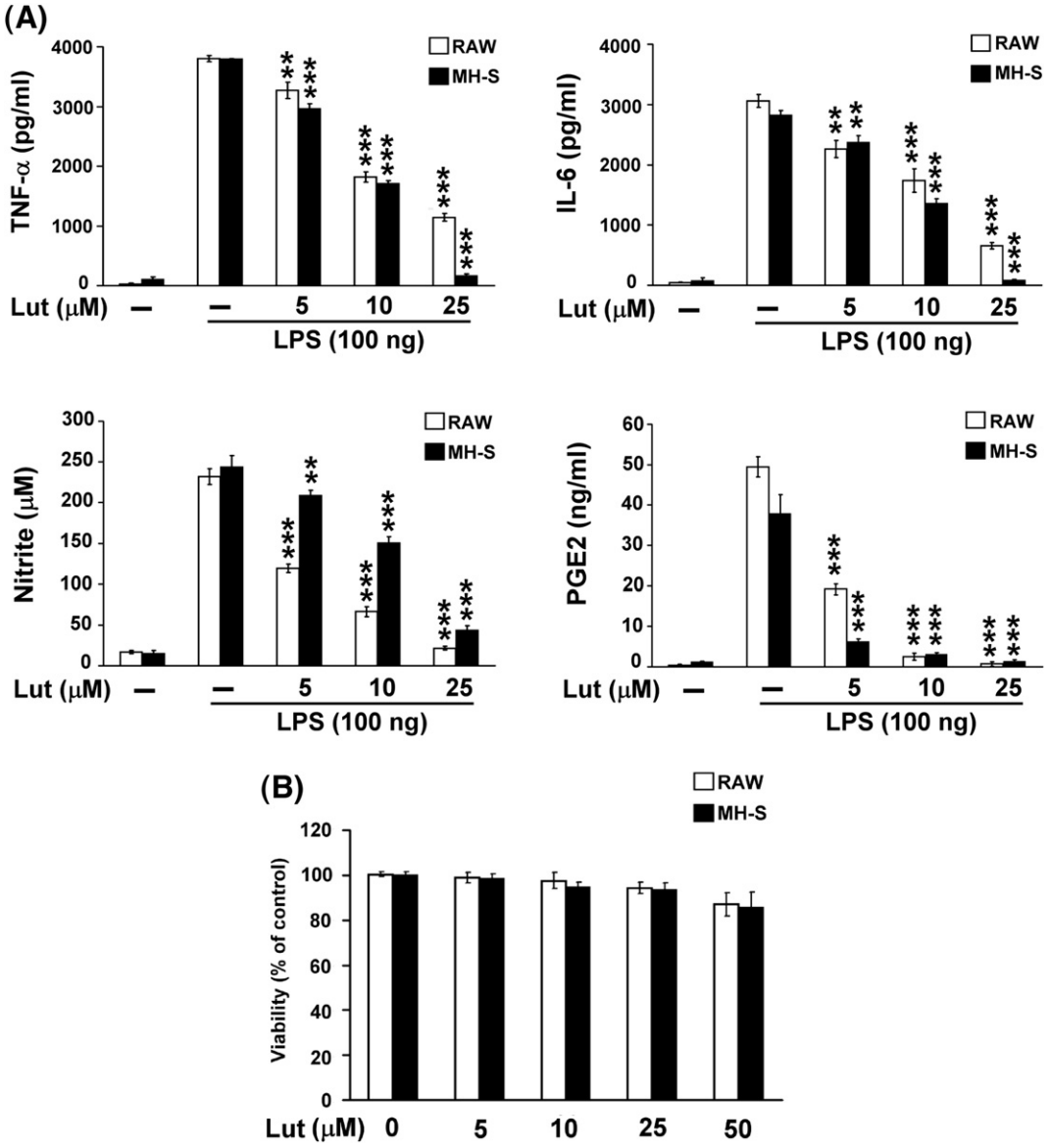


Fig. 1. Luteolin inhibited LPS-induced pro-inflammatory cytokine and mediator production. MH-S and RAW 264.7 cells were pretreated with luteolin (5–25 μM) for 30 min, and then stimulated with LPS (100 ng/ml). (A) Culture media were collected at 8 h for TNF-α and IL-6 analysis, 16 h for NO and PGE_2 analysis. (B) Viability in luteolin-treated cells was evaluated using the MTT assay. Cells were incubated with 5–50 μM luteolin for 24 h. The results are displayed in percentage of control samples. Data are presented as the mean ± SEM ($n=3$) for three independent experiments; $^{**}p<0.01$, $^{***}p<0.001$ as compared with the LPS treatment. Lut, luteolin.

ml) in the absence or presence of luteolin. At each point in time, cells were washed with PBS and then scraped into microcentrifuge tubes and pelleted. The cell pellets were resuspended in extraction lysis buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM NaF, 5 mg/ml each of leupeptin and aprotinin) and incubated for 20 min at 4 °C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined by using the Bio-Rad protein assay reagent according to the manufacturer's instruction. Lysates (20 μg/lane) were separated by SDS-PAGE on polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (PerkinElmer Life Sciences, Inc. Boston, MA, USA). The membranes were blocked in 5% milk in TBS-T solution for 1 h. Then membranes were subsequently probed with monoclonal anti-iNOS antibody (Sigma, St. Louis, MO, USA), anti-COX-2 and IκBα antibody (Santa Cruz Biochemicals, Santa Cruz, CA, USA), anti-phospho-Akt (Thr308) and phospho-Akt (Ser473), anti-IKKα/β, anti-phospho-IKKα/β antibody (Cell Signaling) overnight at 4 °C. The blots were washed with TBS-T three times and incubated with horseradish peroxidase-conjugated rabbit anti-mouse or anti-rabbit IgG (1:20,000; PerkinElmer Life Sciences, Inc. Boston, MA, USA) for 1 h at room temperature. Following three further washings in TBS-T, immunoreactive bands were visualized using the ECL-Plus detection system (PerkinElmer Life Sciences, Inc. Boston, MA, USA).

Semi-quantitative RT-PCR for TNF-α, IL-6, iNOS, COX-2, IL-10 and IL-1Ra

Total cellular RNA was extracted from both the controlled and treated cells in a 6-well plate by Trisolution Reagent Plus (GeneMark, Taiwan) in accordance with the manufacturer's instructions. Then RT-PCR analyses were performed by using the One-step RT-PCR kit (GeneMark, Taiwan). The sequences of the primers are presented in Table 1. The first strand of cDNA synthesis was 1 cycle for reverse transcription (50 °C, 30 min) and 1 cycle for MMLV RTase inactivation (94 °C, 2 min). The second strand cDNA synthesis and PCR reaction were performed as follows: TNF-α, IL-6, IL-10 and IL-1Ra (94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min), iNOS (94 °C for 45 s, 65 °C for 45 s, and 72 °C for 2 min) and COX-2 (94 °C for 45 s, 55 °C for 1 min, 72 °C for 2 min). For each combination of primers, the kinetics of PCR amplification was studied. The number of cycles corresponding to plateau was determined and PCR was performed at exponential range. PCRs were electrophoresed through a 1% agarose gel and visualized by ethidium bromide staining and UV irradiation.

Electrophoretic mobility shift assay

MH-S cells were plated in 3.5-cm dishes (1×10^6 cells). The cells were pretreated without or with luteolin (25 μM) for 30 min and then treated with LPS in the presence or absence of luteolin for 0–120 min. After treatment, cells were washed once with PBS, scraped into 1 ml of cold PBS, and pelleted by centrifugation. Nuclear fractions were extracted by buffer I (1 M Tris–HCl, 0.5 M EDTA, 100 mM EGTA) and Totex buffer. Twenty micrograms of nuclear protein was incubated with 100 pmol of 5′-biotinate double-stranded oligonucleotide probes containing a consensus binding sequence of NF-κB (5′-AGTTGAGGGGACTTTCC-CAGGC-3′) or AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) for 30 min and resolved in 6% nondenaturing polyacrylamide gel. The protein–DNA–biotin complexes were blotted onto a nitrocellulose transfer membrane (PerkinElmer Life Science, Inc. Boston, MA, USA) followed by UV cross-linking. The complexes were revealed with streptavidin–horseradish peroxidase conjugate and SuperSignal chemiluminescent substrate then exposed to X-ray film.

Immunofluorescence

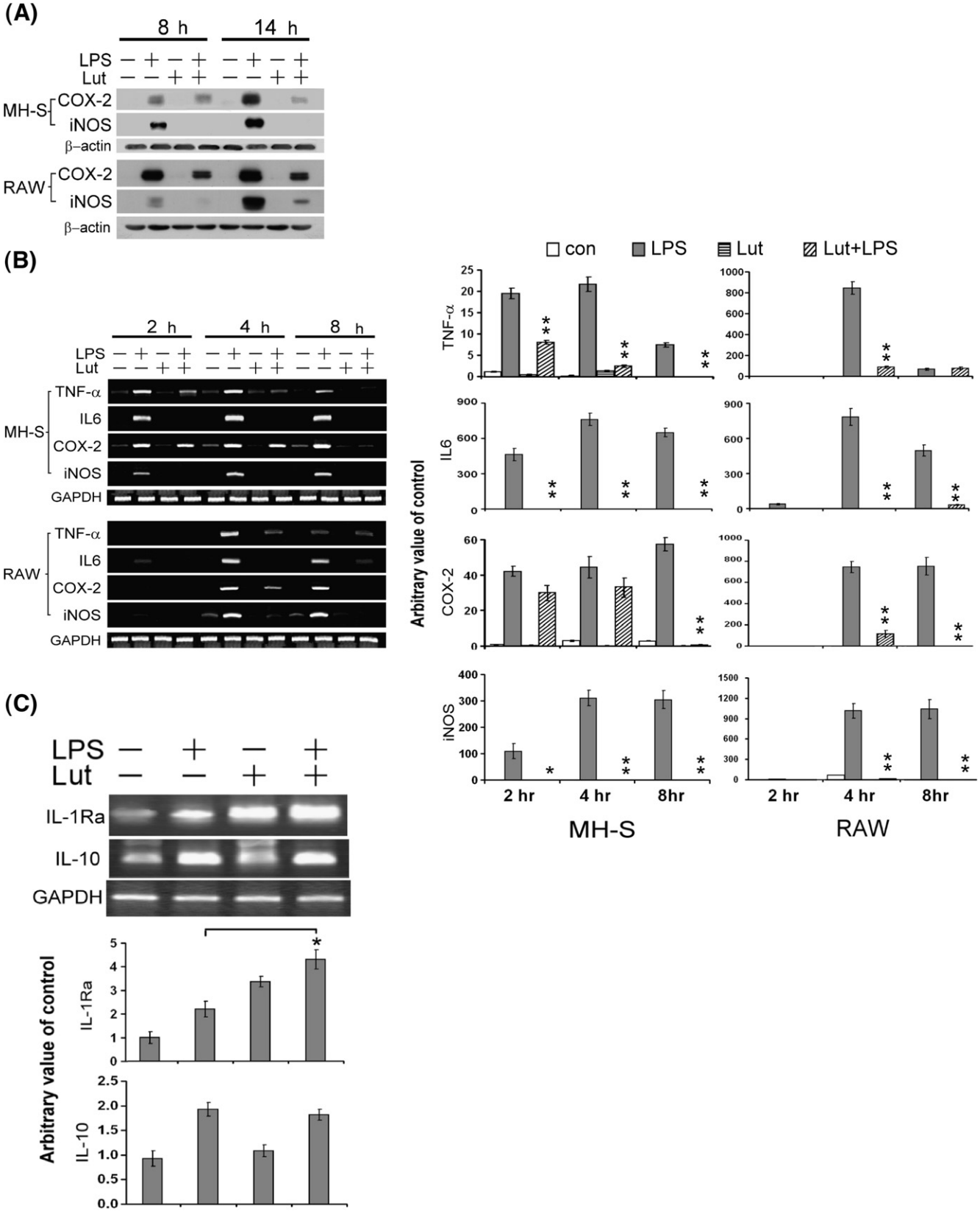
Cells were pretreated without or with luteolin (25 μM) for 30 min and then treated with LPS (100 ng/ml) in the presence or absence of luteolin, and then fixed with 2% paraformaldehyde for 20 min. The cells were incubated with 0.1% Triton X-100 for 30 min then blocked with 1% BSA for 30 min. Cells were probed with mouse anti-p65 antibody (Santa Cruz Biochemicals, Santa Cruz, CA, USA, 1:500) overnight at 4 °C, followed by FITC-conjugated goat anti-mouse IgG antibody (Sigma, St. Louis, MO, USA, diluted 1:200) 1 h at 37 °C, washed with PBS three times and then stained with propidium iodide for 15 min. NF-κB p65 subunit was observed with a laser scanning confocal microscope.

Reactive oxygen species (ROS) production

Intracellular oxidative stress was assayed by measuring intracellular oxidation of dichlorofluorescein (DCFH). The substrate was 2′, 7′-dichlorofluorescein diacetate (H_2DCFDA) which easily diffuses into the cell and was next deacetylated by cellular esterases to the more hydrophilic, nonfluorescent DCFH. H_2O_2 produced in the cell oxidized DCFH to the fluorescent 2′, 7′-dichlorofluorescein (DCF). MH-S cells were pretreated with H_2DCFDA (5 μM, Molecular Probes, Eugene, OR, USA) and luteolin (5–25 μM) for 30 min in a serum-free medium. Next, the cells (except for controls) were exposed to 100 ng/ml LPS at 37 °C for several times. Cells were detached and fluorescence was measured with a FACSCalibur flow cytometer using the Cell Quest software.

Statistical analysis

All results are expressed as means ± SEM of twelve replicates from three independent experiments. Figures shown in this article were obtained from at least three independent experiments with similar patterns. One-way ANOVA followed by a Scheffe post-hoc test and Student's *t*-test was used to determine the level of significance for the statistical analysis of data by using SPSS 10.0 statistical program. A p-value of less than 0.05 was considered significant.


(A)
8 h
14 h
LPS
Lut
MH-S
COX-2
iNOS
β–actin
RAW
COX-2
iNOS
β–actin
(B)
2 h
4 h
8 h
LPS
Lut
MH-S
TNF-α
IL6
COX-2
iNOS
GAPDH
RAW
TNF-α
IL6
COX-2
iNOS
GAPDH
con
LPS
Lut
Lut+LPS
Arbitrary value of control
TNF-α
IL6
COX-2
iNOS
2 hr
4 hr
8hr
MH-S
RAW
(C)
LPS
Lut
IL-1Ra
IL-10
GAPDH
Arbitrary value of control
IL-1Ra
IL-10

Results

Luteolin inhibited LPS-induced pro-inflammatory cytokine expression and NO and PGE_2 production

Pro-inflammatory cytokines (including TNF-α and IL-6) and mediators (NO and PGE_2) play important roles in the inflammatory process. To examine how luteolin regulates the production and expression of these cytokines and mediators, the levels of secreted TNF-α, IL-6, NO and PGE_2 were measured. As demonstrated in Fig. 1A, treatment of mouse alveolar macrophage MH-S and peripheral macrophage RAW 264.7 cells with LPS (100 ng/ml) caused a substantial increase in the production of pro-inflammatory cytokines (TNF-α and IL-6) and mediators (NO and PGE_2). When the cells were incubated with luteolin (5–25 μM) alone, the concentration of TNF-α, IL-6, NO and PGE_2 maintained at a background level similar to that in the unstimulated culture (data not shown). To examine whether luteolin is cytotoxic to these two macrophages cell lines, MH-S and RAW 264.7 cells were incubated with 5–50 μM luteolin for 24 h. Within our tested concentrations, no cytotoxic effect of luteolin was observed in these two cells (Fig. 1B). However, treatment with luteolin for 1 h before being incubated with LPS resulted in a dose-dependent inhibition of the LPS-induced TNF-α, IL-6, NO and PGE_2 production in both MH-S and RAW 264.7 cells (Fig. 1A).

Luteolin decreased the protein and mRNA levels of inflammation-associated genes and increased IL-1Ra mRNA level in LPS-stimulated macrophages

To determine if the inhibitory effect of luteolin on these inflammatory mediators (NO and PGE_2) was related to the regulation of iNOS and COX-2, the levels of these two proteins were examined by Western blot analysis. As indicated in Fig. 2A, the protein levels of iNOS and COX-2 were markedly increased upon LPS treatment, and this induction was effectively inhibited by luteolin treatment.

To address the suppressive effect of luteolin on LPS-induced inflammatory molecules that might result from a transcriptional inhibition, MH-S and RAW 264.7 cells were stimulated with LPS in the presence or absence of luteolin at various indicated time points, and the mRNA levels of iNOS, COX-2, TNF-α and IL-6 were measured by RT-PCR. As presented in Fig. 2B, LPS-stimulated macrophages increased the mRNA levels of iNOS, COX-2, TNF-α, and IL-6. However, luteolin (25 μM) significantly attenuated the LPS-induced TNF-α, IL-6, COX-2 and iNOS mRNA expression in both MH-S and RAW 264.7 macrophages. These results summed up to indicate that the LPS-stimulated iNOS, COX-2 and pro-inflammatory cytokines (TNF-α and IL-6) expression would be suppressed by luteolin at the transcriptional level. Interleukin-10 (IL-10) and IL-1Ra are potent anti-inflammatory cytokines produced predominantly by activated macrophages. The effects of luteolin on the expression of IL-10 and IL-1Ra in LPS-activated MH-S macrophages were examined. As demonstrated in Fig. 2C, luteolin significantly increased the LPS-induced IL-1Ra mRNA level but had no observable effect on the expression of IL-10 mRNA in MH-S macrophages.

Prevention of LPS-induced NF-κB activation by luteolin

NF-κB is an important transcriptional regulator of inflammatory cytokines and plays a crucial role in immune responses (Aderem and Ulevitch, 2000). For the purpose of this study, we hypothesized that luteolin would inhibit the expression of these genes through the suppression of NF-κB activation. We examined the effect of luteolin on the binding activity of NF-κB to its consensus DNA oligonucleotide by employing the electrophoretic mobility shift assay (EMSA). As indicated in Fig. 3A, LPS treatment caused a significant increase in NF-κB–DNA binding activity within 60 min. This binding activity was decreased by pretreatment with luteolin, and the specific interaction between DNA and NF-κB was demonstrated by competitive inhibition with 100-fold excess of unlabeled oligonucleotide. LPS-mediated NF-κB–DNA binding activity was also inhibited by a NF-κB inhibitor, PDTC. Furthermore, we examined the regulatory effect of luteolin on LPS-induced nuclear translocation of the cytosolic NF-κB p65 subunit by immunostaining (Fig. 3B). As expected, luteolin treatment markedly suppressed the LPS-induced NF-κB p65 nuclear translocation. Since nuclear translocation of NF-κB was preceded by the phosphorylation, ubiquitination, and proteosome-dependent degradation of IκB (Ghosh and Baltimore, 1990; Hayden and Ghosh, 2004), we next examined the effect of luteolin on LPS-induced IκB degradation by Western blot analysis. As demonstrated in Fig. 3C, stimulation of MH-S or RAW 264.7 macrophages with 100 ng/ml LPS induced a rapid degradation of cytosolic IκB protein within 10 to 20 min; this effect was drastically blocked by treatment with luteolin (25 μM).

Prevention of LPS-induced AP-1 activation by luteolin

Fig. 3A shows that PDTC inhibited LPS-induced NF-κB–DNA binding activity. In order to determine whether this action

Fig. 2. Luteolin decreased the protein and mRNA levels of inflammation-associated genes in LPS-stimulated macrophages. (A) MH-S and RAW 264.7 cells were pretreated with luteolin (25 μM) for 30 min, and then stimulated with LPS (100 ng/ml) for 8–14 h. Total protein extract was separated by SDS-PAGE, and then Western blot analysis with specific antibodies against COX-2 and iNOS. β-actin was used as an internal loading control. (B) RT-PCR analysis of TNF-α, IL-6, COX-2 and iNOS mRNA expression. Cells were pretreated with luteolin (25 μM) for 30 min, and then stimulated with LPS (100 ng/ml) for 2–8 h. Total RNA was analyzed by RT-PCR as described under Materials and methods. (C) RT-PCR analysis of IL-10 and IL-1Ra mRNA expression. MH-S cells were pretreated with luteolin (25 μM) for 30 min, and then stimulated with LPS (100 ng/ml) for 8 h. Total RNA was analyzed by RT-PCR. The figures were obtained from three independent experiments with similar patterns. Data are presented as the mean±SEM ($n=3$) for three independent experiments; $^{*}p<0.05$, $^{**}p<0.01$ as compared with the LPS treatment.

is attributed to suppressing the expression of inflammatory genes, RT-PCR analysis was performed. LPS-mediated up-regulation of inflammatory genes (iNOS, COX-2, and IL-6) was significantly attenuated by PDTC except TNF-α (Fig. 4A). Based on these results, we hypothesize that there may be other routes participating in the inhibitory effect of luteolin on TNF-α expression. It has been reported that transcription factor activating protein-1 (AP-1) also appears to be activated by LPS and causing production of pro-inflammatory cytokines (Granger et al., 2000; Shaulian and Karin, 2002). Accordingly, subsequent study of LPS-induced AP-1 activation in MH-S cells was performed. Pretreatment of MH-S cells with 10 μM curcumin (an AP-1 inhibitor) resulted in a decrease of the LPS-stimulated TNF-α, IL-6, iNOS mRNA expression except COX-2 mRNA. The inhibitory effect of luteolin on inflammatory gene expression is stronger than that of combined treatment with curcumin and PDTC in LPS-stimulated macrophages (Fig. 4B). To assess the effect of luteolin and curcumin on AP-1–DNA binding activity, EMSA was done on MH-S cells. As indicated in Fig. 4C, treatment with LPS caused an increase in AP-1–DNA binding activity within 60 min; pretreatment with luteolin or curcumin caused a strong inhibition of LPS-induced AP-1–DNA binding activity. These results suggest that the inhibitory effect of luteolin on LPS-stimulated pro-inflammatory cytokines and mediator production might be regulated by both NF-κB and AP-1 transcriptional activation.

Luteolin inhibited the LPS-mediated ROS generation

It has been reported that LPS rapidly induces the generation of ROS especially H_2O_2 in macrophages (Hsu and Wen, 2002; Lu and Wahl, 2005). In order to investigate the effect of luteolin on LPS-induced ROS production, H_2DCFDA was applied in order to detect cell oxygen burst. As illustrated in Fig. 5A, treatment of MH-S cells with LPS rapidly increased intracellular ROS level, especially H_2O_2 and .OH. Preincubation with luteolin (5, 10 and 25 μM) for 30 min attenuated the ROS generation in a dose-dependent manner in the LPS-activated macrophages (Fig. 5B).

Luteolin inhibited the LPS-induced Akt and IKK phosphorylation

Previous study indicated that transcriptional activation of NF-κB can be regulated through IKK or Akt signaling pathway. To further address the upstream signaling molecule in luteolin-mediated NF-κB inactivation, MH-S cells were pretreated with luteolin for 30 min and then stimulated with 100 ng/ml LPS for 10 min, the effect of luteolin on LPS-induced Akt and IKK activation was examined. Pretreatment with luteolin significantly reduced the LPS-elicited Akt (Thr308 and Ser473) and IKKα/β (Ser176/180) phosphorylation. Similarly, pretreatment with a PI3-k selective inhibitor LY294002 (10 μM) resulted in a dramatic reduction of Akt and IKKα/β phosphorylation

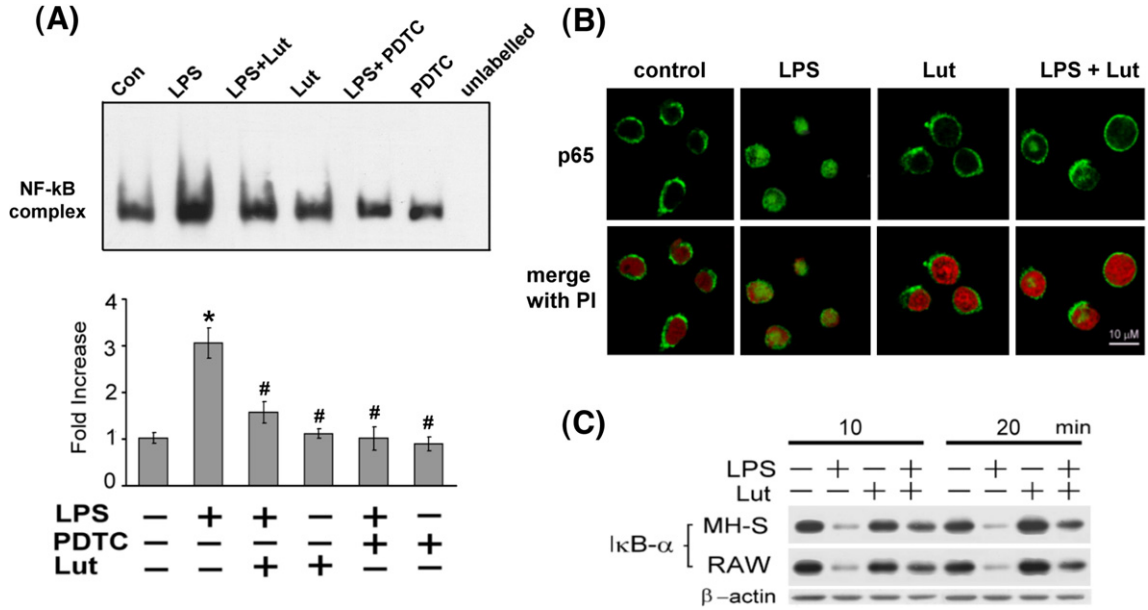


Fig. 3. Attenuation of LPS-induced NF-κB activation by luteolin. (A) Effect of luteolin on LPS-induced NF-κB–DNA binding activity. MH-S cells were pretreated with luteolin (25 μM) or PDTC (200 μM) for 30 min, and then stimulated with or without LPS (100 ng/ml) for 60 min. Nuclear extracts were prepared and electrophoretic mobility shift assay was performed using biotin-labeled NF-κB consensus binding sequence. Competitive EMSA using an unlabeled NF-κB consensus sequence at 100-fold excess confirmed the specificity of NF-κB protein binding. Values were expressed as means ± SEM ($n=3$). *$p<0.05$ as compared with the corresponding vehicle control; #$p<0.05$ as compared with the LPS treatment. (B) Effects of luteolin on LPS-induced nuclear translocation of p65 subunit of NF-κB. MH-S macrophages were preincubated with luteolin (25 μM) for 30 min, and then treated with LPS (100 ng/ml) for 60 min. The subcellular localization of NF-κB p65 subunit was detected by immunofluorescence with antibody against p65 as described in Materials and methods. The same fields were counter stained with PI for location of nuclei. (C) Luteolin blocks LPS-induced IκBα degradation. MH-S and RAW 264.7 cells were pretreated with 25 μM luteolin for 30 min, and then stimulated with LPS (100 ng/ml) for 10 and 20 min. Protein extract was separated by SDS-PAGE followed by Western blot analysis that was performed with specific antibodies against IκBα. Equal loading of proteins was verified by actin immunoblotting. The figures were obtained from three independent experiments with similar patterns.

(Fig. 6). These data suggested that luteolin inhibited NF-κB activation via suppression of Akt and IKK phosphorylated activation.

Discussion

Flavonoids are ubiquitous plant secondary metabolites and have a variety of biological effects, including antioxidant, anti-tumor, anti-microbial, anti-allergic, and anti-angiogenic properties (Shimada et al., 2006). Some natural flavonoids have anti-inflammatory effects in mammalian cells, which may be due to their ability to suppress the production of pro-inflammatory cytokines and mediators (Yuan et al., 2006). Accumulated evidence indicates that dysregulation of cytokines, such as TNF-α and IL-6, plays an essential role in many inflammatory conditions such as septic shock, hemorrhagic shock, rheumatoid arthritis and atherosclerosis. Inhibition of inflammatory cytokine and mediator production or function serves as a key mechanism in the control of inflammation, and agents that suppress the expression of these inflammation-associated genes have therapeutic potential for treatment of inflammatory diseases. Therefore, natural flavonoids have attracted interest as potential therapeutic agents for the treatment of inflammation. The plant flavonoid luteolin exhibits anti-inflammatory effects by inhibiting NF-κB-elicited gene expression and pro-inflammatory cytokine production in vitro (Xagorari et al., 2001), and histologically suppresses the inflammation in bacteria-infected lung tissue in C57BL/6J mice (Tormakangas et al., 2005). On the other hand, alveolar macrophages especially are thought to be the preferential immunoregulatory cell in lung tissue and targeted by bacterial endotoxins such as LPS. In this study, we found that luteolin significantly inhibits LPS-induced TNF-α, IL-6, cyclooxygenase-2, and iNOS expression at the transcriptional level in murine alveolar macrophage MH-S and peripheral macrophage RAW 264.7 cell lines.

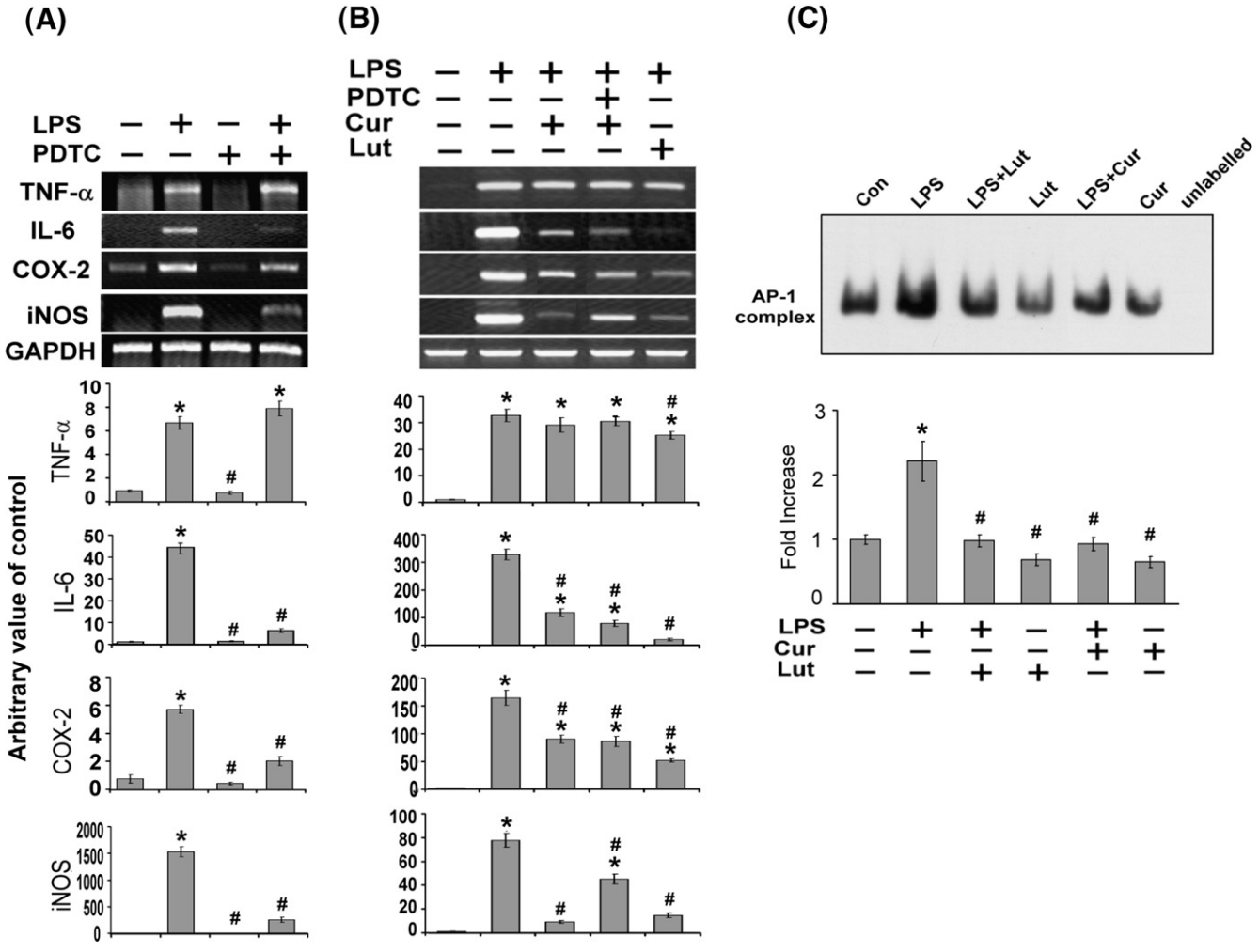


Fig. 4. Suppression of LPS-induced AP-1 activation by luteolin. MH-S cells were pretreated with 200 μM PDTC (A) or combined treatment with PDTC and curcumin (B) for 30 min, and then stimulated with LPS (100 ng/ml) for 4 h. Effects of PDTC and curcumin on LPS-induced TNF-α, IL-6, COX-2 and iNOS mRNA expression were analyzed by RT-PCR as described under Materials and methods. (C) Effect of luteolin on LPS-induced AP-1–DNA binding activity. MH-S cells were pretreated with luteolin (25 μM) or curcumin (10 μM) for 30 min, and then stimulated with or without LPS (100 ng/ml) for 60 min. Nuclear extracts were prepared and EMSA was performed using biotin-labeled AP-1 consensus binding sequence. Competitive EMSA using an unlabeled AP-1 consensus sequence at 100-fold excess confirmed the specificity of AP-1 protein binding. Values are expressed as means±SEM ($n=3$). *$p<0.05$ as compared with the corresponding vehicle control; $^{\#}p<0.05$ as compared with the LPS treatment. The figures were obtained from three independent experiments with similar patterns. Lut, luteolin; Cur, curcumin.

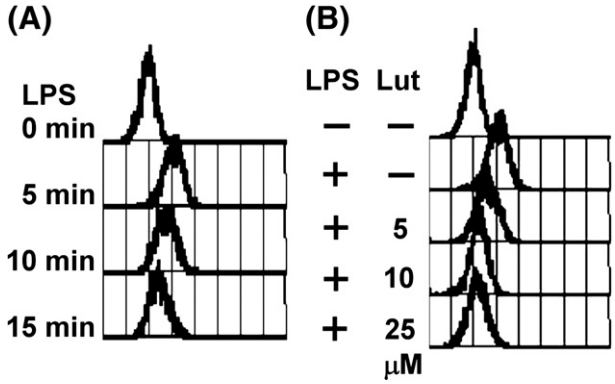


Fig. 5. Effect of luteolin on LPS-induced ROS generation. (A) MH-S macrophages were pretreated with H_2DCFDA (5 μM) for 30 min in serum-free medium and then exposed to LPS (100 ng/ml) at 37 °C for 0, 5, 10, 20 min. (B) MH-S cells were pretreated with H_2DCFDA (5 μM) and luteolin (5, 10, and 25 μM) for 30 min, and then exposed to LPS (100 ng/ml) at 37 °C for another 5 min. Cells were collected and fluorescence was measured with a flow cytometer using Cell Quest software. The figure was obtained from three independent experiments with similar patterns.

Macrophages/monocytes, derived from a bone marrow pluripotent stem cell, are specialized hematopoietic cells distributed throughout different tissues of the body where they play a central role in homeostasis and tissue remodeling. Generation of an immune response may be considered another aspect of macrophage function. When monocytes leave the bone marrow, they do not appear to be programmed for any particular tissue. Rather, they undergo tissue-specific maturation in response to factors in tissues. Different macrophage populations may arise from differing local conditions in the tissue; these populations differ in morphology and functional activity (cytotoxicity, migration, oxygen radical production, pinocytosis and tissue factor production). In the lung, alveolar macrophage function can be influenced by surfactant and other components of lung lining fluid that can be obtained by lung lavage (Lambrecht, 2006). The alveolar macrophages play a key role in inflammatory and immune responses in the lung. However, the alveolar macrophage is thought to be a relatively poor antigen-presenting cell, compared with interstitial macrophages and monocytes. It has been reported that LPS-inducible genes were differentially regulated in macrophages derived from different tissues (Feng et al., 1999). To our knowledge, this is the first report implicating the inhibition of LPS-stimulated inflammatory molecule gene expression by luteolin in both alveolar and peripheral macrophage cell lines. Our observations are consistent with the findings of previous reports demonstrating that luteolin significantly suppressed the LPS-increased expression of TNF-α and iNOS genes in murine primary bone marrow-derived macrophages (Comalada et al., 2006), and of IL-6 and COX-2 in peripheral macrophage cell line RAW 264.7 (Xagorari et al., 2001; Harris et al., 2006). However, our findings show that the different populations of macrophages exhibit differential kinetics of activation in response to LPS. For example, while the activation of MH-S macrophages by LPS is relatively rapid (detectable at 2 h, Fig. 2B). LPS stimulation of RAW 264.7 macrophage activation is more slow (detectable at 4 h, Fig. 2B). Interestingly, the inhibitory effect of luteolin on LPS-induced gene expression (TNF-α, IL-6, COX-2 and iNOS) is stronger in MH-S macrophages than in peripheral macrophages (Fig. 2B). These results imply that alveolar macrophages are more sensitive to tested agents (LPS and luteolin) than peripheral macrophages. The reason for such differences may be due to functional heterogeneity among tissue macrophages as regards their cytokine production and signal transducing properties.

TNF-α, a cytotoxic cytokine, is known to play a key role in inflammatory processes (Nathan, 1987). As LPS stimulation induces a large increase in macrophage TNF-α production (Guha and Mackman, 2001), this production of TNF-α is also known to be responsible for induction of other pro-inflammatory mediators (Chandel et al., 2000). Our results, similar to

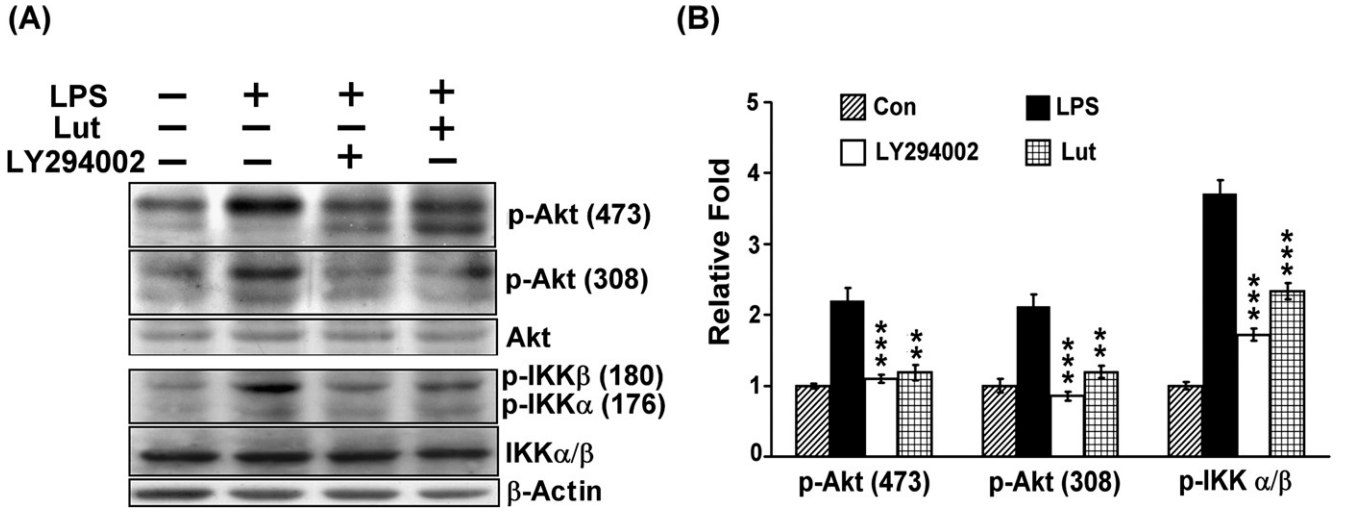


Fig. 6. Luteolin decreased the LPS-induced Akt and IKKα/β phosphorylation. MH-S cells were pretreated with luteolin (25 μM) or LY294002 (10 μM) for 30 min, and then stimulated with LPS (100 ng/ml) for 10 min. Fifty micrograms of each protein was separated by SDS-PAGE, and then Western blot analysis with specific antibodies against phospho-Akt (Thr308), phospho-Akt (Ser473) and phosphor-IKKα/β (Ser176/180) was performed. β-actin was used as a control. Results are expressed as the mean±SEM for three independent experiments; $^{**}p<0.01$, $^{***}p<0.001$ vs. LPS treatment. Lut, luteolin.

those of a previous report (Xagorari et al., 2001), have shown an inhibitory action of luteolin on the LPS-induced production of TNF-α mRNA expression in LPS-activated MH-S macrophages. Moreover, both luteolin and curcumin cause a strong inhibition of LPS-induced AP-1–DNA binding activity (Fig. 4). These observations suggest that luteolin-suppressed TNF-α production most likely occurs via an AP-1-dependent pathway in LPS-stimulated MH-S macrophages.

Interleukin (IL)-6 is a typical pleiotropic cytokine which plays an important role in the homeostasis of the immune system and hematopoietic system, in addition to its physiological functions upon the nervous system, endocrine system and bone metabolism (Kamimura et al., 2003). However, IL-6 production is rapidly increased in acute inflammatory responses associated with infection, injury, trauma and other stress. As such, a dysregulated, high-level production of IL-6 could induce an undesirable inflammatory condition in many organs. With this study we show that luteolin not only inhibits IL-6 production (Fig. 1) but also reduces its mRNA transcript in LPS-stimulated MH-S and RAW 264.7 macrophages, indicating that this compound could transcriptionally down-regulate IL-6 expression. The NF-κB transcription factor has been proved to play a key role in the transcriptional up-regulation of the LPS-inducible IL-6 gene (Guha and Mackman, 2001). We found that although the LPS-induced NF-κB and AP-1–DNA binding activities are inhibited by luteolin (Figs. 3A and 4C), blocking NF-κB activity with PDTC significantly attenuates IL-6 mRNA expression. However, blocking AP-1 activity with curcumin slightly reduced LPS-induced IL-6 expression (Fig. 4). These results suggest that luteolin-mediated NF-κB inactivation is the most important pathway involved in IL-6 down-regulation in LPS-stimulated macrophages.

NO is a free radical playing a pivotal role as a vasodilator, neurotransmitter and immune regulator in a variety of tissues at physiological concentrations. Currently, at least three distinct isoforms of NOS have been isolated and cloned: eNOS (endothelial NOS), iNOS (inducible NOS) and nNOS (neuronal NOS). Many cell types can express iNOS for their function in the host defense against microbial and viral pathogens (Bogdan, 2001), and this leads to the formation of NO radicals or *S*-nitrosothiols or $ONOO^-$ in the host cell or in the microbe itself. On the other hand, iNOS expression in macrophages is activating by particular inducers and participating in the pathology of inflammatory diseases including atherosclerosis, rheumatoid arthritis, diabetes, septic shock, transplant rejection, and multiple sclerosis, leading to cell death (Buttery et al., 1994). Therefore, the regulation of NO production or iNOS expression levels might be an important target in the treatment of inflammatory disorders.

Luteolin has been shown to inhibit the NO production and iNOS expression in LPS-stimulated BV-2 microglial cells (Kim et al., 2006), in primary bone marrow-derived macrophages (Comalada et al., 2006) and in human gingival fibroblasts (Gutierrez-Venegas et al., 2006). Consistently, we found that suppression of iNOS expression by luteolin is in parallel with the comparable inhibition of NO production in LPS-activated MH-S and RAW 264.7 macrophages. Both NF-κB and AP-1 binding sites have been identified on the murine iNOS promoter and play a role in LPS-mediated induction of iNOS in macrophages (Aktan, 2004; Cho et al., 2002). Our results indicate that in addition to the inhibition of the NF-κB activation by luteolin, a slight decrease in the AP-1 activation may also contribute to the suppression of iNOS gene expression.

Cyclooxygenase (COX)-2, an enzyme in the conversion of arachidonic acid to prostaglandins (PGs), prostacyclin, and thromboxane A_2, clearly represents an important step towards relieving the inflammatory process in macrophages (Wadleigh et al., 2000). Thus, selective inhibitors of COX-2 have been considered therapeutically effective for preventing inflammatory reaction and disease. The production of PGs by LPS in macrophages is primarily because of the transcriptional activation of the COX-2 gene. Luteolin has been found to inhibit inflammation-associated COX-2 expression and PGE_2 formation in RAW 264.7 cells (Harris et al., 2006). We demonstrate here that luteolin could suppress PGE_2 production and COX-2 gene expression via inactivation of NF-κB in both MH-S and RAW 264.7 macrophages. Our data also revealed that luteolin might not affect COX-1 enzymatic activity, because this compound did not alter basal PGE_2 production in these two macrophage cell lines (data not shown).

It has been reported that endogenous IL-1Ra produced by both macrophages and infiltrating neutrophils, exhibits an important anti-inflammatory effect in LPS-induced lung-injury model (Ulich et al., 1992). Expression of IL-1Ra may attenuate bronchial hyperreactivity and inflammation in both antigen-induced disease and in allergen-induced late asthmatic reactions in guinea pigs (Okada et al., 1995). Our observations showed that luteolin could up-regulate IL-1Ra mRNA expression in LPS-stimulated MH-S macrophages. These results revealed that the anti-inflammatory effect of luteolin may not only down-regulate the pro-inflammatory cytokines but also up-regulate the anti-inflammatory cytokines.

The intracellular redox (reduction–oxidation) state is physiologically important in maintaining cellular homeostasis and is vital for proper cellular functions. Oxidative stress occurs when this balance is disrupted by excessive production of ROS. ROS are commonly multiplied during inflammatory processes that involve signal transduction and gene activation and can contribute to host cell and organ damage. Accumulating evidence indicates that generation of ROS is involved in regulating the activation status of a variety of transcription factors, including NF-κB and AP-1 (Pham et al., 2004). Intracellular redox changes induced by ROS augment NF-κB activation through the phosphorylation and degradation of IκB by increasing IKK or Akt kinase activity (Gloire et al., 2006). Several agents that activate NF-κB are found to have led to increased intracellular production of ROS. Previous studies proved that antioxidants inhibit NF-κB activation and block the expression of inflammatory cytokines as well as the production of NO and PGE_2. Also, it has been reported that ectopic overexpression of catalase attenuates ROS production and subsequently inactivates AP-1 in endothelial cells (Lin et al., 2004). Structurally distinct antioxidants have been shown to

inhibit the expression of NF-κB- and AP-1-dependent cytokines, iNOS and COX-2 and, thus, reduce inflammation (Comalada et al., 2006; Ma et al., 2003). Therefore, it is suggested that the suppressive effects of these antioxidant compounds on the production of the associated inflammatory mediators are related with their antioxidant activities.

Luteolin exhibits obvious antioxidative and has been shown to be able to scavenge ROS (Harris et al., 2006). In this study, we found the LPS-induced ROS produced within the initial 5 min, degradation of IκBα would occur within 10–20 min, and the NF-κB and AP-1 DNA binding activities would increase within 30 min. It is likely that the LPS induced NF-κB activation via the generation of ROS and possible that luteolin, through scavenging of ROS, may mediate suppression of NF-κB and/or AP-1 signaling, thus reducing the level of downstream products (including TNF-α, IL-6, iNOS and COX-2) in alveolar MH-S macrophages. These results suggest that this pharmacological property of luteolin may have therapeutic potential with respect to modulating redox-sensitive alveolar macrophage functions causally implicated in acute lung injury and other inflammatory disorders within the alveolar compartment during evolving microbial sepsis.

LPS signaling in macrophages involves a series of phosphorylation events leading to transcription factor activation and cytokine production. Stimulation of RAW 264.7 with LPS leads to a time dependent phosphorylation of tyrosine residues of several proteins that is inhibited by luteolin (Xagorari et al., 2001). It has been reported that LPS stimulated NF-κB activation through the phosphorylation and degradation of IκB by increasing IKK or Akt kinase activity (Ardeshna et al., 2000). Studies by Ozes et al. and Madrid et al. showed that a signaling pathway culminating in phosphorylation both IKKα and β by Akt are necessary for activation of NF-κB (Madrid et al., 2001; Ozes et al., 1999). Our results show that luteolin significantly decreases the phosphorylation of Akt on Ser473 and Thr308 and IKKα/β on Ser176/180 (Fig. 6) similarly to results obtained with LY294002, and this inhibitory event is accompanied by the suppression of IκB degradation and NF-κB p65 subunit nuclear translocation in alveolar MH-S macrophages, indicating that Akt and IKK inactivation might be involved in luteolin-mediated anti-inflammatory actions. Experiments are underway to further dissect the molecular mechanism that involves kinase in the inhibitory effects of luteolin on the LPS-induced cytokine and mediator production in MH-S cells.

Luteolin belongs to the flavonoid family of compounds. Flavonoids are composed of two aromatic rings linked through three carbon atoms that form an oxygenated heterocycle. Variations in the basic structure of flavonoids yield different classes of flavonoids. The structure variations may mediate the differences in the bioactivity of these related compounds. Xagorari and co-workers used RAW 264.7 macrophages to evaluate the effect of flavonoids on macrophage physiology. Their results showed that among the tested flavonoids, quercetin and luteolin are the most active compounds in reducing the action of LPS on pro-inflammatory cytokine release in macrophage and luteolin showed a lower IC50 than quercetin (Xagorari et al., 2001). These data are in line with the findings of Comalada et al. (2006) who showed that luteolin and quercetin effectively inhibited LPS-stimulated TNF-α release in primary bone marrow-derived macrophages. Analysis of the structure–activity relationship showed that four hydroxylations at positions 5, 7, 3′ and 4′, together with the double bond at C2–C3 and the position of the B ring at 2, seem to be necessary for the highest anti-inflammatory effect. The major determinants of antioxidative capacity have been classically considered to be the presence of a catechol group in the B ring, the presence of a 3-OH group, and the C2–C3 double bond (Silva et al., 2002). Consistent with this, our preliminary study also showed that luteolin is more potent than baicalein in suppression of LPS-induced macrophage activation. The anti-inflammatory capacity of flavonoids has long been put to use in Chinese medicine as crude plant extracts. Some flavonoids have been found to inhibit chronic inflammation in animal models (Camuesco et al., 2004; Kawaguchi et al., 2004). Current clinical approaches showed that flavonoids are promising anti-inflammatory drugs. However, the pharmacokinetic characteristics of flavonoids have to be considered before these agents may be used successfully in vivo. Most flavonoids are found glycosylated in plants and cannot be absorbed by the intestinal epithelium in that form. Hydrolysis of the glycosides by the intestinal flora yields free flavonoids that are absorbed efficiently. Comalada et al. suggested that flavonoids must be administered orally in glycosidic form in order to be applied as anti-inflammatory agents (Comalada et al., 2006).

In conclusion, our observations suggest that luteolin has a strong antioxidant capacity to inhibit inflammation-associated gene expression (TNF-α, IL-6, iNOS, and COX-2) by suppressing redox-dependent NF-κB and AP-1 activation, resulting in its anti-inflammatory effects, and imply that luteolin may be a potential drug for treating inflammatory diseases. Our observations also demonstrate that the anti-inflammatory property of *L. japonica* may be partly due to the luteolin, providing support for the traditional use of this plant in Chinese medicine against alveolar inflammatory disorders. However, further in vivo investigation of this activity is necessary to qualify its mechanism and medicinal potential.

Acknowledgements

This work was supported by grants from the National Science Council (NSC-94-2320-B-075A-004) and Taichung Veterans General Hospital (TCVGH-957314C), Taiwan, Republic of China.

References

Aderem, A., Ulevitch, R.J., 2000. Toll-like receptors in the induction of the innate immune response. Nature 406, 782–787.

Aktan, F., 2004. iNOS-mediated nitric oxide production and its regulation. Life Sciences 75, 639–653.

Ardeshna, K.M., Pizzey, A.R., Devereux, S., Khwaja, A., 2000. The PI3 kinase, p38 SAP kinase, and NF-kappaB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. Blood 96, 1039–1046.

Bogdan, C., 2001. Nitric oxide and the immune response. Nature Immunology 2, 907–916.

Buttery, L.D., Evans, T.J., Springall, D.R., Carpenter, A., Cohen, J., Polak, J.M., 1994. Immunochemical localization of inducible nitric oxide synthase in endotoxin-treated rats. Laboratory Investigation 71, 755–764.

Camuesco, D., Comalada, M., Rodriguez-Cabezas, M.E., Nieto, A., Lorente, M.D., Concha, A., Zarzuelo, A., Galvez, J., 2004. The intestinal anti-inflammatory effect of quercitrin is associated with an inhibition in iNOS expression. British Journal of Pharmacology 143, 908–918.

Chandel, N.S., Trzyna, W.C., McClintock, D.S., Schumacker, P.T., 2000. Role of oxidants in NF-kappa B activation and TNF-alpha gene transcription induced by hypoxia and endotoxin. Journal of Immunology 165, 1013–1021.

Cho, M.K., Suh, S.H., Kim, S.G., 2002. JunB/AP-1 and NF-kappa B-mediated induction of nitric oxide synthase by bovine type I collagen in serum-stimulated murine macrophages. Nitric Oxide 6, 319–332.

Christofidou-Solomidou, M., Muzykantov, V.R., 2006. Antioxidant strategies in respiratory medicine. Treatments in Respiratory Medicine 5, 47–78.

Comalada, M., Ballester, I., Bailon, E., Sierra, S., Xaus, J., Galvez, J., de Medina, F.S., Zarzuelo, A., 2006. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: analysis of the structure–activity relationship. Biochemical Pharmacology 72, 1010–1021.

Das, M., Ram, A., Ghosh, B., 2003. Luteolin alleviates bronchoconstriction and airway hyperreactivity in ovalbumin sensitized mice. Inflammation Research 52, 101–106.

Feng, G.J., Goodridge, H.S., Harnett, M.M., Wei, X.Q., Nikolaev, A.V., Higson, A.P., Liew, F.Y., 1999. Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: *Leishmania* phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. Journal of Immunology 163, 6403–6412.

File Jr., T.M., Tsang, K.W., 2005. Severe acute respiratory syndrome: pertinent clinical characteristics and therapy. Treatments in Respiratory Medicine 4, 95–106.

Ghosh, S., Baltimore, D., 1990. Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B. Nature 344, 678–682.

Gloire, G., Legrand-Poels, S., Piette, J., 2006. NF-kappaB activation by reactive oxygen species: fifteen years later. Biochemical Pharmacology 72, 1493–1505.

Granger, R.L., Hughes, T.R., Ramji, D.P., 2000. Gene, stimulus and cell-type specific regulation of activator protein-1 in mesangial cells by lipopolysaccharide and cytokines. Biochimica et Biophysica Acta 1492, 100–107.

Guha, M., Mackman, N., 2001. LPS induction of gene expression in human monocytes. Cellular Signalling 13, 85–94.

Guha, M., O'Connell, M.A., Pawlinski, R., Hollis, A., McGovern, P., Yan, S.F., Stern, D., Mackman, N., 2001. Lipopolysaccharide activation of the MEK–ERK1/2 pathway in human monocytic cells mediates tissue factor and tumor necrosis factor alpha expression by inducing Elk-1 phosphorylation and Egr-1 expression. Blood 98, 1429–1439.

Gutierrez-Venegas, G., Kawasaki-Cardenas, P., Arroyo-Cruz, S.R., Maldonado-Frias, S., 2006. Luteolin inhibits lipopolysaccharide actions on human gingival fibroblasts. European Journal of Pharmacology 541, 95–105.

Harris, G.K., Qian, Y., Leonard, S.S., Sbarra, D.C., Shi, X., 2006. Luteolin and chrysin differentially inhibit cyclooxygenase-2 expression and scavenge reactive oxygen species but similarly inhibit prostaglandin-E2 formation in RAW 264.7 cells. The Journal of Nutrition 136, 1517–1521.

Hayden, M.S., Ghosh, S., 2004. Signaling to NF-kappaB. Genes and Development 18, 2195–2224.

Hsu, H.Y., Wen, M.H., 2002. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. The Journal of Biological Chemistry 277, 22131–22139.

Kamimura, D., Ishihara, K., Hirano, T., 2003. IL-6 signal transduction and its physiological roles: the signal orchestration model. Reviews of Physiology, Biochemistry and Pharmacology 149, 1–38.

Kawaguchi, K., Kikuchi, S., Hasunuma, R., Maruyama, H., Yoshikawa, T., Kumazawa, Y., 2004. A citrus flavonoid hesperidin suppresses infection-induced endotoxin shock in mice. Biological and Pharmaceutical Bulletin 27, 679–683.

Kim, J.S., Lee, H.J., Lee, M.H., Kim, J., Jin, C., Ryu, J.H., 2006. Luteolin inhibits LPS-stimulated inducible nitric oxide synthase expression in BV-2 microglial cells. Planta Medica 72, 65–68.

Kobayashi, H., Kobayashi, M., Heming, T.A., Bidani, A., Pollard, R.B., Suzuki, F., 1999. Cytokine production by rabbit alveolar macrophages: differences between activated and suppressor cell phenotypes. Immunology Letters 69, 339–346.

Kotanidou, A., Xagorari, A., Bagli, E., Kitsanta, P., Fotsis, T., Papapetropoulos, A., Roussos, C., 2002. Luteolin reduces lipopolysaccharide-induced lethal toxicity and expression of proinflammatory molecules in mice. American Journal of Respiratory and Critical Care Medicine 165, 818–823.

Lai, S.T., 2005. Treatment of severe acute respiratory syndrome. European Journal of Clinical Microbiology & Infectious Diseases 24, 583–591.

Lambrecht, B.N., 2006. Alveolar macrophage in the driver's seat. Immunity 24, 366–368.

Lee, J.H., Ko, W.S., Kim, Y.H., Kang, H.S., Kim, H.D., Choi, B.T., 2001. Anti-inflammatory effect of the aqueous extract from *Lonicera japonica* flower is related to inhibition of NF-kappaB activation through reducing I-kappaBalpha degradation in rat liver. International Journal of Molecular Medicine 7, 79–83.

Lew, T.W., Kwek, T.K., Tai, D., Earnest, A., Loo, S., Singh, K., Kwan, K.M., Chan, Y., Yim, C.F., Bek, S.L., Kor, A.C., Yap, W.S., Chelliah, Y.R., Lai, Y.C., Goh, S.K., 2003. Acute respiratory distress syndrome in critically ill patients with severe acute respiratory syndrome. JAMA: The Journal of the American Medical Association 290, 374–380.

Lin, S.J., Shyue, S.K., Liu, P.L., Chen, Y.H., Ku, H.H., Chen, J.W., Tam, K.B., Chen, Y.L., 2004. Adenovirus-mediated overexpression of catalase attenuates oxLDL-induced apoptosis in human aortic endothelial cells via AP-1 and C-Jun N-terminal kinase/extracellular signal-regulated kinase mitogen-activated protein kinase pathways. Journal of Molecular and Cellular Cardiology 36, 129–139.

Liu, X., Zhang, M., He, L., Li, Y.P., Kang, Y.K., 2006. Chinese herbs combined with Western medicine for severe acute respiratory syndrome (SARS). Cochrane Database of Systematic Reviews, CD004882.

Lu, Y., Wahl, L.M., 2005. Oxidative stress augments the production of matrix metalloproteinase-1, cyclooxygenase-2, and prostaglandin E2 through enhancement of NF-kappa B activity in lipopolysaccharide-activated human primary monocytes. Journal of Immunology 175, 5423–5429.

Ma, Q., Kinneer, K., Ye, J., Chen, B.J., 2003. Inhibition of nuclear factor kappaB by phenolic antioxidants: interplay between antioxidant signaling and inflammatory cytokine expression. Molecular Pharmacology 64, 211–219.

Madrid, L.V., Mayo, M.W., Reuther, J.Y., Baldwin Jr., A.S., 2001. Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. The Journal of Biological Chemistry 276, 18934–18940.

Matsunaga, K., Klein, T.W., Friedman, H., Yamamoto, Y., 2001. Alveolar macrophage cell line MH-S is valuable as an in vitro model for *Legionella pneumophila* infection. American Journal of Respiratory Cell and Molecular Biology 24, 326–331.

Nathan, C.F., 1987. Secretory products of macrophages. The Journal of Clinical Investigation 79, 319–326.

Okada, S., Inoue, H., Yamauchi, K., Iijima, H., Ohkawara, Y., Takishima, T., Shirato, K., 1995. Potential role of interleukin-1 in allergen-induced late asthmatic reactions in guinea pigs: suppressive effect of interleukin-1 receptor antagonist on late asthmatic reaction. Journal of Allergy and Clinical Immunology 95, 1236–1245.

Ozes, O.N., Mayo, L.D., Gustin, J.A., Pfeffer, S.R., Pfeffer, L.M., Donner, D.B., 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 401, 82–85.

Park, E., Kum, S., Wang, C., Park, S.Y., Kim, B.S., Schuller-Levis, G., 2005. Anti-inflammatory activity of herbal medicines: inhibition of nitric oxide production and tumor necrosis factor-alpha secretion in an activated macrophage-like cell line. The American Journal of Chinese Medicine 33, 415–424.

Pham, C.G., Bubici, C., Zazzeroni, F., Papa, S., Jones, J., Alvarez, K., Jayawardena, S., De Smaele, E., Cong, R., Beaumont, C., Torti, F.M., Torti, S.V., Franzoso, G., 2004. Ferritin heavy chain upregulation by NF-kappaB inhibits TNFalpha-induced apoptosis by suppressing reactive oxygen species. Cell 119, 529–542.

Shaulian, E., Karin, M., 2002. AP-1 as a regulator of cell life and death. Nature Cell Biology 4, E131–E136.

Shimada, H., Miura, K., Imamura, Y., 2006. Characteristics and inhibition by flavonoids of 20alpha-hydroxysteroid dehydrogenase activity in mouse tissues. Life Sciences 78, 2931–2936.

Silva, M.M., Santos, M.R., Caroco, G., Rocha, R., Justino, G., Mira, L., 2002. Structure–antioxidant activity relationships of flavonoids: a re-examination. Free Radical Research 36, 1219–1227.

Tormakangas, L., Vuorela, P., Saario, E., Leinonen, M., Saikku, P., Vuorela, H., 2005. In vivo treatment of acute *Chlamydia pneumoniae* infection with the flavonoids quercetin and luteolin and an alkyl gallate, octyl gallate, in a mouse model. Biochemical Pharmacology 70, 1222–1230.

Ulich, T.R., Guo, K., Yin, S., del Castillo, J., Yi, E.S., Thompson, R.C., Eisenberg, S.P., 1992. Endotoxin-induced cytokine gene expression in vivo. IV. Expression of interleukin-1 alpha/beta and interleukin-1 receptor antagonist mRNA during endotoxemia and during endotoxin-initiated local acute inflammation. American Journal of Pathology 141, 61–68.

Wadleigh, D.J., Reddy, S.T., Kopp, E., Ghosh, S., Herschman, H.R., 2000. Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. The Journal of Biological Chemistry 275, 6259–6266.

Wu, C.Y., Jan, J.T., Ma, S.H., Kuo, C.J., Juan, H.F., Cheng, Y.S., Hsu, H.H., Huang, H.C., Wu, D., Brik, A., Liang, F.S., Liu, R.S., Fang, J.M., Chen, S.T., Liang, P.H., Wong, C.H., 2004. Small molecules targeting severe acute respiratory syndrome human coronavirus. Proceedings of the National Academy of Sciences of the United States of America 101, 10012–10017.

Xagorari, A., Papapetropoulos, A., Mauromatis, A., Economou, M., Fotsis, T., Roussos, C., 2001. Luteolin inhibits an endotoxin-stimulated phosphorylation cascade and proinflammatory cytokine production in macrophages. The Journal of Pharmacology and Experimental Therapeutics 296, 181–187.

Xagorari, A., Roussos, C., Papapetropoulos, A., 2002. Inhibition of LPS-stimulated pathways in macrophages by the flavonoid luteolin. British Journal of Pharmacology 136, 1058–1064.

Yuan, G., Wahlqvist, M.L., He, G., Yang, M., Li, D., 2006. Natural products and anti-inflammatory activity. Asia Pacific Journal of Clinical Nutrition 15, 143–152.