Intracellular Signal Transduction Mechanisms Regulating the Activation of Human Bronchial Epithelial Cells by Interleukin-17A, Interleukin-27, Tumor Necrosis Factor-alpha and Human Basophils in Inflammatory Diseases

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy
in
Chemical Pathology

The Chinese University of Hong Kong
July 2010

Acknowledgements

This thesis is to report my own research work from August 2007 to May 2010. I would like to thank everyone who has helped me during this period.

Firstly, I would like to deliver my most heartful gratitude to my supervisor, Professor C.W.K. Lam, for his continuous encouragement and supervision throughout my three-year PhD program. I learnt a lot from Professor Lam about not only how to be a scientist, but also how to be a man. His kindness, enthusiasm and diligence will continue to motivate me to make better achievements in my future.

I am also deeply grateful to my supervisor, Professor C.K. Wong, for his unconditional support, valuable discussions and detailed instructions. He has always guided me to solve every problem confronted in my research work, presentations and publications. This thesis could not be completed without his kind patience and ceaseless help. It was my pleasure to conduct research work under his supervision.

I would like to thank my former colleague Dr. P.F.Y. Cheung who provided me technical assistance patiently and shared her experimental experience unreservedly. She was always selfless when I asked for her help. I am also grateful to Dr. I.H.S.

Chan, Dr. P.M.K. Poon and Dr. L.C.W. Lit, for their valuable ideas and discussion. Besides, I would like to say thanks to all the current and former colleagues in this research group: Ms. S.S.M. Ng, Ms. J.Y.S. Chow, Dr. S.W.M. Lun, Ms. A.W.Y. Ho, Ms. P.T.Y. Wong, Ms. Y.Y.O. Lam, Dr. S.Q. Hu, Mr. D.P. Chen, Dr. S.K.W. Lee, Ms. S.L. Yu and Ms. A.O.K. Choi for their encouragement, guidance and friendship.

My gratitude also goes to all laboratory technicians and staff of the Department of Chemical Pathology, for providing me a harmonious working environment.

Last but not least, I would like to express my deepest appreciation to my family, especially my parents and my wife, for their boundless support and endless love, which will always be my courage to overcome all of the difficulties.

ABBREVIATIONS

AHR	Airway hypersensitivity response
Akt	Protein kinase B
APC	Antigen presenting cells
BAFF	B-cell–activating factor of the TNF family
BAL	Bronchoalveolar lavage
BLyS	B lymphocyte stimulator
BSA	Bovine serum albumin
CBA	Cytometric beads array
CCR	CC chemokine receptor
CD	Cluster of differentiation
COPD	Chronic obstructive pulmonary disease
CS	Corticosteroids
DC	Dendritic cells
D-MEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethy sulfoxide
ECL	Enhanced chemiluminescence
ECP	Eosinophil cationic protein
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated protein kinase
FBS	Fetal bovine serum
FcεR	High affinity IgE receptor
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte macrophage colony-stimulating factor
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor kappa B
IL	Interleukin
JAK	Janus kinase
JNK	c-Jun N-terminal kinases

LPS	Lipopolysaccharide
LFA-1	Leukocyte function-associated antigen-1
LTC4	Leukotriene C4
Mac-1	Macrophage antigen-1
MACS	Magnetic cell sorting system
MAPK	Mitogen-activated protein kinases
MAPKK	MAPK kinase
MBP	Major basic protein
MCP-1	Monocyte chemotactic protein-1
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
min	Minute
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinases
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MTT	Thiazolyl blue tetrazolium bromide
MUC	Mucin
NF-κB	Nuclear factor kappa B
NLR	Nod-like receptor
NO	Nitric oxide
PAF	Platelet-activating factor
PAMP	Pathogen-associated molecular pattern
PBEC	Primary bronchial epithelial cells
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PG	Prostaglandin
PGN	Peptidoglycan
PI3K	Phosphoinositide 3-kinase
PRR	Pattern-recognition receptor
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
RIG-I	Retinoic acid-inducible gene I
ROR	Retinoic acid receptor-related orphan receptor
RPMI	Roswell Park Memorial Institute
SD	Standard deviation

SH2	Src-homology 2
STAT	Signal transducers and activators of transcriptions
TACE	TNF-α-converting enzyme
TARC	Thymus and activation-regulated chemokine
TGF	Transforming growth factor
Th	T helper lymphocyte
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	TNF-α receptors
Treg	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
VCAM-1	Vascular-cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

ABSTRACT

Airway bronchial epithelial cells play important roles in host defense, inflammation and regulation of immune responses. Activated bronchial epithelial cells are potent sources of a wide variety of soluble and cell-surface molecules that can alter the biological functions of inflammatory cells in the airways. Molecular mechanisms regulating the production of inflammatory mediators from bronchial epithelial cells remain to be fully elucidated.

In the present study, we investigated the mechanisms of the activation of human bronchial epithelial cells induced by various stimuli including interleukin (IL)-17A, IL-27, tumor necrosis factor (TNF)-α and human basophils. The activation of human bronchial epithelial cells was studied in terms of the expression of cytokines, chemokines and adhesion molecules. Using intracellular staining with flow cytometry and selective pharmacological inhibitors, we further investigated the underlying intracellular signaling mechanisms regulating the activation of human bronchial epithelial cells.

Basophils are the accessory cell type required for T helper (Th)2 induction and initiators in IgE-mediated chronic allergic inflammation in response to allergens. Number of basophils and Th17 cells increases at the sites of allergic inflammation in

the airways of allergic asthmatic patients. To elucidate the interaction among the activation of human bronchial epithelial cells, Th17 cells, and basophils, we investigated the activation effects of Th17 hallmark cytokine IL-17A on the human primary bronchial epithelial cells/BEAS-2B bronchial epithelial cells and human primary basophils/ KU812 basophilic cells. Human bronchial epithelial cells and basophils were cultured either together or separately in the presence or absence of IL-17A stimulation. Co-culture of human bronchial epithelial cells and basophils could significantly increase the release of inflammatory cytokine IL-6 and mononuclear chemoattractant protein-1 (MCP-1/CCL2), a chemokine for basophils, eosinophils and monocytes, while human bronchial epithelial cells were the main source for releasing IL-6 and CCL2. Such induction was synergistically enhanced upon the activation of IL-17A. The use of transwell inserts in the co-culture system demonstrated that the direct interaction between these two cell types was necessary for IL-6 and CCL2 release induced by IL-17A. Surface expression of intercellular adhesion molecule-1 (ICAM-1) on the human bronchial epithelial cells was also up-regulated upon their interaction. The interaction of human bronchial epithelial cells and basophils under IL-17A stimulation was differentially regulated by extracellular signal-regulated kinase (ERK), c-Jun N-terminal protein kinase (JNK), p38 mitogen activated protein kinase (MAPK) and nuclear factor (NF)-kappaB

pathways. Our findings therefore suggest a novel immunopathological role of human Th17 cells and basophils in allergic asthma through the activation of granulocytes-mediated inflammation initiated by the direct interaction between human basophils and bronchial epithelial cells.

IL-27 is a novel member of the IL-6/IL-12 family cytokines that are produced early by antigen-presenting cells (APCs) during immune responses. IL-27 can drive the commitment of naive T cells to a Th1 phenotype and inhibit inflammation in later phases of infection. Recent evidence has suggested that human bronchial epithelial cells with the expression of IL-27 receptor complex are potential target cells of IL-27. Here we investigated the *in vitro* effects of IL-27, alone or in combination with inflammatory cytokine TNF-α on the pro-inflammatory activation of human bronchial epithelial cells, and the underlying intracellular signaling molecules were also studied. IL-27 was found to up-regulate ICAM-1 expression on the surface of human bronchial epithelial cells, and a synergistic effect was observed in the combined treatment of IL-27 and TNF-α on the surface expression of ICAM-1. Although IL-27 did not alter the basal IL-6 secretion from human bronchial epithelial cells, it could significantly enhance TNF-α-induced IL-6 production. The synergistic effects on the induction of ICAM-1 and IL-6 were partially due to the up-regulated expression of TNF-α receptor (p55TNFR) on the surface of human bronchial

epithelial cells induced by IL-27. Further investigations showed that the enhanced production of ICAM-1 and IL-6 in human bronchial epithelial cells activated by IL-27 and TNF-α was differentially regulated by phosphatidylinositol 3-OH kinase (PI3K)-Akt, p38 MAPK and NF-kappaB pathways. Our study therefore suggests a potential role of IL-27 and TNF-α in the pathogenesis of airway infection or inflammatory diseases.

All of the above findings suggest that human bronchial epithelial cells could be activated by a variety of stimuli in airway inflammatory reactions. Besides, different intracellular signaling pathways could regulate the activation of human bronchial epithelial cells in response to different stimuli. Our results therefore provide new insight into the molecular mechanisms involved in airway inflammatory diseases and may have important therapeutic implications.

摘要

人體呼吸道支氣管上皮細胞在宿主防禦，炎症反應和免疫應答中伴演著重要的角色。活化的支氣管上皮細胞可以大量產生一系列可溶性的和結合於細胞表面的炎症介質分子，這些炎症介質從而能夠改變呼吸道炎症反應中各效應細胞的生物學功能。然而，調節支氣管上皮細胞釋放各種炎症介質的分子機制目前尚未闡述清楚。

在本項研究中，我們重點探討了不同的細胞因子或者效應細胞活化支氣管上皮細胞的效應機制，它們包括近期發現的細胞因子白介素-17A（IL-17A），白介素-27（IL-27）和腫瘤壞死因子（TNF-α）以及嗜鹼性粒細胞。我們從細胞因子和趨化因子的分泌以及細胞表面粘附分子的表達等方面對支氣管上皮細胞的活化效應進行了詳細的研究。同時，運用現代流式細胞內染色技術和特異性的藥物抑製劑干預措施，我們深入闡述了調節支氣管上皮細胞活化效應的細胞內信號傳導通路。

嗜鹼性粒細胞是輔助性 2 型 T 淋巴細胞免疫應答反應中重要的效應細胞。同時，嗜鹼性粒細胞是免疫球蛋白 E 介導的慢性過敏性炎症反應中的起始效應細胞。研究發現，在過敏性哮喘病人的呼吸道炎症組織中，嗜鹼性粒細胞和輔助性 17 型 T 淋巴細胞的數量增多。為了進一步闡明人支氣管上皮細胞，嗜鹼性粒細胞和輔助性 17 型 T 淋巴細胞三者之間的相互作用關係，

我們本研究中運用人支氣管上皮細胞細胞株（BEAS-2B）和嗜鹼性粒細胞細胞株（KU812）以及相應的原代支氣管上皮細胞和外周血分離的嗜鹼性粒細胞細胞, 系統分析了輔助性 17 型 T 淋巴細胞標誌性細胞因子 IL-17A 活化支氣管上皮細胞和嗜鹼性粒細胞的效應機制。我們將支氣管上皮細胞和嗜鹼性粒細胞共同或者分離培養，結果發現支氣管上皮細胞和嗜鹼性粒細胞相互作用可以誘導炎性細胞因子白介素-6（IL-6）和趨化因子單核細胞趨化蛋白-1(MCP-1/CCL2)的釋放, 後者可以招募嗜鹼性粒細胞，嗜酸性粒細胞和單核細胞的聚集。在這種共同細胞培養模型中，活化的支氣管上皮細胞是釋放 IL-6 和 CCL2 的主要效應細胞。IL-17A 的刺激可以協同誘導釋放更多的 IL-6 和 CCL2。利用半透膜(transwell insert)建立上下雙層細胞共培養體系，我們發現支氣管上皮細胞和嗜鹼性粒細胞的直接接觸對於 IL-17A 協同刺激釋放 IL-6 和 CCL2 具有重要意義。同時，支氣管上皮細胞和嗜鹼性粒細胞的共同培養可以誘導支氣管上皮細胞表面的細胞間粘附分子-1 (ICAM-1)的上調表達。進一步的研究表明細胞外信號調解激酶（ERK），C-Jun 氨基端激酶(JNK)，p38 絲裂原活化蛋白激酶(p38MAPK)和胞核因子-kappaB (NF-κB)這些細胞內信號通路調節著 IL-17A 刺激下的支氣管上皮細胞和嗜鹼性粒細胞的活化效應。我們的研究結果因此闡明了輔助性 17 型 T 淋巴細胞在過敏性哮喘發病中一種新穎的的免疫病理機制，即支氣管上皮細胞和嗜鹼性粒細胞的直接接觸可以誘發呼吸道炎症反應，輔助性 17 型 T 淋巴細胞的浸潤可

以進一步加重這種炎症效應。

IL-27 是新近發現的 IL-6/IL-12 家族的一種細胞因子，它在免疫應答早期由抗原遞呈細胞生成。IL-27 能夠誘導初始型 T 淋巴細胞向輔助性 1 型 T 淋巴細胞表型轉換並且能夠抑制後期的炎症反應。近期的研究證據表明人體支氣管上皮細胞表達 IL-27 的受體複合物，因此是 IL-27 潛在的靶作用細胞。本文中我們探討了 IL-27 和另外一種炎症細胞因子 TNF-α 對於支氣管上皮細胞的體外活化效應，同時深入研究了調節其活化效應的細胞內信號傳導途徑。結果顯示 IL-27 可以顯著提高支氣管上皮細胞表面細胞間粘附分子-1 的表達，IL-27 和 TNF-α 的聯合刺激能夠協同誘導細胞間粘附分子-1 的表達。儘管 IL-27 不能誘導支氣管上皮細胞釋放 IL-6，但是它可以顯著增強 TNF-α 刺激下的支氣管上皮細胞釋放更多的 IL-6。IL-27 和 TNF-α 這種協同刺激支氣管上皮細胞生成 IL-6 和細胞間粘附分子-1 的效應可初步由 IL-27 可誘導 TNF-α 受體 p55TNFR 的上調表達所解釋。進一步的研究發現 IL-27 和 TNF-α 誘導支氣管上皮細胞產生 IL-6 和細胞間粘附分子-1 主要通過激活磷脂酰肌醇-3-OH 激酶(PI3K)-Akt，p38MAPK 和 NF-κB 這三條細胞內信號通路所調節。本項研究因此說明了細胞因子 IL-27 和 TNF-α 在呼吸道感染和炎症中扮演著重要的角色。

總之，以上研究表明在呼吸道炎症疾病中多種因素可以導致人體支氣管上皮細胞的活化，並且多種不同的細胞內信號傳導通路調節著支氣管上皮細

胞的活化。本論文的研究結果對呼吸道炎症發病的分子機製做出了新的解釋，將有助於發展新的治療呼吸道炎症疾病的措施。

Publications

Publications arising from work of this thesis

Refereed publications

1. Wong CK*, **Cao J*(*Equal first author)**, Yin YB and Lam CW. IL-17A activation on bronchial epithelial cells and basophils: a novel inflammatory mechanism. *Eur Respir J.* 2010; 35: 883-893.

2. **Cao J**, Wong CK, Yin Y, Lam CW. Activation of human bronchial epithelial cells by inflammatory cytokines IL-27 and TNF-alpha: Implications for immunopathophysiology of airway inflammation. *J Cell Physiol.* 2010; 223: 788-797.

3. Wong CK, Ng SS, Lun SW, **Cao J**, Lam CW. Signalling mechanisms regulating the activation of human eosinophils by mast-cell-derived chymase: implications for mast cell-eosinophil interaction in allergic inflammation. *Immunology.* 2009;126:579-587.

4. 林偉基, 黃振國, **曹 炬**. 細胞因子和趨化因子——急性感染的炎症指標. *澳門科技大學學報.* 2008; 2: 43-48.

Published conference abstracts:

1. **Cao J**, Wong CK, Lun SW, Lam CW. Activation of T helper lymphocytes by mast cell-derived chymase: immunopathological implication in allergic diseases. Presented at the Annual Scientific Meeting of Hong Kong Society for Flow Cytometry, 03/2008.

2. **Cao J**, Wong CK, Lun SW, Lam CW. Activation of T helper lymphocytes by mast

cell-derived chymase: immunopathological implication in allergic diseases. Presented at the Annual Scientific Meeting of Hong Kong Society for Immunology, 04/2008.

3. **Cao J**, Wong CK, Lam CW. Elevated induction of IL-6, CCL2 and ICAM-1 upon interaction between human basophilic and bronchial epithelial cells activated by IL-17A: a novel immunopathological mechanism of allergic asthma. Presented at the Annual Scientific Meeting of Hong Kong Society for Flow Cytometry, 03/2009. (Oral presentation)

4. **Cao J**, Wong CK, Lam CW. Elevated induction of IL-6, CCL2 and ICAM-1 upon interaction between human basophilic and bronchial epithelial cells activated by IL-17A: a novel immunopathological mechanism of allergic asthma. Presented at the Annual Scientific Meeting of Hong Kong Society for Immunology, 04/2009.

5. **Cao J**, Wong CK, Lam CW. Activation of human bronchial epithelial cells by inflammatory cytokines IL-27 and TNF-α: implications for immunopathophysiology of airway inflammation. Presented at the Annual Scientific Meeting of Hong Kong Society for Flow Cytometry, 3/2010.

6. **Cao J**, Wong CK, Lam CW. Activation of human bronchial epithelial cells by inflammatory cytokines IL-27 and TNF-α: implications for immunopathophysiology of airway inflammation. Presented at the Annual Scientific Meeting of Hong Kong Society for Immunology, 4/2010.

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Chapter 1

General Introduction

1.1 Human bronchial epithelial cells in airway inflammation

Human bronchial epithelial cells are the first mechanical barriers against deleterious factors, such as air pollutants, allergens, viruses, bacteria and cigarette smoke, and they are actively involved in initiating, maintaining, and regulating both innate and adaptive immune responses in human airways (Khair *et al.*, 1996). Upon encountering these inhaled stimuli, human bronchial epithelial cells can produce and release a variety of antimicrobial substances and inflammatory mediators which serve in a paracrine or autocrine manner to regulate airway host defense and immune responses (Martin *et al.*, 1997). The activation of human bronchial epithelial cells can also lead to the recruitment and influx of other inflammatory cells in the airways such as dendritic cells (DC), monocytes, T cells, B cells, neutrophils, basophils and eosinophils through distinct molecular mechanisms (Schleimer *et al.*, 2007). The cross-talk between human bronchial epithelial cells and these inflammatory cells will result in augmentation of host immune responses and initiate and sustain airway inflammation and remodeling (Kato and Schleimer, 2007). Therefore, human bronchial epithelial cells play a central role in the pathogenesis of airway

inflammatory diseases, and they have become a target for therapeutic intervention for asthma, chronic bronchitis, chronic obstructive pulmonary disease (COPD), hyperoxic lung injury and other airway inflammatory diseases (Takizawa, 1998; 2005).

1.1.1 Characteristics of human bronchial epithelial cells

The human bronchial epithelium is composed of the surface human bronchial epithelial cells and mucus glands, and it forms an interface between the airways and the inspired air (Velden and Versnel, 1998). Human bronchial epithelium consists of three major cell types: basal, goblet, and ciliated cells. Well-differentiated human bronchial epithelial cells form a suprabasal columnar structure for mucociliary clearance. The bronchial epithelium normally forms a continuous layer, and the integrity of bronchial epithelial cells not only makes an effective physical barrier protecting the underlying tissue against noxious substances, but also a complicated host defense system by producing a number of mediators serving as an active participants in immune responses (Levine, 1995; Velden and Versnel,1998). Any damage to bronchial epithelial cells would make them lose biological functions and cause a variety of airway inflammatory diseases.

Human bronchial epithelium is a dynamic tissue which normally undergoes a

slow and constant renewal. At present, basal cells are recognized as the stem cells of human bronchial epithelial cells. However, there is no detailed and sophisticated understanding of progenitor cell–progeny relationships in human bronchial epithelium (Crystal *et al.*, 2008). Interleukin (IL)-4 and IL-13 have been shown to modulate the functions of bronchial epithelial cells such as cell proliferation, ciliary beating, and mucous secretion (Kikuchi *et al.*, 2004). Epidermal growth factor receptor (EGFR) signaling regulates various cellular processes including cell proliferation, differentiation and mucin production (Kim *et al.*, 2005).

1.1.2 Activation of human bronchial epithelial cells in airway inflammation

There are increased evidences that human bronchial epithelial cells play pivotal roles in the pathogenesis of airway inflammatory diseases. As the first line of human body tissues in the airways, bronchial epithelial cells have evolved a sophisticated protective mechanism. They respond to external inhaled microorganisms by production of mucus and ciliary beating (Khair *et al.,* 1996). Human bronchial epithelial cells can also generate a number of substances to neutralize or kill invading pathogens, including enzymes, collectins, pentraxins, permeabilizing peptides, protease inhibitors and other small molecules (Schleimer *et al,*. 2007). In addition, they can produce several families of cytokine and chemokines. The production of

many of these mediators is regulated by the pattern-recognition receptors (PRRs) expressed by human bronchial epithelial cells, including Toll-like receptors (TLRs; from TLR1 to TLR10), nucleotide-binding oligomerization domain [NOD]–like receptor [NLR] family receptors (eg, NOD1 and NOD2) and ribonucleic acid (RNA) helicases (melanoma differentiation-associated gene 5 [MDA5] and retinoic acid-inducible gene I [RIG-I]) (Sha *et al.*, 2004; Fritz *et al.,* 2006; Opitz *et al.,* 2007; Schleimer *et al.,* 2007). Therefore, human bronchial epithelial cells are critical participants in innate immune response for rapid recognition and protection of human body from invading pathogens.

Not only do human bronchial epithelial cells have a role in the primary innate immune response to inhaled pathogens, they also regulate adaptive immunity in the airways. They are able to recruit a variety of inflammatory cells by secretion of a number of chemokines, and then they interact with these inflammatory cells through cell-cell adhesion and interaction via direct contact or surface-exposed molecules, such as adhesion molecules and major histocompatibility complex (MHC) molecules (Velden and Versnel, 1998). Besides, human bronchial epithelial cells can also modify the functions of inflammatory cells by production of multiple cytokines in the airways. At present, it is well known that human bronchial epithelial cells are capable of inducing and regulating the differentiation and biological functions of

DCs, B cells, T cells and eosinophils (Schleimer *et al.*, 2007; Kato and Schleimer, 2007).

Since DC express chemokine receptor CCR6, human bronchial epithelial cells would attract infiltrating DC via chemokine CCL20 (macrophage inflammatory protein 3a [MIP-3a]) and β-defensins production induced by different stimuli, such as allergens including house dust mite allergen *Dermatophagoides pteronyssinus* (Der p) 1 and extracellular nucleotides, pathogen-associated molecular patterns (PAMPs) including dsRNA, peptidoglycan (PGN) and the outer membrane protein A from *Klebsiella pneumoniae* (KpOmpA), and cytokines such as tumor necrosis factor (TNF)-α, IL-1, IL-4, IL-13 and IL-17 (Kato and Schleimer, 2007; Reibman *et al.*, 2003; Kao *et al.*, 2005; Pichavant *et al.*, 2006; Marcet *et al.*, 2007; Yang *et al.*, 1999). Human bronchial epithelial cells can produce another important cytokine granulocyte/macrophage colony stimulating factor (GM-CSF) that would promote the survival and induce maturation of DC (Schleimer *et al.* 2007). In response to the stimulation of TLR3 ligand double-stranded RNA, TNF-α, IL-1β, and T helper (Th) 2 cytokines including IL-4 and IL-13, human bronchial epithelial cells can express thymic stromal lymphopoietin (TSLP) (Kato *et al.*, 2007). TSLP is a 140-amino-acid IL-7-like four-helix-bundle cytokine which binds to a specific receptor complex comprised of the IL-7 receptor (IL-7R) and the TSLP receptor (TSLP-R) (Liu *et al.*,

2007). TSLP has been shown to skew DC to prime naive $CD4^+$ T cells to differentiate into Th2 phenotype (Soumelis *et al.*, 2002; Liu, 2006). Besides, other bronchial epithelial cells-derived inflammatory factors can also affect the functions of DC in airway inflammatory reactions, and they are type I interferon (IFN) (IFN-α and IFN-β), type III IFN (IFN-λ1, IFN-λ2 and IFN-λ3), IL-1β, IL-33, osteopontin and IL-25 (Hammad and Lambrecht, 2008; Rogge *et al.,* 1998; Mennechet and Uzé, 2006).

Human bronchial epithelial cells are able to influence the characteristics of T cells in airway inflammation. They can recruit Th1 cells by the production of chemokines including CXCL10/IFN-inducible protein (IP)-10, CXCL9/monokine induced by IFN-γ (MIG) and CXCL11/ interferon-inducible T cell α-chemoattractant (I-TAC), and can attract the migration of Th2 cells by the production of CCL1/I-309, CCL22/macrophage-derived chemokine (MDC) and CCL17/thymus and activation-regulated chemokine (TARC) (Kato and Schleimer, 2007; Spurrel *et al.*, 2005; Panina-Bordignon *et al.,* 2001; Heijink *et al.*, 2007). Besides, human bronchial epithelial cells can express co-stimulatory molecules including B7 family members B7-H1 and B7-DC which would regulate the biological functions of T cells (Kurosawa *et al.,* 2003; Kim *et al.,* 2005; Stanciu *et al.,* 2006). IL-33 produced by human bronchial epithelial cells is a ligand for ST2 (a member of the IL-1 receptor

family) expressed by Th2 cells, and it plays an important role in Th2 responses (Schmitz *et al.*, 2005).

In the airways, bronchial epithelial cells have the capacity to produce chemokine CCL28/mucosae-associated epithelial chemokine (MEC) that can recruit B cells (John *et al.*, 2005; Lazarus *et al.*, 2003). IL-6 and transforming growth factor (TGF)-β released from bronchial epithelial cells can activate B cells (Schleimer *et al.*, 2007). Besides, human bronchial epithelial cells may produce B-cell–activating factor of the TNF family (BAFF)/B lymphocyte stimulator (BLyS) which can regulate the development of B cells and promote B cell class-switch recombination (CSR) (Kato *et al.*, 2006; Xu *et a*l., 2007).

In allergic asthma, human bronchial epithelial cells can induce the migration of eosinophils via the secretion of CCL5/regulated upon activation normal T cell expressed and secreted (RANTES) and CCL2 / monocyte chemoattractant protein 1 (MCP-1). The interaction of human bronchial epithelial cells and eosinophils could up-regulate the expression of cytokine IL-6 and chemokines CXCL8/IL-8, CCL2, CXCL9, CCL5 and CXCL10 (Wong *et al.*, 2005; 2006; Wang *et al.*, 2005), and mediate the release of leukotriene C4 (LTC4) (Dent *et al.*, 2000). Besides, the contact of human bronchial epithelial cells and eosinophils could induce eosinophil cationic protein (ECP) release from eosinophils under the stimulation of IL-5 and TNF-α

(Takafuji *et al.,* 1996). Therefore, the cross-talk between human bronchial epithelial cells and eosinophils may play an important role in the pathogenesis of asthma.

In summary, human bronchial epithelial cells play important roles in regulating innate and adaptive responses to inhaled pathogens in the air ways (Figure 1.1). Human bronchial epithelial cells that express PRRs recognize pathogen-associated molecular patterns of pathogens, and then the activation of innate immunity induces recruitment and activation of a variety of effector cells, such as DC, T cells, B cells, neutrophils, monocytes, mast cells, eosinophils and basophils via distinct cytokines and chemokines produced from human bronchial epithelial cells. The cross-talk between bronchial epithelial cells and these inflammatory cells regulates specific adaptive immune responses to inhaled pathogens.

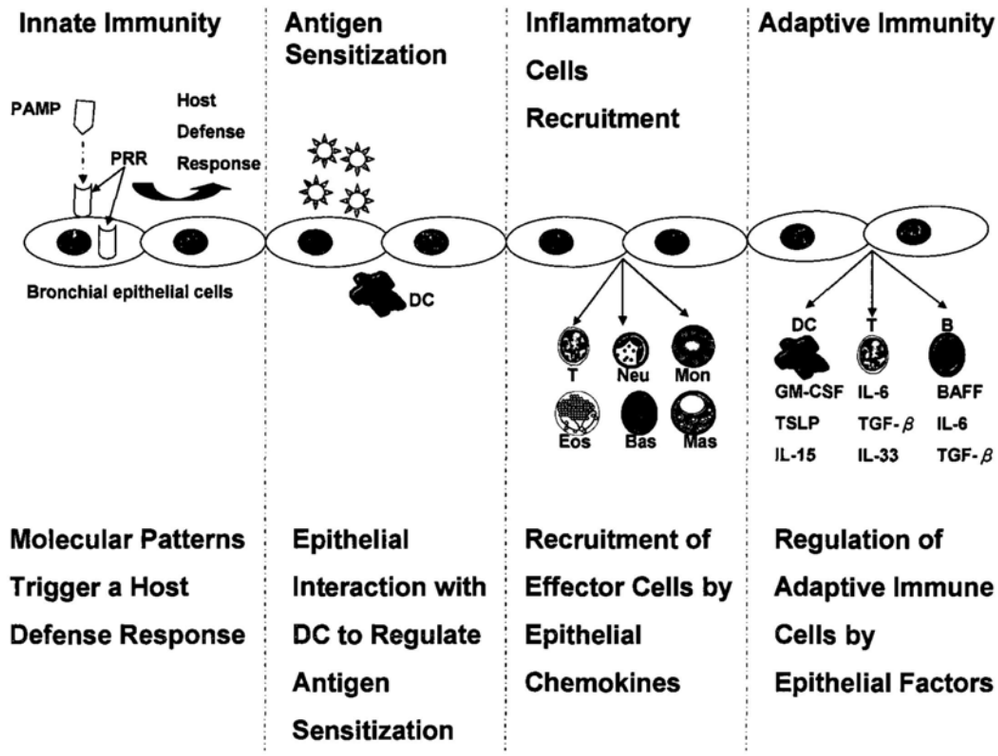


Figure 1.1 Model summarizing the roles of bronchial epithelial cells in airway inflammation. PAMP: Pathogen-associated molecular pattern; PRR: Pathogen-recognition receptor; DC: Dendritic cells; T: T cells; Neu: Neutrophils;

Mon: Monocytes; Eos: Eosinophils: Bas: Basophils; Mas: Mast cell; BAFF: B-cell–activating factor of the TNF family. (modified from Schleimer *et al.*, 2007).

1.1.3 Mediators of human bronchial epithelial cells

In airway inflammation, human bronchial epithelial cells produce a large number of mediators: lipid mediators, including leukotrienes B4 and C4 (LTB4, LTC4), prostanoids (prostaglandin D2, E2 and F2α [PGD2, PGE2, PGF2α]), platelet-activating factor (PAF), 5-,12-,15-hydroxyeicosatetraenoic acid (HETEs), and 8,15di-HETE and 12-hydroxyheptadecatrienoic acid (HHT) (Raeburn and Webber, 1994), and these lipid mediators regulate the recruitment of inflammatory cells, mucus secretion, microvascular leak and bronchoconstriction; cytokines: GM-CSF, TNF-α, TGF-β, IL-1β, IL-3, IL-6, IL-10, IL-11, IL-16, IL-25 and IL-33, and these epithelial cytokines play crucial roles in driving immune responses in the airways (Buc *et al.*, 2009; Martin *et al.*, 1997); chemokines: CXCL1/growth related oncogene (Gro)-α, CXCL2/macrophage inflammatory protein (MIP) 2, CXCL8, CCL2, CCL5, CCL11/eotaxin and these chemokines are able to attract neutrophils, monocytes, T cells, eosinophils and basophils infiltrating into inflammatory sites in the airways (Reed and Kita, 2004; Doherty and Broide, 2007). Human bronchial epithelial cells have also been demonstrated to generate nitric oxide (NO) which mediates plasma exudation, mucus and ciliary motion (Martin *et al.,* 1997), and they can also produce other mediators including endothelin and platelet-derived growth factor (PDGF), which can promote bronchospasm, or fibroblast/smooth muscle

proliferation (Velden and Versnel, 1998). Furthermore, human bronchial epithelial cells express principal adhesion molecules including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), endothelial-leucocyte adhesion molecule-1 (ELAM-1) and granule-associated membrane protein-140 (GMP-140) on cell surface, and these adhesion molecules are capable of regulating cell-cell adhesion and interaction by recognizing their specific ligands expressed by other inflammatory cells on their surface in airway inflammation (Calderón and Lockey. 1992; Raeburn and Webber, 1994).

1.2 Th17 cells in airway inflammation

Th17 cells are a newly discovered $CD4^+$ T cell subset that produces hallmark effector cytokines IL-17A and IL-17F (Schmidt-Weber *et al.* 2007). IL-17A was firstly described and cloned by Rouvier *et al.* (1993) and it has the highest overall amino acid sequence identity (50%) with IL-17F (Weaver *et al.* 2007). The genes encoding them are proximally located on chromosome 6 (Gaffen, 2008). It has been reported that IL-17A and IL-17F can be secreted as both homodimers and heterodimers (Liang *et al.*, 2007; Wright *et al.*, 2007). Both IL-17A and IL-17F appear to bind to a receptor complex composed of IL-17RA and IL-17RC to signal, of which IL-17RC binds to both IL-17A and IL-17F with the same affinity while

IL-17RA binds to IL-17A with a much stronger affinity than to IL-17F (Kuestner *et al.*, 2007; Hymowitz *et al.*, 2001). Recent studies also suggested that adaptor protein Act1 (transcription factor NF-κB activator 1) was essentially involved in IL-17 receptor signaling (Qian *et al.*, 2007). The retinoic acid receptor-related orphan receptor (ROR)γt is now accepted as the key lineage defining transcription factor for Th17 cells (Ivanov *et al.*, 2006). In human naïve $CD4^+$ T cells, IL-1β and IL-6 promote RORγ expression and Th17 polarization (Acosta-Rodriguez *et al.*, 2007), while IL-23 is required for growth, survival, and effector functions of Th17 cells (Zhou *et al.*, 2007; Iwakura *et al.*, 2008).

It is well recognized that Th17 cells play vital roles in the pathogenesis of airway inflammatory diseases. In rat models, intratracheal administration of recombinant IL-17A has been found to significantly increase the absolute number of neutrophils in bronchial alveolar lavage fluids (BALFs) (Laan *et al.*, 1999). In mouse models, overexpression of IL-17F by adenoviral gene transfer strategy could also result in an increase in the number of neutrophils in BALFs (Hurst *et al.*, 2002). In human beings, it has been demonstrated that allergic asthmatic patients had higher plasma IL-17A concentration than normal healthy controls (Wong *et al.*, 2001), and IL-17A level in sputum of patients with allergic asthma correlated with airway hyperresponsiveness to methacholine (Barczyk *et al.*, 2003).Also, an increased expression of IL-17F has

been found in allergic asthma patients following allergen challenge (Kawaguchi *et al.*, 2001). In *in vitro* studies, IL-17A could stimulate airway mucin (MUC5AC and MUC5B) gene expression in human tracheobronchial epithelial cells (Chen *et al.*, 2003). IL-17A and IL-17F could enhance the production of cytokines (GM-CSF, granulocyte colony-stimulating factor [G-CSF], IL-6 and IL-11), chemokines (CXCL1, CXCL8 and CCL2), and matrix metalloproteinases (MMP-1 and MMP-3) from human bronchial epithelial cells and fibroblasts (Iwakura *et al.*, 2008; Yagi *et al.*, 2007). Besides, IL-17A and IL-17F were able to induce the production of chemokines CXCL1, CXCL8 and CCL4 from human eosinophils, while IL-17F could also increase the release of cytokines IL-1β and IL-6 (Cheung *et al.*, 2008).

At present, it has been accepted that IL-17A and IL-17F produced from Th17 cells could recruit and activate neutrophils through the induction of cytokines and chemokines (IL-6, G-CSF, GM-CSF, CXCL1 and CXCL8) from human bronchial epithelial cells and fibroblasts, thereby increasing myeloperoxidase (MPO) and elastase activities in BALFs (Figure 1.2).Moreover, IL-17A and IL-17F may also attract the influx of other inflammatory cells, and amplify airway inflammation and ultimately lead to tissue injury and airway remolding (Kawaguchi *et al.*, 2004).

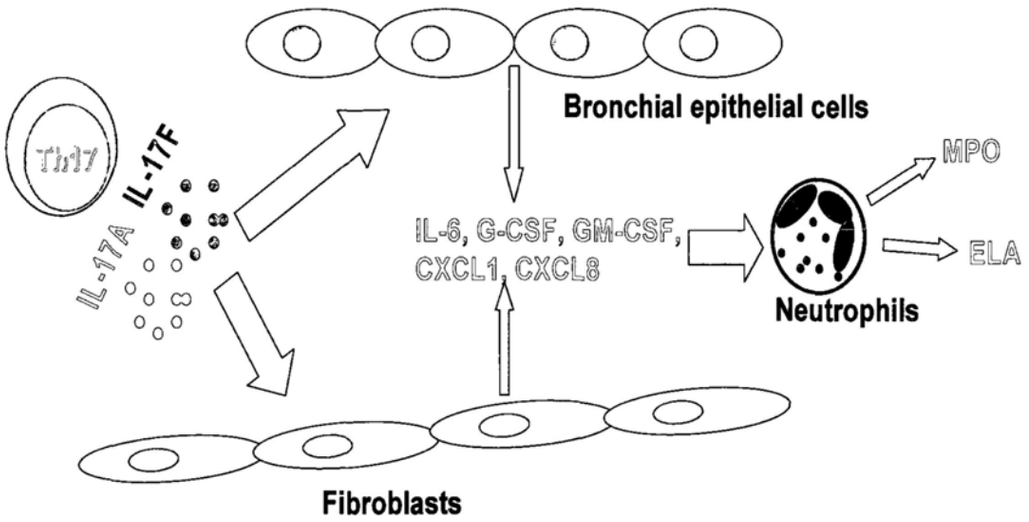


Figure 1.2 The proposed roles of Th17 cells in the airway inflammation. ELA: elastase; MPO: myeloperoxidase. (Modified from Lindén, 2001).

1.3 Basophils in airway inflammation

Basophils were first described by Paul Ehrlich, and they represent less than 1% of circulating blood leukocytes in human beings as the rarest of blood granulocytes (Marone *et al.,* 2002; Galli *et al.,* 2005). Basophils exhibit a segmented nucleus with their cytoplasmic granules containing multiple prominent amorphous granules. They are derived from $CD34^+$ pluripotent stem cells, differentiate and mature in hematopoietic tissues, and then circulate in the peripheral blood until they infiltrate into tissues or undergo apoptosis (Galli and Franco, 2008). IL-3 is the principal cytokine required for the growth and development of basophils (Valent, 1995). Basophils share several common features with mast cell, such as the expression of the high-affinity receptor for immunoglobulin E (IgE) (FcεRI), metachromatic staining, and both of them can produce similar inflammatory mediators including Th2 cytokines and histamine that play important roles in allergic inflammation (Prussin and Metcalfe, 2003; 2006). However, basophils are considered as a separate cell lineage from mast cells, and either basophils or mast cells have their own distinct roles in the pathogenesis of inflammatory diseases.

Mature basophils express a large number of surface molecules (Figure 1.3): immunoglobulin receptors, such as FcεRI and two subtypes of IgG receptors (FcγRII A and B); complement receptors, such as CD11b, CD11c, CD21, CD35, CD46 and

CD55; adhesion molecules, such as selectins (CD15s and CD62L) and integrins (CD11a, CD18, CD49a and CD49d); cytokine receptors, such as CD25, CD116, CD123, CD124, CD125 CD128 and IL-18R; chemokine receptors: CCR1, CCR2, CCR3, CCR5, CXCR1 and CXCR2 (Arock *et al.*, 2002; Anselmino *et al.*, 1989; Valent, 1995; Bodger *et al.*, 1989). These surface molecules regulate the phenotype, recruitment and activation of basophils.

Upon activation, basophils are able to produce a variety of mediators (Figure 1.3). They are cytokines (IL-3, IL-4 and IL-13), chemokines(CCL3 and CXCL8), histamine, proteoglycans (heparin and chondroitin sulphate), lipid mediators (leukotriene C4 and platelet activation factor), proteases (tryptase, elastase and cathepsin G), basic proteins (basogranulin, 2D7 antigen, eosinophil cationic protein [ECP] and eosinophil-derived neurotoxin [EDN] and other enzymes (β-hexosaminidase, β-glucuronidase and eosinophil peroxidase) (Simons *et al.*, 2007). These basophil-derived mediators make basophils as the critical effectors cells in inflammatory diseases such as systemic anaphylaxis.

There is a growing body of evidences suggesting that basophils contribute to aberrant Th2 immune responses and airway inflammation in asthmatics. The ability of basophils to produce IL-4 can facilitate or even induce Th2 development under certain circumstances (Barrett and Austen, 2009). Basophils can be activated in both

Fc3εI-dependent and -independent manner to release their mediators (Sullivan and Locksley, 2009), and they can also be activated by some complement proteins, cytokines or chemokines (Mitre and Nutman, 2006). Crosslinking of Fc3εI by antigens and immunoglobulin E (IgE) can trigger the production of histamine, cytokines, lipid mediators that play an important role in late phase of allergic inflammation in asthma (Barrett and Austen, 2009). In a recent study, it was demonstrated that baosphils could release platelet-activating factor (PAF) upon surface FcγRIII binding to immune complexes of IgG1 and antigens, which were actively involved in the pathophysiology of systemic anaphylaxis in mouse models (Tsujimura *et al.,* 2008). In human patients with allergic asthma, there was an increased number of sputum basophils in the airways of asthmatics after allergen inhalation challenge, and sputum basophils correlated with airway hyperresponsiveness (AHR) to methacholine (Gauvreau *et al.*, 2000). Basophils were shown to be the predominant IL-4- and IL-13-expressing cells after activation in allergic asthmatic (Devouassoux *et al.,* 1999). Besides, basophils could be recruited to the bronchial mucosa and produce IL-4 after allergen provocation in sensitive asthma patients, thereby contributing to both IgE production and allergic airway inflammation (Nouri-Aria *et al.,* 2001). Increased infiltration of basophils has also been detected in the lung sections of asthma patients (Kepley *et al.,* 2001). Own to

active participation of basophils in allergic asthma, flow cytometric analysis of *in vitro*-activated peripheral blood basophils by using activation antigens CD13, CD164 (behaving as CD203c) and CD107a (paralleling CD63 expression) is being developed to diagnose allergic asthma (Ebo *et al.*, 2006).

Basophil surface markers

Immunoglobulin receptors

Fc ε RI, Fc γ RIIA and B

Complement receptors

CD11c, CD11b, CD21, CD35, CD46 and CD55

Adhesion molecules

CD11a, CD15s, CD18, CD49a, CD49d and CD62L

Cytokine receptors

CD25, CD116, CD123, CD124, CD125 CD128 and IL-18R

Chemokine receptors

CCR1, CCR2, CCR3, CCR5, CXCR1 and CXCR2

Other surface molecules

CD40, CD40L, CD13 and CD203c

Basophil products

Histamine

Cytokines

IL-3, IL-4 and IL-13

Chemokines

CCL3 and CXCL8

Proteoglycans

heparin and chondroitin sulphate

Lipid mediators

leukotriene C4 and platelet activation factor

Proteases

tryptase, elastase and cathepsin G

Basic proteins

basogranulin, 2D7 antigen, eosinophil major basic protein, ECP and eosinophil-derived neurotoxin

Other enzymes

β-hexosaminidase, β-glucuronidase and eosinophil peroxidase

Figure 1.3 Surface molecules on basophils and basophile-derived mediators. (modified from Simons *et al.,* 2007 and Arock *et al.,* 2002).

1.4 Dendritic cells (DC) in airway inflammation

DC were described as they are now recognized by Ralph Steinman in the 1970s as potent professional antigen-presenting cells (APC) (Heath and Carbone, 2009). DC are essentially involved in integrating innate and adaptive immune responses. They develop from bone marrow-derived precursor cells and are usually located at the body sites where external pathogens encounter, such as skin, airway, lung and gut (Lambrecht *et al.,* 2001). DC can be subdivided in to two subtypes: myeloid DC (also referred to as conventional DC) and plasmacytoid DC (Yoshida, 2009; Grouard *et al.,* 1997).

DC are able to dynamically sense "danger" signals, such as bacterial or viral infection, tissue damage and necrosis as a unique sentinel (Lambrecht *et al.,* 2001). After antigen uptake by DC, DC would migrate to draining lymph nodes where they would undergo maturation and direct the differentiation of naïve T cells to effector T cells. In this stage, activated DC express high levels of major histocompatibility complex (MHC) molecules (MHC class I and II) that present antigen-derived peptides to naïve T cells, and activated DC also express co-stimulatory molecules CD80 and CD86 which can bind to CD28 on the surface of naïve T cell, Besides, activated DCs would produce a variety of cytokines (such as IL-6, IL-12, IL-18, IL-23, IL-27 and IFN-γ) that play an important role in instructing naïve T cells.

Taken together, MHC and costimulatory molecules as well as cytokines produced by DC determine the differentiation and development of naïve T cells (Macatonia *et al.*, 1995; Joffre *et al.*, 2009; Kuipers and Lambrecht, 2004).

Dendritic cells are well positioned in the airways to respond to any foreign invaders (Figure 1.4). The density of DC in the airways has been reported to be related to the extent of antigen exposure (Upham, 2003). In response to different types of inflammatory stimuli, including bacteria, virus and allergen, there is a marked increase of the number of DC in the airways. It has been demonstrated that exposure to the Mycobacterium *Bacillus Calmette-Gue'rin* (BCG), *Bordetella pertussis*, influenza virus and house-dust mite Der p1 could result in the recruitment and activation of airway DC (Havenith *et al.,* 1992, McWilliam *et al.,* 1997; Hamilton-Easton *et al.,* 1995; Hammad and Lambrecht, 2008). Activated airway DC then rapidly present antigens to naïve T cells and elicit specific immune response to pathogens. In *Mycobacterium tuberculosis* infection, DC could directly phagocytose *Mycobacterium tuberculosis*, secrete elevated levels of inflammatory cytokines including TNF-α, IL-1, and IL-12, and prime mycobacterium-specific $CD4^+$ and $CD8^+$T cell responses *in vitro,* thereby facilitating the initiation of immune responses to this pathogen (Henderson *et al.,* 1997). For viral infection in the airways, the main function of DC is to process and present viral peptides to $CD8^+$ and $CD4^+$ T cells,

which can induce antigen-specific response to virus. In addition, DC can produce certain cytokines and chemokines to regulate the antiviral response. The most important cytokines are type I interferons (IFNs) including IFN-α and IFN-β, and both of them can induce the expression of a series of genes that orchestrate host defense against viral infection (Theofilopoulos *et al.,* 2005). DC can also produce chemokines CCL3, CCL4, CCL5, CXCL1, CXCL2, CXCL3, and CXCL8, which can attract neutrophils, cytotoxic T cells, and NK cells. These inflammatory cells then participate in anti-viral response (Piqueras *et al.,* 2006; Luster, 2002). In allergic airway diseases, DC play an important role in allergic inflammation and tissue remodeling. It has been demonstrated that the number of respiratory mucosal DC was increased and these DC could express the high affinity IgE receptor on cell surface by which allergens can be internalized and targeted to MHC molecules (Fokkens *et al.,* 1989; Tunon-De-Lara *et al.,* 1996; Upham, 2003). In contrast to DC of healthy donors, Der p 1 could up-regulate the expression of CD86 only on DC from house dust mite--sensitive patients, and this was associated with an enhanced production of inflammatory cytokines including TNF-α, IL-1β and IL-10. DC in house dust mite--sensitive patients may amplify Th2 response upon the exposure to house dust mite (Hammad *et al.,* 2001). Besides, allergen-pulsed DC could induce naive as well as memory autologous $CD4^+$ T cells from atopic but not from nonatopic individuals

to produce Th2 cytokines, such as IL-4, IL-5 and IL-10 (Bellinghausen *et al.,* 2000) and DC are suggested to be involved in the recruitment of Th2 cells to the airways by the production of chemokines CCL17 and CCL22 (Hammad and Lambrecht, 2006). Therefore, DC activation in the airways not only modulate allergic sensitization, but also maintain allergic inflammation. Corticosteroids (CS) predictably decrease the increased numbers of DC in the bronchial mucosa of atopic asthmatic patients (Möller *et al.,* 1996). Inhibition of the activation of DC in the airways may be the potential strategy to treat airway inflammatory diseases.

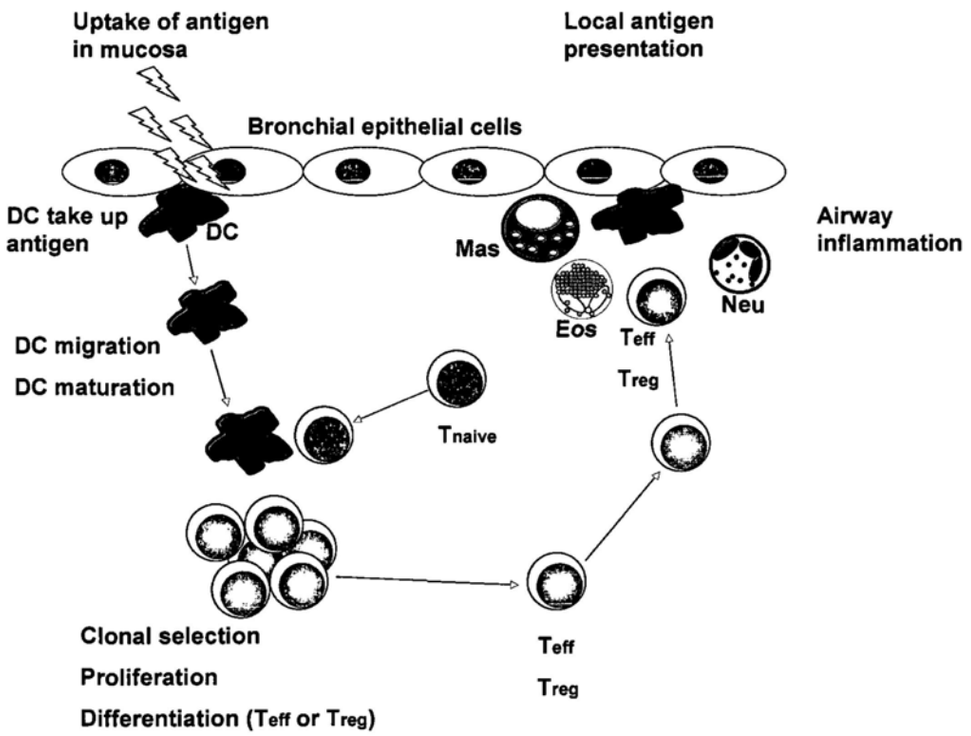


Figure 1.4 The roles of DC in airway inflammatory disease. DC take up antigen and then migrate to the draining mediastinal nodes where they undergo maturation and become potent antigen-presenting cells. Depending on the nature of the antigen and cytokine milieu at the inflammatory cite, antigen-specific T cells differentiate into T effector cells (Teff - Th1, Th2 or Th17) or regulatory T cells (Treg). Differentiated Teff or Treg subsequently migrate back to the inflammatory site to regulate inflammatory responses with other inflammatory cells (Neu: neutrophils, Mas: mast cells, Eos: eosinophils), and they would be re-activated by local airway DC. (modified from Kuipers and Lambrecht, 2004).

1.5 IL-6 in airway inflammation

IL-6 is one of the most important cytokines in infection, inflammation and immunity. Human IL-6 gene is located on chromosome 7 at 7p21 and the major messenger RNA (mRNA) for human IL-6 is 1.3 kb in length (Tamm, 1989). It can be synthesized and released by a series of different cell types including macrophages, fibroblasts, lymphocytes, epithelial and endothelial cells. Normal cells usually do not produce IL-6 constitutively in the steady state. However, it can be induced by a large number of stimuli, such as viruses, bacteria, cytokines and growth factors (Van, 1990). At present, it is well established that IL-6 plays a crucial role in regulating acute phase response, hematopoiesis and human immune responses (Akira *et al.*, 1993).

IL-6 is an important regulator in airway inflammation. Human bronchial epithelial cells from patients with airway inflammatory diseases such as asthma showed increased levels of IL-6 (Takizawa, 1998), and allergen challenge could induce increased release of IL-6 from alveolar macrophages of patients with asthma (Broide *et al.*, 1992). Besides, in patients with chronic obstructive pulmonary disease, an increased level of IL-6 has been detected in sputum, with a further increase during exacerbations (Chung, 2001). IL-6 is able to stimulate the growth, differentiation and activation of T and B cells. Recent findings demonstrated that IL-6 could collaborate

with IL-β to promote the polarization of human Th17 cells (Acosta-Rodriguez *et al.*, 2007). IL-6 is also involved in IgE synthesis as a co-factor for IL-4 (Chung, 2001), and it contributes to the development of Th2-dominated inflammation, suggesting that IL-6 may play a role in initiating and/or amplifying airway allergic inflammation (Elias *et al.*, 1999). Besides, IL-6 could induce the production of respiratory mucous glycoprotein and MUC2 in human airway epithelia cells (Martin *et al.*, 1997). IL-6 may be associated with airway remodeling, since it has been demonstrated that IL-6 could increase the proliferation of pig airway smooth muscle cells (De *et al.*, 1995). Using transgenic mice which overexpressed human IL-6 in the airway epithelial cells, it was suggested that IL-6 was involved in airway lymphocytic inflammation, airway hyperresponsiveness and altered airway physiology (Dicosmo *et al.*, 1994).

1.6 TNF-α in airway inflammation

TNF-α is a polypeptide hormone of 157-amino-acid peptide (molecular mass 17 kDa) and its gene is located on the short arm of human chromosome 6 (Vilcek and Lee, 1991). TNF-α is generated as a pro-peptide of 233 amino acids and activated by cleaving a 76-amino-acid signal peptide by TNF-α-converting enzyme (TACE) (Kriegler, *et al.*, 1988). Both soluble and membrane-exposed forms of TNF-α have their biological functions. The biological effects of TNF-α are mediated by TNF-α

receptors (TNFR) Type I (TNFR1; p55 or CD120a) or Type II (TNFR2; p75 or CD120b) expressed on the surface of many cell types (Tartaglia *et al.,* 1991). TNF-α can be produced by a wide range of inflammatory cells (mainly macrophages, monocytes, lymphocytes, mast cells, DC, neutrophils and eosinophils) and structural cells (epithelial cells, smooth muscle cells, fibroblasts and keratinocytes) in response to a large number of different stimuli including cytokines, endotoxin, calcium flux, oxygen free-radical mechanisms and phorbol esters (Russo and Polosa, 2005; Martin *et al.,* 1997). It is suggested that TNF-α is an important cytokine implicated in a lot of human diseases including sepsis, autoimmune diseases, allergy and cancer (Vassalli, 1992)

TNF-α has been suggested to play a critical role in many aspects of the airway inflammatory diseases. It is a key regulator in initiating airway inflammation and generating airway hyper-responsiveness. Elevated expression level of TNF-α has been observed in patients with asthma or COPD (Russo and Polosa, 2005; De, 2002). Besides, TNF-α gene promoter polymorphism that could result in constitutively higher expression of TNF-α was reported to be associated with COPD (Sakao *et al.,* 2001; Wilson *et al.,* 1997). Once released in the airways, TNF-α could up-regulate the expression of cell surface adhesion molecules, such as intercellular cell-adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and E-selectin.

These adhesion molecules contribute to the migration of neutrophils and eosinophils to inflammatory sites in the airways (Thomas, 2001; Russo and Polosa, 2005). TNF-α is able to induce neutrophil degranulation and stimulate eosinophils and macrophages to release MMP (Schwingshackl *et al.,* 1999; Lim *et al.,* 2000). In addition, TNF-α could alter cell migration and induce mucus, tenascin, IL-6, IL-8 and GM-CSF secretion from airway epithelial cells (Martin *et al.,* 1997; Chung, 2001; Russo and Polosa, 2005). TNF-α is also capable of inducing airway mucous cell metaplasia and hypersecretion (De, 2002) and it has the ability to activate myofibroblasts lie below the bronchial basement membrane, thereby contributing to airway wall fibrosis and remodeling (Palombella *et al.,* 1988; Palombella and Vilcek; 1989). Because of these important roles of TNF-α in the pathogenesis of airway inflammation, anti–TNF-α therapy may be a useful approach for treating asthma and COPD, and clinical trials are currently underway (Brightling *et al.*, 2008).

1.7 IL-27 in airway inflammation

IL-27 is a novel member of the IL-6/IL-12 family of cytokines, and it is a heterodimeric cytokine composed of EBI3 and IL-27p28 (Hunter *et al.*, 2004). IL-27 is produced by antigen-presenting cells following the stimulation of inflammatory stimuli such as lipopolysaccharide (LPS) or Gram-positive bacteria (Pflanz *et al.,*

2004; Smits *et al.*, 2004). Besides, endothelial cells have also been reported to be capable of producing IL-27 (Villarino *et al.*, 2004). T cell cytokine receptor (TCCR)/WSX-1 and glycoprotein 130 (gp130) form a functional signaling receptor complex for IL-27, and WSX-1 is a class I cytokine receptor with homology to the IL-12 receptor, while gp130 is a common receptor chain shared by other cytokines, such as IL-6 (Hunter *et al.*, 2004). Co-expression of WSX-1 and gp130 has been found on many cell types including monocytes, dendritic cells, T and B lymphocytes, NK cells, mast cells, keratinocytes, epithelial cells and endothelial cells (Pflanz *et al.*, 2004). Upon binding to its receptor, IL-27 exerts its pleitropic functions in innate and adaptive immunity.

Initial investigations demonstrated that IL-27 could increase the production of IFN-γ by naive $CD4^+$ T cells, and IL-27 played a key role in driving the development of naive T cells into the Th1 at the initial step of differentiation (Yoshida and Miyazaki, 2008). In later phase of inflammatory responses, IL-27 also has an anti-inflammatory property in inhibiting Th1 immune response (Stumhofer and Hunter, 2008). Th1 immune response is critical for the host defense against *Mycobacterium tuberculosis*, and it has been reported that *Mycobacterium tuberculosis* infection could induce the expression of IL-27, and IL-27 then modulated the control the inflammatory responses in *Mycobacterium tuberculosis*

infection (Pearl *et al.,* 2004; Robinson and Nau, 2008).

IL-27 is a potent inhibitor of Th2 responses. There was an enhanced induction of Th2 cytokines (IL-4, IL-5 and IL-13) in WSX-1-deficient mice infected with *Trypanosoma cruzi* (Hamano *et al.,* 2003). In mouse models of asthma, IL-27/WSX-1 signaling contributed to the down-regulation of airway hyper-reactivity and lung inflammation (Miyazaki *et al.*, 2005), and intranasal administration of IL-27 could inhibit allergic airway hyperresponsiveness and inflammation, suggesting that IL-27 may provide us a novel therapeutic approach for treating allergic asthma (Yoshimoto *et al.*, 2007). Besides, IL-27 has been shown to antagonize the development of Th17 cells, which play an important role in allergic inflammation, autoimmunity and bacterial infection (Stumhofer and Hunter, 2008).

Since WSX-1 and gp130 are co-expressed by other inflammatory cells, IL-27 can modulate the functions of a range of immune cell types. For example, IL-27 could stimulate $CD8^+$ T cells to produce IFN-γ and enhance their cytotoxic activity against tumors (Mayer *et al.*, 2008; Hisada *et al.,* 2004). IL-27 has also been reported to activate natural killer (NK) cells and induce anti-tumor responses (Matsui *et al.,* 2009). In addition, IL-27 is able to stimulate monocytes and mast cells to produce several inflammatory cytokines (Pflanz *et al.*, 2004).

The roles of IL-27 in immune responses are still obscure, and the pro- or

anti-inflammatory signaling of IL-27 in airway inflammation remains to be clarified for the development of new therapeutic approaches for airway inflammatory diseases.

1.8 CCL2 /monocyte chemoattractant protein-1 (MCP-1) in airway inflammation

Chemokines are a large family of small heparin-binding proteins whose major functions are to mediate cell trafficking. Based on the number and location of the cysteine residues at the N-terminus of the molecule, chemokines can be divided into four subfamilies: CXC, CC, CX3C, and C (Rollins, 1997). CCL2 is a member of the C-C chemokine family, and it can regulate the chemotaxis and transendothelial migration of monocytes, memory T lymphocytes, NK cells, basophils and eosinophils, but not neutrophils (Deshmane *et al.,* 2009). There is also some controversy about the ability of CCL2 to recruit DC (Gu *et al.,* 1997). CCL2 is located on chromosome 17 (chr.17, q11.2), and it is encoded by a single gene (Deshmane *et al.,* 2009). CCL2 is typically secreted in two predominant forms (with molecular weights of 9 and 13 kDa) (Melgarejo *et al.,* 2009), and it is thought to exert its biological effects as a non-covalently-bound dimeric protein derived by proteolytic cleavage from a larger precursor (Graves, 1999). CCL2 can be produced by a wide range of cell types, such as T lymphocytes, monocytes, fibroblasts,

endothelial and epithelial cells, smooth muscle cells and other inflammatory cells either constitutively or after induction by a variety of stimulatory agents including stress factors, cytokines, or growth factors (Melgarejo *et al.,* 2009). CCL2 mediates its biological effects through the binding to G-protein-coupled receptor CCR2 whose expression is relatively restricted to certain types of cells, such as monocytes, activated T lymphocytes and NK cells (Deshmane *et al.,* 2009; Melgarejo *et al.,* 2009). There are increasing evidences that CCL2 are essentially involved in the development of atherosclerosis, tumor, autoimmune diseases or infectious diseases (Deshmane *et al.,* 2009).

In airway inflammatory diseases, elevated CCL2 has been detected in the bronchial epithelium of idiopathic pulmonary fibrosis and asthma patients, and CCL2 was also significantly elevated in tuberculosis effusions and during pleural infections (Melgarejo *et al.,* 2009). The increased expression of CCL2 is associated with the recruitment and infiltration of monocytes and other inflammatory cells, which can maintain and amplify airway inflammation. CCL2 is a potent activator of human basophils and mast cells and can stimulate them to release histamine or leukotriene (Gu *et al.,* 1997; Rose *et al.,* 2003), and CCL2 is an inducer of endothelial migration, angiogenesis and vascular smooth-muscle hyperplasia (De, 2002). Besides, CCL2 can stimulate fibroblasts to produce TGF-β and procollagen, and it also plays a role

in the enhancement of Th2 polarization (Rose *et al.*, 2003). All these studies support an important role of CCL2 in airway inflammation and remodeling. A recent study also demonstrated that polymorphism in the gene regulatory region of CCL2 was associated with an increased level of blood eosinophils and asthma susceptibility and severity (Szalai *et al.*, 2001). Furthermore, in mouse models of allergic airway inflammation, treatment with antibodies against CCL2 could drastically diminish bronchial hyperresponsiveness and lung inflammation, which correlated with a pronounced decrease of inflammatory mediators derived from monocytes and lymphocytes (Gonzalo *et al.*, 1998). These data suggest that CCL2 may represent a promising target for therapeutic intervention of airway inflammatory disease.

1.9 Adhesion molecule ICAM-1 in airway inflammation

ICAM-1(CD54) is a member of the immunoglobulin supergene family and contains five extracellular immunoglobulin-like domains. It is a transmembrane glycoprotein consisted of 505 amino acids. Depending on the degree of glycosylation, ICAM-1 has a molecular mass ranging from 80 to 114 kDa in different cell types (Roebuck and Finnegan, 1999). ICAM-1 is one of the most important adhesion molecules that mediate cell adhesive interactions by binding to β2 integrins, leucocyte function associated molecule (LFA)-1 (CD11a/CD18) and macrophage

antigen (Mac)-1 (CR3,CD11b/CD18). ICAM-1 can be constitutively expressed by a variety of hematopoietic or non-hematopoietic cells, such as fibroblasts, keratinocytes, endothelial and epithelial cells, as well as T-cells, B-cells, dendritic cells, macrophages, and eosinophils (Stanciu and Djukanovic, 1998). Upon the stimulation with a number of inflammatory mediators, including hormones, retinoic acid, viral infection, cellular stresses and proinflammatory cytokines (IL-1, TNF-α, IFN-γ), ICAM-1 expression on these cell types can be up-regulated (Roebuck and Finnegan, 1999). ICAM-1-regulated cell interactions are very important for transendothelial migration of leukocytes to the sites of inflammation and the activation and homing of T cells as a co-stimulatory molecule (Zuckerman *et al.*, 1998). ICAM-1 can be shed and detected in plasma as soluble ICAM-1 (sICAM-1). sICAM-1 is increased in some human diseases, but its role and significance are still unclear at present (Witkowska and Borawska, 2004).

ICAM-1 expression plays an important role in a number of inflammatory diseases such as asthma, inflammatory bowl disease, atherosclerosis, acute respiratory distress syndrome, hyperoxic lung injury, and autoimmune disease (Roebuck and Finnegan, 1999). In asthma patients, prominent increase of ICAM-1 expression has been observed in asthmatic airways and the significant up-regulation of ICAM-1 has been detected on a variety of effector cells in asthma, such as

epithelial and endothelial cells, eosinophils and T cells (Stanciu and Djukanovic, 1998). The increased ICAM-1 expression mediated migration of inflammatory cells from the peripheral blood into the airways, which could contribute to airway inflammation and airway hyperresponsiveness. In patients with a variety of fibrotic lung diseases, increased ICAM-1 expression has been found on alveolar macrophages compared with cells from normal lungs, which indicates that ICAM-1 expression may be involved in the development of pulmonary fibrosis (Paine and Ward, 1999). In mouse models of LPS-induced airway inflammatory diseases, treatment with ICAM-1 antibodies could reduce the recruitment of inflammatory cells to the alveolar space (Moreland *et al.*, 2002). On the other hand, blockade of ICAM-1 expression could attenuate inflammation in experimental models of other inflammatory disease (Xu and Li, 2009). These results therefore support the concept of a therapeutic strategy for airway inflammatory diseases by directing against ICAM-1 expression.

1.10 Signal transduction pathways in inflammatory reactions and pharmacological inhibitors

1.10.1 Signal transduction pathways in inflammatory reactions

There are increasing evidences that different signal transduction pathways are

involved in the regulation of inflammation. These include RAS-RAF-mitogen-activated protein kinases (MAPK), Janus kinases (JAK)-signal transducers and activators of transcription (STAT), phosphatidylinositol 3-kinase (PI3K) and nuclear factor-kappa B (NF-κB) pathways.

1.10.1.1 RAS-RAF-MAPK pathway

MAPK are a group of evolutionarily conserved enzymes using phosphorylation cascades to generate a coordinated cellular response which regulates proliferation, differentiation, apoptosis and inflammation. It is well known that MAPK can be activated by a large number of different stimuli including growth factors and cytokines (Brown and Sacks, 2008). There are three major subgroups in the MAPK family that have been well studied in mammals. They are extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK).The different MAPK pathways control the specificity of cellular signaling and have their distinct roles in cellular function (Figure1.5). ERK pathway is the best characterized MAPK pathway. It is activated by numerous cytokines and growth factors including epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF). ERK pathway is triggered by guanosine triphosphate (GTP)-bound GTPase Ras. When bound to GTP, Ras recruits

and activates the kinase Raf. The activated Raf then induces the phosphorylation and activation of MAPK/ERK kinase 1 and 2 (MEK1 and MEK2). MEK1 and MEK2 in turn activate ERK1/2. Activated ERK1/2 subsequently dimerise and translocate to the nucleus, where they phosphorylate the transcription factors including c-fos and Ets like transcription factor-1(ELK1), and the protein kinases MAP kinase signal-integrating kinase (MNK), mitogen and stress-activated protein kinase (MSK), and ribosomal S6 kinase (RSK) (Brown and Sacks, 2008; Schindler *et al.*, 2007). The principal functional sequelae of ERK pathway are thought to control cell growth and proliferation. However, it is suggested that ERK pathway may be also involved in T cell activation and cytokine production (Schindler *et al.*, 2007).

JNK family contains three isoforms, JNK1, JNK2, and JNK3, and these members are encoded by distinct but highly homologous genes, and their expression levels vary in different tissues (Schindler *et al.*, 2007). JNK pathway is strongly activated by different stimuli including cytokines, selected G-protein coupled receptors (GPCR) and cell stress, but it is weakly activated by growth factor, activated Ras or phorbol esters (Brown and Sacks, 2008; Ravingerová *et al.*, 2003). The phosphorylation and activation of JNK require the activation of two specific MAPKK kinase enzymes, MKK4 and MKK7 by other kinases. Activated JNK is subsequently translocated to the nucleus and then phosphorylates and activates

transcription factors. The transcription factors for JNK include c-Jun, activating transcription factor (ATF)-2, Elk-1, signal transducers and activators of transcription (STAT)-3, heat shock transcription factor (HSF)-1, and/or anti-apoptotic Bcl-2 protein (Brown and Sacks, 2008; Ravingerová *et al.,* 2003). JNK activation is involved in cell proliferation, apoptosis, DNA repair, motility and metabolism (Johnson and Nakamura, 2007)

There are four isoforms of the p38 kinase family (α, β, γ and δ) that have been identified to date. Extracellular stimuli of the p38MAPK pathway include cytokines, hormones, G-protein coupled receptors and cell stress (Brown and Sacks, 2008). Kinases MKK3 and MKK6 selectively phosphorylate and activate p38-MAPK, and activated p38MAPK then phosphorylate and activate several transcription factors including ATF-1, ATF-2, Elk-1 and serum response factor (SRF). p38 MAPK pathway is involved in regulating angiogenesis, cell proliferation, cytokine production and inflammation (Ravingerová *et al.*, 2003; Brown and Sacks, 2008).

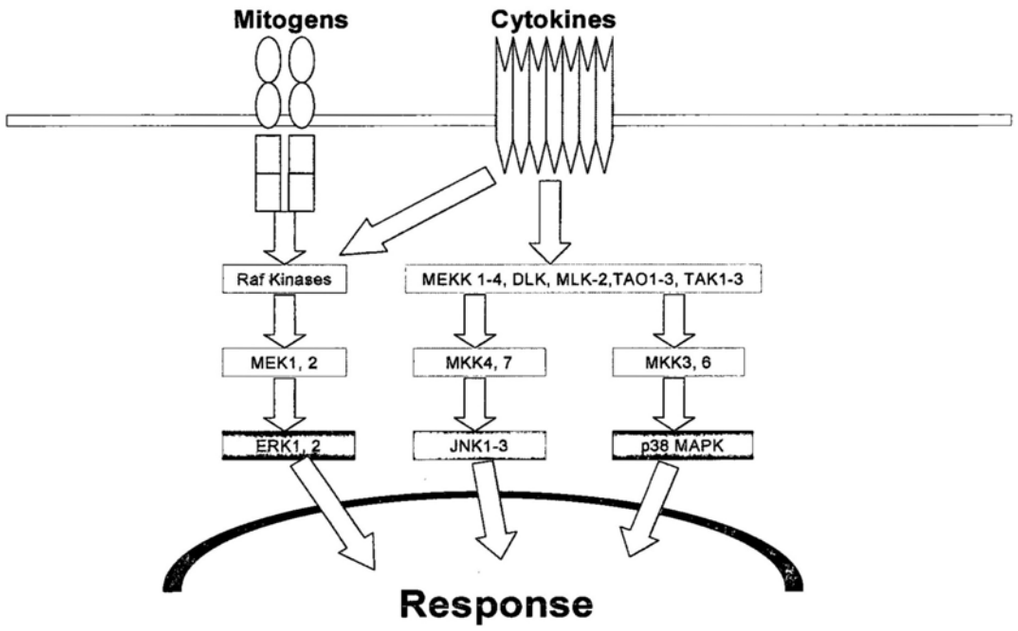


Figure 1.5 The MAPK signalling cascades. DLK: dual leucine zipper-bearing kinase; MLK2: mixed lineage kinase; TAO: thousand-and-one amino acids; TAK: transforming growth factor-beta-activated kinase. (modified from Brown and Sacks, 2008).

1.10.1.2 1JAK-STAT pathway

JAKs represent a family of four cytoplasmic non-receptor tyrosine kinases including Jak1, Jak2, Jak3 and Tyk2. JAKs are constitutively associated with cytoplasmic domains of cytokine receptors and they have an N-terminal Four-pointone/Ezrin/Radixin/Moesin (FERM) domain, a central JAK homology (JH) 2 pseudokinase domain and a C-terminal JH1 kinase domain (O'Sullivan *et al.,* 2007). STATs are a family of seven structurally and functionally related proteins including Stat1, Stat2, Stat3, Stat4, Stat5a and Stat5b, Stat6. Each Stat possess a four helix bundle transactivation domain, a central β-barrel Ig-like DNA binding domain, a helical linker domain, an Src-homology (SH) 2 domain and an effector domain (Kisseleva *et al.,* 2002; O'Sullivan *et al.*, 2007). JAKs selectively phosphorylate and activate STATs, while STATs can also be activated by other several members of the G-protein-coupled receptors (Kisseleva *et al.*, 2002).

JAK/STAT signaling pathway is initiated upon cytokine binding to its speicific receptor, leading to aggregation and conformational changes of cytoplasmic receptor subunits. JAKs then bind specifically to cytoplasmic cytokine receptor signaling subunits and autophosphorylate themselves. The activated JAKs in turn phosphorylate specific intracellular receptor tyrosine residues, creating STAT docking sites. The phosphorylated tyrosine residues subsequently recruit Stats

through their Src-homology (SH2) domains. Once recruited to the cytokine receptor, Stats are phosphorylated and activated rapidly by JAKs. Activation of Stats results in dissociation from the receptor, and then these activated Stats dimerize, translocate to the nucleus and bind to target promoters, leading to the expression of target genes (Aaronson and Horvath, 2002; Kisseleva *et al.*, 2002).

JAK/STAT signaling pathway plays a key role in regulating numerous aspects of innate and adaptive response, while hyperactive JAK/STAT can cause inflammatory disorders. Suppressors of cytokine signaling (SOCS) proteins, protein inhibitors of activated stats (PIAS) and protein tyrosine phosphatases (PTPs) are principal negative regulators of JAK/STAT pathway. These classes of negative regulators play an important role in regulating the activity of JAK/STAT signaling pathway by several ways (Rawlings *et al.*, 2004).

1.10.1.3 PI3K pathway

There are three classes of PI3Ks (class IA and IB, II and III) according to their structure and lipid substrate specificity. The most extensively studied is class IA PI3K. IA PI3K is composed of a regulatory (p85) and an enzymatic subunit (p110). The regulatory subunit functions to stabilize p110 and to limit its activity in quiescent cells. In response to extracellular stimuli including growth factors, insulin

and cytokines, IA PI3K is activated by binding of the regulatory subunit p85 to autophosphorylated tyrosine-kinase receptors or G-protein coupled receptors. Upon activation, IA PI3K converts the phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] in the membrane to phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3]. PI(3,4,5)P3 then acts as a second messenger by directly binding to downstream signaling molecules with pleckstrin-homology (PH) domains such as protein serine/threonine kinases (protein kinase B/Akt) and phosphoinositide-dependent kinase (PDK)-1 (Ehrhardt and Ludwig, 2009; Cantley, 2002; Neri *et al.*, 2002).

Akt is not phoshorylated directly by IA PI3K. However, it is phosphorylated at threonine 308 by PDK1 that is also an IA PI3K effector signaling molecule and at serine 473 by a still undefined kinase. Phosphorylation at Thr308 and Ser473 is necessary for full activation of Akt. Therefore, the phosphorylation and activation of Akt are dependent on the activation of IA PI3K. Detecting the phosphorylation of Akt at ser473 is usually applied to determine the activation state of IA PI3K (Ehrhardt and Ludwig, 2009). Activation of Akt in turn leads to the phosphorylation of a variety of other proteins, which plays a crucial role in cell growth, proliferation, differentiation, survival and activation. Moreover, there are increasing evidences that Akt are actively involved in tumorigenesis and resistance to chemotherapeutic drugs (Neri *et al.*, 2002; Martelli *et al.*, 2006)

1.10.1.4 NF-κB pathway

NF-κB is a transcription factor expressed in a number of cell types, which plays a critical role in regulating immune responses, cell proliferation and survival. There are five NF-κB family members that have been identified in mammalian cells, including Rel A (p65), c-Rel, RelB, p50/NF-κB1and p52/NF-κB2, which are assembled to form various heterodimeric and homodimeric complexes. The predominant form of NF-κB member is the heterodimer composed of p50 and RelA subunits that are ubiquitously expressed, while other three members of NF-κB family are commonly restricted to specific cell types (Santoro *et al.,* 2003).

In quiescent cells, NF-κB dimers are placed in the cytoplasm by binding to inhibitory proteins of the IκB family, including IκBα, IκBβ, IκBε and IκBγ. Upon the activation of different stimuli, such as cytokines, bacterial and viral infections, mitogens and growth factors, and stress-inducing agents, NF-κB is activated by IκB kinase (IKK) complex, which is composed of two catalytic subunits (IKK-α and IKK-β) and a regulatory subunit (IKK-γ/NEMO). In the classic IKK/ NF-κB pathway, IKK-α/IKK-β/IKKγ complex mediated phosphorylation- and proteasome-dependent degradation of IκBs relies on serine phosphorylation of the IKK-β, and this pathway leads to the activation of NF-κB1/Rel A (p50/p65) and/or p50/c-Rel and plays an important role in inflammatory and immune responses. In the alternative pathway

activated by different stimuli including lymphotoxin-β and CD40 ligand. IKK-α homodimers mediated phosphorylation and processing of the p52 precursor p100, which binds to RelB through amino-terminal Rel homology domain in unstimulated cells. Activation of IKK-α homodimers leads to the degradation of C-terminal ankyrin repeat in p100 and nuclear translocation of p52:RelB dimmers. This way regulates secondary lymphoid organogenesis and adaptive immunity (Jimi and Ghosh, 2005; Van, 2007; Karin, 2006).

1.10.2 Signaling molecule inhibitors as new drugs for inflammatory diseases

As mentioned above, RAS-RAF-MAPK, JAK-STAT, PI3K and NF-κB pathways play important roles in the pathogenesis of inflammatory diseases by controlling cell proliferation, differentiation, apoptosis, migration and mediator expression. Several specific signaling molecule inhibitors are under development for treating inflammatory diseases as potential drugs (Ito *et al.,* 2007). PD98059 is a non-competitive inhibitor of ERK and can inhibit the phosphorylation of ERK. SP600125 is a competitive inhibitor of ATP binding of JNK and can competitively and reversibly inhibit the phosphorylation of JNK1, 2 and 3. SB203580 is a competitive inhibitor of ATP binding of p38 MAPK and can selectively inhibit the enzymatic activity of p38MAPK. AG490 is a JAK inhibitor to inhibit the

phosphorylation of STAT. LY294002 could selectively inhibit the phosphorylation of PI3K. BAY11-7082 could selectively and irreversibly inhibit the phosphorylation of IκB-α (O'Neill, 2006). Although a growing number of reports have demonstrated that these signaling molecule inhibitors are effective in treating inflammatory diseases in animal models, a better understanding of the disease mechanisms is required to support the potential of signaling molecule inhibitors as new therapeutic drugs.

1.11 Aims and Scope of the Study

Airway inflammatory diseases, such as bronchial asthma and COPD, represent a major public health problem associated with significant morbidity and mortality in the world (Ito *et al.,* 2007). Bronchial epithelial cells play a crucial role in the pathogenesis of airway inflammation, and they have been recognized as the potential therapeutic targets for airway inflammatory diseases. The molecular mechanisms of the activation of bronchial epithelial cells suggest that the participation of bronchial epithelial cells in airway inflammation is regulated by multiple mediators and inflammatory cells (Cristal *et al.,* 2008). Therefore, clarifying the complex interactions between bronchial epithelial cells and inflammatory cytokines or effector cells would contribute to develop novel drugs for airway inflammatory diseases.

This thesis addresses the activation of human bronchial epithelial cells stimulated

by a variety of stimuli including human basophils, IL-17A, IL-27 and TNF-α. Basophils are rare circulating granulocytes and they serve as initiator and accessory cells for the Th2 cell polarization, and they could produce diverse inflammatory mediators that cause the symptoms of acute and chronic allergic airway inflammation (Sullivan and Locksley, 2009). IL-17A is the hallmark cytokine of Th17 cells, and it is elevated in the patients of airway inflammatory diseases and can provoke pulmonary inflammation (Chakir *et al.*, 2003). IL-27 is a novel member of the IL-6/IL-12 family cytokines, and it has pleiotropic biological functions in immune responses (Stumhofer and Hunter, 2008). TNF-α is a critical inflammatory cytokine which plays a critical role in many aspects of airway inflammatory diseases (Barnes, 2001).

In this study, the activation of human bronchial epithelial cells by these stimuli was investigated in terms of the expression of adhesion molecules and release of cytokines and chemokines. The underlying signaling pathways regulated the activation of human bronchial epithelial cells were studied using specific signaling molecule inhibitors and intracellular staining assays by flow cytometry. We hope that our study will provide a better understanding of the role of bronchial epithelial cells in airway inflammation and contribute to the development of better therapeutic strategies for airway inflammatory diseases.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Reagents and buffers for purification of human basophils

2.1.1.1 Human buffy coat

Human buffy coat was obtained from the Hong Kong Red Cross Blood Transfusion Service and basophils were purified from buffy coat within 24 hours after donation.

2.1.1.2 Sodium chloride (NaCl) solution (1.5 M)

Sodium chloride solution (1.5M) was prepared by dissolving 87.6 g NaCl (SigmaSigma–Aldrich Corp, St. Louis, MO, USA) in 1L double distilled water and sterilized by autoclaving at 121°C for 15 minutes. The concentrated stock solution was further diluted to 1.8 % w/v and 0.9% w/v sterile normal saline. The solutions were kept at 4°C.

2.1.1.3 Ficoll^{TM}-Paque solution

Ficoll-Paque containing 5.7 g Ficoll 400, 9 g sodium diatrizoate and calcium EDTA in 100 ml water (density = 1.077 g/ml) was purchased from Amersham Pharmacia Biotech Ltd, Uppsala, Sweden. It was used for the isolation of peripheral blood mononuclear cells (PBMC) *in vitro*. This solution was kept at room temperature.

2.1.1.4 Magnetic cell sorting system (MACS) and basophil isolation Kit II

The magnetic cell sorting system and Basophil Isolation Kit II were purchased from Miltenyi Biotec, Bergisch Gladbach, Germany. The MACS consisted of a MidiMACS Separation Unit, a MACS MultiStand and a LS Separation Column which could separate maximum of 2 x 10^9 total cells and 10^8 magnetically labeled cells. The Basophil Isolation Kit II consisted of 2 mL FcR blocking reagent (human IgG), 2 mL basophil biotin-antibody cocktail (a cocktail of biotin-conjugated antibodies against CD3, CD4, CD7, CD14, CD15, CD16, CD36, CD45RA, HLA-DR and CD235a), and 2 x 2 mL anti-biotin microbeads (microbeads conjugated to a monoclonal anti-biotin antibody). The Basophil Isolation Kit II was an indirect magnetic labeling system for the isolation of untouched basophils from PBMCs. Non-basophils (i.e. T cells, NK cells, B cells, monocytes, dendritic cells, erythroid cells, platelets, neutrophils, and eosinophils) were indirectly magnetically labeled using a cocktail of biotin-conjugated antibodies and anti-biotin microBeads. Isolation of highly pure basophils was achieved by depletion of the magnetically labeled cells.

2.1.1.5 Phosphate-buffered-saline (PBS) solutions

PBS powder (Sigma–Aldrich Corp) containing 0.2 g monobasic potassium phosphate, 0.2 g potassium chloride, 8 g sodum chloride and 1.15 g dibasic sodium phosphate, was prepared by dissolving in one-liter double distilled water. The pH was adjusted

to 7.4 by 1 M hydrochloric acid (HCl) or 1 M sodium hydroxide (NaOH) and then sterilized by autoclaving at 121°C for 15 min. Another sterilized PBS solution at pH 7.4 was purchased from Gibco™ Invitrogen Corporation, Carlsbad, CA, USA. The solutions were kept at 4°C.

2.1.1.6 Wash buffer

Sterilized Phosphate buffered saline (PBS, Sigma–Aldrich Corp) was supplemented with HI-FCS (2 %), and this solution was served as washing buffer in MACS. It was kept at 4 °C.

2.1.2 Primary cells and cell lines

2.1.2.1 KU812

The KU812 cell line was established from the peripheral blood of a patient in blast crisis of chronic myelogenous leukemia. The cells contained at least one Ph1 (Philadelphia) chromosome. The cell line had some characteristics of basophilic leukocytes (Fc receptors, basophilic granules, histamine production), and are negative for lymphoid markers. It was purchased from the American Type Culture Collection (ATCC), USA.

2.1.2.2 BEAS-2B

BEAS-2B are adenovirus 12-SV40 virus hybrid (Ad12SV40) transformed epithelial

cells, which were isolated from normal human bronchial epithelium obtained from autopsy of non-cancerous individuals. The cells stained positively for keratins and SV40 T antigen, and they retained the ability to undergo squamous differentiation in response to serum. It was purchased from the American Type Culture Collection (ATCC), USA.

2.1.2.3 Primary human bronchial epithelial cells (PBEC)

PBEC isolated from human lung tissue were purchased from ScienCell Research Laboratories, Carlsbad, USA. PBEC were cryopreserved at primary culture and delivered frozen. Each vial contains >5 x 10^5 cells in 1 ml volume. PBEC were characterized by immunofluorescent method with antibodies CK-18, -19, and vimentin. PBEC were negative for HIV-1, HBV, HCV, mycoplasma, bacteria, yeast and fungi. PBEC were guaranteed to further culture at the conditions provided by ScienCell Research Laboratories, USA.

2.1.2.4 Culture medium

Rosewell Park Memorial Institute (PPMI) 1640 medium and Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM/F12) medium containing 15 mM N-2-hydroxy-ethyl-piperazine-N'-2-ethene-sulfonic acid (HEPES), 2 mM L-glutamine and pyridoxine HCL at pH7.2 were purchased from Gibco^{TM} Invitrogen Corp, CA, USA. PBEC medium containing 0.5 ng/mL human recombinant epidermal growth factor, 52 μg/mL bovine pituitary extract, 0.1 ng/mL retinoic acid, 0.5 μg/mL hydrocortisone, 5 μg/mL insulin, 10 μg/mL transferrin, 0.5 μg/mL epinephrine, 6.5

ng/mL triiodothyronine, 50 μg/mL gentamicin, and 50 pg/mL amphotericin-B was purchased from ScienCell Research Laboratories, USA. It was HEPES and bicarbonate buffered and had a pH of 7.4 when equilibrated in an incubator with an atmosphere of 5% CO2/95% air. This serum-free medium was formulated (quantitatively and qualitatively) to provide a defined and optimally balanced nutritional environment that selectively promotes proliferation and growth of primary human bronchial epithelial cells *in vitro*. No antibiotic was added and all the media were free of detectable LPS (< 0.1 EU/ml) as determined by the *Limulus amoebocyte* lysate assay (sensitivity limit 12 pg/ml; Associates of Cape Cod, Woods Hole, MA, USA). All other solutions used in culture were prepared using pyrogen-free water and sterile polypropylene plasticware without any contained detectable LPS.

2.1.2.5 Serum supplements

Fetal Calf Serum (FCS) was purchased from Gibco^{TM} Invitrogen Corporation, Carlsbad, CA, USA. It was of low endotoxin (<10 EU/ml) and hemoglobin (<10 mg/ml) level by test. Heat-Inactivated Fetal Calf Serum (HI-FCS) was prepared as 50 ml aliquots by heating at 56°C for 30 minutes and stored at -20°C until use.

2.1.2.6 Paraformaldehyde (1 %) in PBS for cell fixation

Paraformaldehyde powder (10 g) purchased from Sigma-Aldrich Corp was dissolved

in PBS (1 L). It was titrated to pH 7.4 and kept at 4 °C until use.

2.1.2.7 Trypan blue solution

Trypan blue solution (Sigma-Aldrich Corp) contains 0.4% (w/v) trypan blue dissolved in 0.817% sodium chloride and 0.06% dibasic potassium phosphate.

2.1.2.8 Giemsa staining solutions

Giemsa staining solutions (Sigma-Aldrich Corp) were used to stain cells after cytocentrifugation. Giemsa stain is a mixture of methylene blue and eosin. It is a member of the Romanowski group of stains, which are defined as being the black precipitate formed from the addition of aqueous solutions of methylene blue and eosin, dissolved in methanol. All staining solutions were light-protected and stored at room temperature.

2.1.2.9 TLR ligands

Ultra-purified LPS from *E.coli* K12 strain without any contamination by lipoprotein, R837 (Imiqumod, a synthetic antiviral molecule) for TLR4 and TLR7 were purchased from invivoGen Corp, CA, USA. Double-stranded polyinosinic-polycytidylic acid [poly (I:C)] for TLR3 was purchased from Sigma, and peptidoglycan for TLR2 from Fluka Chemie AG, Geneva, Switzerland.

2.1.3 Recombinant human cytokines

2.1.3.1 Recombinant human IL-17A and IL-17F

Recombinant human IL-17A and IL-17F were purchased from R&D Systems Inc. Minneapolis, MN, USA. They were lyophilized recombinant proteins derived from *E. coli*, and the activity of them had been measured by its ability to induce IL-6 secretion by NIH/3T3 mouse embryonic fibroblasts. Both of IL-17A and IL-17F had a purity >97% by SDS-PAGE under reducing conditions and visualized by silver stain, and the endotoxin level of them was <1.0 EU per 1 μg of the protein by the LAL method. Stock solutions of 50 μg/ml were prepared in sterilized PBS, supplemented with 0.5% BSA, and stored as 50 μl aliquots at -80℃ until use.

2.1.3.2 Recombinant human IL-12

Recombinant human IL-12 was purchased from R&D Systems. It was lyophilized recombinant protein derived from *Spodoptera frugiperda*, Sf 21 (baculovirus). Its activity had been measured in a cell proliferation assay using PHA-stimulated human T lymphoblasts. It had a purity >97% by SD-SPAGE under reducing conditions and visualized by silver stain, and the endotoxin level of it was <1.0 EU per 1 μg of the protein by the LAL method. Stock solutions of 50 μg/ml were prepared in sterilized PBS, supplemented with 0.5% BSA, and stored as 50 μl aliquots at -80℃ until use.

2.1.3.3 Recombinant human IL-23

Recombinant human IL-23 was purchased from R&D Systems. It was lyophilized

recombinant protein derived from *Spodoptera frugiperda*, Sf 21 (baculovirus). Its activity had been measured by its ability to induce IL-17 secretion by mouse splenocytes. It had a purity >97% by SDS-PAGE under reducing conditions and visualized by silver stain, and the endotoxin level of it was <1.0 EU per 1 μg of the protein by the LAL method. Stock solutions of 100 μg/ml were prepared in sterilized PBS, supplemented with 0.5% BSA, and stored as 50 μl aliquots at -80℃ until use.

2.1.3.4 Recombinant human IL-27

Recombinant human IL-27 was purchased from R&D Systems. It was lyophilized recombinant protein expressed in a mouse myeloma cell line, NSO. Its activity had been measured in a cell proliferation assay using mouse $CD4^+$ T cells in the presence of anti-CD3 and anti-CD28. It had a purity >90% by SDS-PAGE under reducing conditions and visualized by silver stain, and the endotoxin level of it was <1.0 EU per 1 μg of the protein by the LAL method. Stock solutions of 100 μg/ml were prepared in sterilized PBS, supplemented with 0.5% BSA, and stored as 50 μl aliquots at -80℃ until use.

2.1.3.5 Recombinant human IFN-γ

Recombinant human IFN-γ was purchased from R&D Systems. It was lyophilized recombinant protein derived from *E. coli*. Its activity had been measured in antiviral

assays using HeLa human cervical epithelial carcinoma cells infected with encephalomyocarditis (EMC) virus. It had a purity >97% by SDS-PAGE under reducing conditions and visualized by silver stain, and the endotoxin level of it was <1.0 EU per 1 μg of the protein by the LAL method. Stock solutions of 50μg/ml were prepared in sterilized PBS, supplemented with 0.5% BSA, and stored as 50 μl aliquots at -80℃ until use.

2.1.3.6 Recombinant human TNF-α

Recombinant human TNF-α was purchased from R&D Systems. It was lyophilized recombinant protein derived from *E. coli*. Its activity had been measured a cytotoxicity assay using L929 mouse fibrosarcoma cells in the presence of the metabolic inhibitor actinomycin D. It had purity >97% by SDS-PAGE under reducing conditions and visualized by silver stain, and the endotoxin level of it was <1.0 EU per 1 μg of the protein by the LAL method. Stock solutions of 100 μg/ml were prepared in sterilized PBS, supplemented with 0.5% BSA, and stored as 50 μl aliquots at -80℃ until use.

2.1.4 Reagents for protein array

2.1.4.1 Protein array kits

Antibody-based RayBio human cytokine array V kits were purchased from

RayBiotect Inc, Norcross, GA, USA, which could rapidly and accurately identify the expression profiles of 79 different cytokines at relatively inexpensive cost. One kit contained 8 Array Membranes, 500X Detection Antibodies (20ul), 20,000X HRP-conjugated streptavidin (10ul), 2X Blocking Buffer (25 ml), 20X Wash Buffer I (20 ml), 20X Wash Buffer II (20 ml), Detection Buffer C (5 ml) , Detection Buffer D (5 ml) and Eight-well Tray (1 each).

2.1.4.2 Enhanced chemiluminescent (ECL) system

ECL detection kit was purchased from Amersham Biosciences, USA. The kit contained a detection reagent 1(62.5 ml), detection reagent 2 (62.5 ml), anti-mouse Ig secondary antibody (horseradish peroxidase-linked whole antibody from sheep, 100μl), anti-rabbit Ig secondary antibody (horseradish peroxidase-linked whole antibody from donkey, 100μl), and 5 g blocking reagent. The kit was used to detect either mouse or rabbit membrane bound primary antibodies and stored at 4 °C.

2.1.4.3 ECL films (Hyperfilm™ ECL™)

The Hyperfilm™ ECL™ was a high performance chemiluminescence film used for protein array analysis. It was purchased from Amersham Biosciencs, USA.

2.1.5 Reagents and buffer solutions for flow cytometry

2.1.5.1 FACSFlow sheath fluid

The sheath fluid is a ready-to-use product purchased from BD Pharmingen Corp, San Diego, CA, USA. It is a balanced electrolyte solution containing sodium chloride, potassium chloride, disodium EDTA, sodium fluoride and anti-microbial agent. The solution was kept at room temperature.

2.1.5.2 FACS staining medium

The buffer contained 0.5% BSA and 0.01% sodium azide (NaN_3) in 1X PBS. It was stored at 4°C and used for washing in immunofluorescent staining.

2.1.5.3 BD Cytofix™ fixation buffer

BD Cytofix fixation buffer used to fix unstained cells for subsequent immunofluorescent staining of intracellular molecules was purchased from BD Biosciences. It contained Dulbecco's Phosphate-Buffered Saline (DPBS) pH 7.4 with 4% w/v paraformaldehyde (0.22 μm pore-filtered), which was stored at 4°C and protected from light.

2.1.5.4 BD Perm buffer III

BD Perm Buffer III used for the permeabilization of fixed cells was purchased from BD Biosciences. It was stored undiluted at room temperature.

2.1.5. 5 Monoclonal antibodies for immunofluorescent staining

2.1.5.5.1 FITC-conjugated mouse IgG_1 anti-human ICAM-1 (CD54) monoclonal antibody

The antibody (clone BBIG-I1), at a concentration of 100 μg/ml, was purchased from R&D Systems. It was used in direct immunofluorescent staining assay with 4 μl per 5 x 10^5 cells. It was stored at 4°C and protected from prolonged exposure to light.

2.1.5.5.2 FITC-conjugated mouse IgG_{2b} anti-human ICAM-3 (CD50) monoclonal antibody

The antibody (clone TU41) from BD Biosciences was buffered in PBS (pH 7.4) with 0.09 % (w/v) NaN_3 and 0.2 % (w/v) bovine serum albumin (BSA). It was used in direct immunofluorescent staining assay with 4 μl per 5 x 10^5 cells. It was stored at 4 °C and protected from prolonged exposure to light.

2.1.5.5.3 FITC-conjugated mouse IgG_1 anti-human CD18 monoclonal antibody

The antibody (clone 6.7) from BD Biosciences was kept in buffered solution containing BSA and 0.09 % (w/v) NaN_3. It was used in direct immunofluorescent staining assay with 4 μl per 5 x 10^5 cells. It was stored at 4°C and protected from prolonged exposure to light.

2.1.5.5.4 FITC-conjugated mouse IgG_1 anti-human CD62L monoclonal antibody

The antibody (clone Dreg 56) from BD Biosciences was kept in buffered solution containing BSA and 0.09 % (w/v) NaN_3. It was used in direct immunofluorescent staining assay with 4 μl per 5 x 10^5 cells. It was stored at 4°C and protected from prolonged exposure to light.

2.1.5.5.5 FITC-conjugated mouse IgG_1 monoclonal immunoglobulin isotype control

The antibody (clone MOPC-21) purchased from BD Biosciences, was buffered in PBS (pH 7.4) with 0.09 % (w/v) NaN_3 and 0.2 % (w/v) BSA. It was used as an isotypic control in direct immunofluorescent staining assay with 4 μl per 5 x 10^5 cells. It was stored at 4°C and protected from prolonged exposure to light.

2.1.5.5.6 FITC-conjugated mouse IgG_{2b} monoclonal immunoglobulin isotype control

The antibody (clone 27-35) purchased from BD Biosciences, was buffered in PBS (pH 7.4) with 0.09 % (w/v) NaN_3 and 0.2 % (w/v) BSA. It was used as an isotypic control in direct immunofluorescent staining assay with 4 μl per 5 x 10^5 cells. It was stored at 4°C and protected from prolonged exposure to light.

2.1.5.5.7 FITC-conjugated goat anti-mouse IgG antibody

Alexa Fluor® 594 F(ab')2 fragment of goat anti-mouse IgG (H+L) purchased from Invitrogen, was buffered in 10 mM PBS (pH 7.4) containing 1% BSA, 40 % glycerol and 0.05 % NaN3. It was used as a secondary antibody for indirect immunofluorescent staining assay at a dilution of 1:250 in flow cytometry analysis. It was stored at 4°C and protected from prolonged exposure to light.

2.1.5.5.8 Mouse anti-human TCCR/WSX-1 monoclonal antibody

This antibody (clone 191106) purchased from R&D Systems, was produced from a hybridoma resulting from the fusion of a mouse myeloma with B cells obtained from a mouse immunized with purified, NS0-derived, recombinant human T cell cytokine receptor (rhTCCR) extracellular domain. The IgG fraction of the tissue culture supernatant was purified by Protein G affinity chromatography. It was used in indirect immunofluorescent staining assay with 10 μl per 5 x 10^5 cells. It was stored at -20°C.

2.1.5.5.9 Mouse anti-human gp130 monoclonal antibody

This antibody (clone 28126) purchased from R&D Systems, was produced by immunizing a mouse with purified, insect cell line Sf 21-derived, recombinant

human gp130 (rhgp130) extracellular domain. It was used in indirect immunofluorescent staining assay with 10 μl per 5 x 10^5 cells. It was stored at -20°C.

2.1.5.5.10 Mouse anti-human p55TNFR monoclonal antibody

This antibody (clone 16803) purchased from R&D Systems, was produced by immunizing a mouse with purified, *E. coli*-derived, recombinant human tumor necrosis factor receptor 1 (rhTNF RI; Accession # NP_001056) extracellular domain. It was used in indirect immunofluorescent staining assay with 10 μl per 5 x 10^5 cells. It was stored at -20°C.

2.1.5.5.11 Mouse anti-human p75TNFR monoclonal antibody

This antibody (clone 22235) purchased from R&D Systems, was produced by immunizing a mouse with purified, *E. coli*-derived, recombinant human tumor necrosis factor receptor II (rhTNF RII) extracellular domain. It was used in indirect immunofluorescent staining assay with 10 μl per 5 x 10^5 cells. It was stored at -20°C.

2.1.5.5.12 Mouse anti-human IL-17 RA monoclonal antibody

The antibody (clone 133617) purchased from R&D Systems, was produced by immunizing a mouse with purified, NS0-derived, recombinant human IL-17 R

extracellular domain (rhIL-17 R; aa 33-320; Accession # Q96F46). It was used in indirect immunofluorescent staining assay with 10 μl per 5 x 10^5 cells. It was stored at -20°C.

2.1.5.5.13 Mouse anti-human IL-17 RC monoclonal antibody

This antibody (clone 309822) purchased from R&D Systems, was produced by immunizing with a mouse with purified, NS0-derived, recombinant human IL-17 RC (rhIL-17 RC; aa 21-454; Accession #AAH06411). It was used in indirect immunofluorescent staining assay with 10 μl per 5 x 10^5 cells. It was stored at -20°C.

2.1.5.5.14 Mouse anti-human IL-17RA adaptor protein Act1 monoclonal antibody

This antibody (WW-18) purchased from Santa Cruz Biotechnology, CA. USA, was produced by immunizing a mouse with recombinant Act1 of human origin. It was used in indirect immunofluorescent staining with 10 μl per 5 x 10^5 cells. It was used in indirect immunofluorescent staining assay with 10 μl per 5 x 10^5 cells. It was stored at 4°C.

2.1.5.5.15 Mouse anti-JNK/SAPK (pT183/pY185) monoclonal antibody

This antibody clone 41/JNK/SAPK (pT183/pY185) purchased from BD Biosciences, was produced by immunizing a mouse with phosphorylated human JNK/SAPK (pT183/pY185) Peptide. It was used in indirect immunofluorescent staining assay with 10 µl per 5 x 10^5 cells. It was stored at -20°C.

2.1.5. 5.16 Mouse anti-ERK1/2 (pT202/pY204) monoclonal antibody

This antibody (clone 20A) purchased from BD Biosciences, was produced by immunizing a mouse with phosphorylated rat ERK1 (T202/Y204) peptide. The 20A monoclonal antibody recognizes the phosphorylated threonine 202 and tyrosine 204 (pT202/pY204) of human ERK1 and pT184/pY186 of human ERK2. It was used in indirect immunofluorescent staining assay with 10 µl per 5 x 10^5 cells. It was stored at -20°C.

2.1.5.5.17 Mouse anti-p38 MAPK (pT180/pY182) monoclonal antibody

This antibody clone 30/p38 MAPK (pT180/pY182) purchased from BD Biosciences, was produced by immunizing a mouse with human p38 MAPK (pT180/pY182). It was used in indirect immunofluorescent staining assay with 10 µl per 5 x 10^5 cells. It was stored at -20°C

2.1.5.5.18 Mouse anti-Human IKBα (pS32/pS36) monoclonal antibody

This antibody (clone 39A1413) purchased from BD Biosciences, was produced by immunizing a mouse with a synthetic peptide containing phosphorylated serines at amino acid residues of 32 and 36 of human IκBα. It was used in indirect immunofluorescent staining assay with 10 μl per 5 x 10^5 cells. It was stored at 4°C.

2.1.5.5.19 Mouse anti-Akt (pS472/pS473) monoclonal antibody

This antibody (clone 104A282) purchased from BD Biosciences, was produced by immunizing a mouse with phosphorylated human Akt1 (pS473) peptide. The 104A282 antibody recognizes Akt1 phosphorylated at S473 and Akt3 phosphorylated at S472. It was used in indirect immunofluorescent staining assay with 10 μl per 5 x 10^5 cells. It was stored at 4°C.

2.1.5.5.20 Mouse anti-Stat1 (pY701) monoclonal antibody

This antibody (clone 14/P-STAT1) purchased from BD Biosciences, was produced by immunizing a mouse with phosphorylated human Stat1 (pY701) peptide. The 14/P-STAT1monoclonal antibody recognizes the phosphorylated Y701 in Stat1α and Stat1β. It was used in indirect immunofluorescent staining assay with 10 μl per 5 x 10^5 cells. It was stored at -20°C.

2.1.5.5.21 Mouse IgG_1 isotype control

The mouse IgG_1 isotype control purchased from BD Biosciences, was reconstituted with buffer contained 0.5% BSA and 0.01% sodium azide (NaN_3) in 1X PBS. It was used as an isotype control in indirect immunofluorescent staining assay at a concentration of 50 μg/ml and added in 10 μl to each sample tube in flow cytometry.

2.1.5.5.22 Human serum

Human serum was obtained from venous blood of Chinese healthy volunteers, and it was used for blocking procedure in immunofluorescent staining assay. It was stored at -20°C.

2.1.6 Enzyme linked immunosorbent assay (ELISA) kits

ELISA kits for determining the concentrations of IL-6, CXCL1, CXCL8, CCL2, G-CSF and TGF-β were purchased from BD Biosciences. They were stored at 4°C and protected from prolonged exposure to light.

2.1.7 Cytokine cytometric bead array (CBA) kits

CBA kits for measurement of IL-10, TNF-α, CCL5 and CXCL9/interferon-γ-inducible protein-10 (IP-10) were purchased from BD Pharmingen Corp, San Diego, CA, USA. The kits contained cytometer setup beads,

PE positive control detector, FITC positive control detector for compensation settings. They also contained five populations of capture beads, PE conjugated detection reagents, standards and wash buffer. They are stores at 4°C.

2.1.8 Limulus Amoebocyte Lysate (LAL) kit

LAL Kits purchased from Cambrex Bio Science Biowhittaker Inc, MD, USA (sensitivity limit 12 pg/ml), was used to determine LPS in all culture solutions. The kit contained the LAL prepared from circulating amebocytes of the horseshoe crab *Limulus polyphemus, E.coli* endotoxin and chromogenic substrate.

2.1.9 Signaling transduction inhibitors

All the signaling molecule inhibitors were purchased from Calbiochem, San Diego, CA, USA.U0126 and SP600125 are selective inhibitors of ERK pathway and JNK pathway respectively. SB203580 is the selective inhibitor of p38MAPK pathway. LY294002 is the selective inhibitor of PI3K pathway. BAY117082 is the inhibitor of NF-κB pathway. AG490 is the inhibitor of JAK-STAT pathway. SB203580 was dissolved in endotoxin-free water, while other inhibitors were dissolved in dimethy sulfoxide (DMSO). Aliquots of inhibitors were stored at -80 °C until use. In all studies, the concentration of DMSO was 0.1 % (vol/vol).

2.1.10 Protein synthesis inhibitors

Actinomycin D and cycloheximide are RNA transcription inhibitor and mRNA translation inhibitor respectively, and they were purchased from SigmaSigma–Aldrich Corp, St. Louis, MO, USA. They were dissolved in DMSO and stored at -80°C until use.

2.1.11 Micro BCA protein assay kit

The kit was purchased from Pierce Chemical Co., IL, USA and used to determine the concentration of proteins. It was kept at 4°C.

2.2 Methods

2.2.1 Purification of human basophils and culture of baophils

2.2.1.1 Purification of human basophils

Fresh human buffy coat obtained from the non-atopic healthy volunteers of Hong Kong Red Cross Blood Transfusion Service was diluted 1:2 with PBS at 4 ℃ and centrifuged using an isotonic Ficoll solution (density 1.077 g/ml; Amersham and Pharmacia Biotech) for 30 min at 1,000 x g. The PBMC fraction was collected and washed twice with cold PBS containing 2 % FBS (Invitrogen Corp.). Basophils were then purified from the PBMC fraction using basophil isolation kit (Miltenyi Biotec.) with by magnetic depletion of non-basophils by passing through a LS+ column (Miltenyi Biotec) within a magnetic field. With this preparation, the drop-through

fraction contained purified basophils with a purity of at least 95 % as assessed by Giemsa staining solution (Sigma-Aldrich Corp), and the basophils had a viability of 98% as assessed by trypan blue exclusion assay. The isolated basophils were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10 % FBS and 20 mM Hepes (Gibco).

2.2.1.2 Study of human basophils morphology

Cytocentrifuge preparation was commonly used to examine the morphological characteristics of human basophils after purification. Basophils (5 x 10^4) were centrifuged at 300 rpm for 3 minutes onto a microscopic slide by the Shandon Cytospin 3 Centrifuge (Shandon Scientific Ltd.). The cells were air-dried, stained with Giemsa staining solutions (Sigma-Aldrich Corp), and visualized using Nikon Eclipse E800 microscope (Nikon Corp., Tokyo, Japan).

2.2.1.3 Culture of basophils

The isolated basophils were cultured in RPMI 1640 medium (Gibco) supplemented with 10 % FBS and 20 mM Hepes (Invitrogen).

2.2.2 Culture of BEAS-2B cells

BEAS-2B cells were grown in D-MEM/F12, with L-glutamine, 15 mM HEPES and pyridoxine HCl, supplemented with 10 % HI FCS (Gibco) in 6-well cell culture plates. The cells were incubated at 37°C in a 95% humidified incubator supplied with

5% CO_2 and subcultured on every 2-3 days.

2.2.3 Culture of primary human bronchial epithelial cells

Primary human bronchial epithelial cells purchased from ScienCell Research Laboratories, CA, USA, were seeded in plastic T-75 flasks (Costar) and grown in serum-free bronchial epithelial cell medium containing 0.5 ng/mL human recombinant epidermal growth factor, 52 μg/mL bovine pituitary extract, 0.1 ng/mL retinoic acid, 0.5 μg/mL hydrocortisone, 5 μg/mL insulin, 10 μg/mL transferrin, 0.5 μg/mL epinephrine, 6.5 ng/mL triiodothyronine, 50 μg/mL gentamicin, and 50 pg/mL amphotericin-B (ScienCell) at 37°C in a humidified 5% CO2 atmosphere. Media were changed every 48 h until PBEC were 90% confluent.

2.2.4 Culture of KU812 cells

The human basophilic leukemia cell line, KU812 cells, was from ATCC, USA. KU812 cells were maintained in RPMI1640 medium (Invitrogen) with 10 % FBS. The cells were incubated at 37°C in a 95% humidified incubator supplied with 5% CO_2 and subcultured on every 3-4 days.

2.2.5 Co-culture of primary human bronchial epithelial Cells / BEAS-2B cells and human peripheral blood basophils / KU812 cells

Primary human bronchial epithelial cells / BEAS-2B cells were cultured in a 24-well culture plate until confluence. The medium of primary bronchial epithelial cells/BEAS-2B cells was replaced with RPMI 1640 medium containing 10 % FBS

(Invitrogen) with or without basophils/KU812 cells. For inhibition experiments, BEAS-2B and KU812 cells were pretreated with signaling molecule inhibitors for 1 h. The cells were then stimulated with different cytokines or activators for 24h before harvesting.

2.2.6 Co-culture of fixed primary human bronchial epithelial cells/BEAS-2B cells and human peripheral blood basophils/KU812 cells

Confluent primary human bronchial epithelial cells/BEAS-2B cells or basophils/KU821 cells were treated with 1% paraformaldehyde in PBS on ice for 1 h to prevent the release of mediators from cells while preserving the cell membrane integrity to maintain intercellular interaction. After fixation, cells were washed at least 10 times with PBS containing 2% FBS, and fixed or unfixed primary bronchial epithelial cells/BEAS-2B cells or basophils/KU812 cells were co-cultured in RPMI 1640 medium supplemented with 10 % FBS.

2.2.7 Co-culture of primary human bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells in the presence of transwell inserts

To prevent direct interaction between primary bronchial epithelial cells/BEAS-2B cells and basophils/KU821 cells in the co-culture, transwell inserts (pore size: 0.4

μM) (BD Biosciences Corp) were used to separate the cells into two compartments. Confluent primary bronchial epithelial cells/BEAS-2B cells and basophils/KU821 cells were cultured together in the presence of transwell inserts, in which basophils/KU821 cells were placed in the upper compartment and primary bronchial epithelial cells/BEAS-2B cells were in the lower one.

2.2.8 Detection of cell viability

Cell viability was detected by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Primary human bronchial epithelial cells/BEAS-2B or KU812 cells (2×10^4 cells/0.2 ml) were inoculated into a 96-well plate. Various inhibitors at serial concentrations were added to the cells. After 48 h incubation, MTT (50 μg, Sigma-Aldrich Corp) was added to each well and incubated for 2 h. Viable cells took up MTT and reduced it into dark blue, water-insoluble formazan by mitochondrial dehydrogenase, which reflected the normal function of mitochondria and cell viability. The cells were then lysed with dimethyl sulfoxide (DMSO; 200 μl) to yield the color solution. The absorbance at an optical density of 550 nm was measured to quantify the viable cells.

2.2.9 Protein array analysis of cytokines release

Culture supernatants collected after 24 h treatment at 37 ℃. The expression profiles of 79 different cytokines were assessed semiquantitatively using antibody-based RayBio human cytokine array V (RayBiotect Inc, Norcross, GA, USA). The cytokine map of array was listed in Appendix.

2.2.10 ELISA

Concentrations of human IL-6, CXCL1, CXCL8, CCL2, G-CSF and TGF-β in the culture supernatants after treatments were measured using the corresponding ELISA kit (BD Biosciences). Culture supernatants (100 μl) were incubated with capture antibodies on the plates and detection conjugate-antibodies (100 μl). The mixture formed a sandwich immune complex after incubation. After washing, the enzymatic products were measured at 450 nm corrected by the reading at 540 nm using VICTOR^3 Multilabel Counter model 1420-032 (Perkin Elmer, USA).

2.2.11 CBA assay

The concentrations of IL-10, TNF-α, CCL5 and CXCL9/interferon-γ-inducible protein-10 (IP-10) in the culture supernatants were determined by CBA kits (BD Biosciences) using flow cytometer (FACSCalibur flow cytometer, BD Biosciences, CA, USA). Culture supernatants (50 μl) were incubated with different capture beads

mixtures (50 μl) and PE-conjugated detection antibodies (50 μl). The mixture formed a sandwich immune complex after incubation of 3 h, the capture beads were washed and re-suspended for sample data acquisition using Cell Quest (FACSCalibur). The sample results were finally generated in graphical format using the BD CBA analysis software.

2.2.12 Flow cytometric analysis of cell-surface expression of target molecules

Primary human bronchial epithelial cells/BEAS-2B cells or basophils/KU812 cells were harvested and resuspended in cold PBS. After blocking with 2% human pooled serum at room temperature for 15 min, followed by washing with PBS. In direct immunofluorescent staining, the cells were incubated either with FITC-conjugated mouse anti-human monoclonal antibodies or FITC-conjugated mouse IgG_1 and IgG_{2b} isotype for 45 min at 4℃. In indirect immunofluorescent staining, the cells were first incubated with either purified mouse anti-human monoclonal antibodies or mouse IgG_1 isotype for 1 h at 4℃, followed by incubating with a FITC-conjugated anti-mouse secondary antibody (1: 250) for further 45 min at 4 ℃. After final washing, the cells were then resuspended in 1% paraformaldehyde in 1 x PBS as fixative. Expression of cell surface of target molecules on 5,000 viable cells was then

quantitatively analyzed by flow cytometry (FACSCalibur flow cytometer, BD Biosciences) in terms of mean fluorescence intensity (MFI).

2.2.13 Flow cytometric analysis of activated intracellular target molecules

The intracellular expression of target molecules was determined using established intracellular staining assay. Briefly, cells were fixed with pre-warmed BD Cytofix Buffer (4 % paraformaldehyde) for 10 min at 37°C after treatment. After centrifugation, cells were permeabilized in BD Perm Buffer III for 30 min and then stained with mouse anti-human monoclonal antibodies or corresponding mouse IgG1 isotype (BD Pharmingen) for 60 min followed by FITC conjugated goat anti-mouse IgG1 secondary antibody for another 45 min at 4°C in dark. Cells were then washed, resuspended and subjected to analysis. Expression of intracellular phosphorylated signaling molecules of 5,000 viable cells was analyzed by flow cytometry (FACSCalibur, BD Biosciences) as MFI.

2.2.14 Endotoxin level assay

The endotoxin level in the recombinant human cytokines was determined by LAL test. Recombinant human cytokines in serial concentrations was added to 96-well plate and mixed with the LAL for 10 min at 37°C. The substrate solution was then

mixed with the LAL-sample and incubated at 37°C for additional 6 min before stopping by stop reagent. In this process, Gram-negative bacterial endotoxin catalyzed the activation of a proenzyme in the LAL. The activated enzyme then catalyzed the splitting of p-nitroaniline (pNA) from the colorless substrate Ac-lle-Glu-Ala-Arg-pNA. The yellow coloured-pNA could be measured photometrically after the reaction was stopped.

2.2.15 Statistical analysis

The statistical significance of differences was determined by one-way ANOVA or Student's t- test. The values are expressed as mean ± SD from three independent experiments. Any difference with p values less than 0.05 was considered significant. When ANOVA indicated a significant difference, Bonferroni post hoc test was then used to assess the difference between groups. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) statistical software for Windows, version 16.0 (SPSS Inc, IL, USA).

Chapter 3

Elevated Induction of IL-6, CCL2 and ICAM-1 upon Interaction between Human Bronchial Epithelial Cells and Basophils Activated by IL-17A

3.1 Introduction

Basophils are the least (<0.5 %) granulocytes in peripheral blood that differentiate from CD34+ hematopoietic progenitors in the bone marrow. They play critical roles in mediating responses to Th2-related parasite infection and allergic inflammation (Min, 2008). A growing number of studies have suggested that basophils could contribute to the development of Th2 immunity by up-regulating the expression of Th2 cytokines in allergic inflammation, such as IL-4, IL-13, IL-25 and TSLP (Min *et al.*, 2004; Schroeder *et al.*, 2001; Wang *et al.*, 2007; Sokol *et al.*, 2008; Min and Paul, 2008). Basophils therefore serve as initiator and accessory cells for the Th2 cell polarization in response to protease allergens and by recruiting other effector cells such as eosinophils or neutrophils (Mukai *et al.*, 2005; Obata *et al.*, 2007). Basophils are rarely found in normal tissues, but their number increases at allergic inflammatory sites in the airways of asthmatic patients or in the skin of patients with atopic dermatitis (Macfarlane *et al.*, 2000; Marone *et al.*, 2005; Irani *et*

al., 1998; Sullivan and Locksley, 2009; Karasuyama *et al.,* 2009). The Number of basophils is increased in the airways of asthmatic when compared with normal subjects, and it is further increased during asthma exacerbations and in response to allergen inhalation challenge. The allergen-induced increased number of basophils in airway sputum exceeds that of mast cells, and basophils increased by approximately 200-fold from baseline values at 7 h after allergen inhalation challenge (Gauvreau *et al.,* 2000). Moreover, basophil number in the airway is correlated with the bronchial responsiveness of asthmatics (Gauvreau *et al.,* 2000). Basophils are one of the main cells that release IL-4 and IL-13 in the peripheral blood of asthmatic patients after allergen activation (Devouassoux *et al.,* 1999). Basophils also produce leukotriene C4 and histamine that can cause the symptoms of acute and chronic allergic inflammation. Recent studies suggested that chronic allergic inflammation required basophils as initiator, but not mast cells or T cells, through the interaction of antigen, IgE, and FcRI (Schroeder *et al.,* 2001; Sokol *et al.,* 2008; Min and Paul, 2008).

Activated bronchial epithelial cells are potent sources of a wide variety of proinflammatory cytokines and chemokines such as IL-6, IL-8 CXCL8, and monocyte chemoattractant protein (MCP-1/CCL2) (Ip *et al.,* 2007). Secretion of inflammatory mediators together with the recruitment and interaction of different immune effector cells such as eosinophils with the bronchial epithelium would

eventually contribute to bronchial inflammation, tissue damage and remodeling of pulmonary structure in asthma (Wong *et al.*, 2005). However, the detailed immunopathological mechanisms of basophils upon the interaction with bronchial epithelium in asthma remain to be determined.

Th17 cells are a new class of effector $CD4^+$ T cells. They are distinct from Th1 and Th2 lineages, and produce hallmark cytokines IL-17A, IL-17F and IL-22 (Sato *et al.*, 2007; Liang *et al.*, 2006). IL-1β, IL-6 and IL-23, but not TGF-β can drive the differentiation and development human IL-17 producing T cells via the activation of interferon-regulatory factor 4 and transcription factor retinoic acid receptor-related orphan receptor (ROR)γt (Acosta-Rodriguez *et al.*, 2007; Wilson *et al.*, 2007; Brustle *et al.*, 2007). Several *in vitro* studies have demonstrated that IL-17A were able to induce the release of IL-6, CXCL-8, GM-CSF and CXCL1 from various cell types including epithelial and vascular endothelial cells, fibroblasts and keratinocytes (Wilson *et al.*, 2007; Kawaguchi *et al.*, 2004). In animal models *in vivo*, IL-17 could recruit and activate neutrophils in the airways via the induction of CXCL8. Besides, plasma concentration of IL-17A was found to be significantly higher in allergic asthmatic patients than that of normal control subjects (Wong *et al.*, 2001; Hashimoto *et al.*, 2005). Apart from the circulation, the increased numbers of cells expressing IL-17A were found in sputum and bronchoalveolar lavage fluids of subjects with

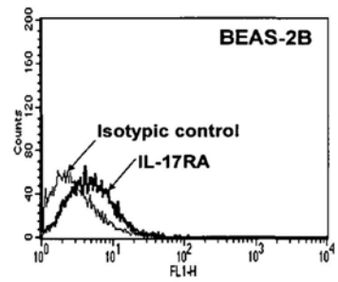
asthma in comparison with control subjects, and the level of IL-17A in eosinophils purified from peripheral blood was significantly higher in asthma subjects than in normal controls (Tesmer *et al.,* 2008; Molet *et al.,* 2001). IL-17A can provoke pulmonary inflammation by facilitating infiltration of inflammatory cells, and amplify the inflammatory responses through the induction of a similar profile of CXC chemokines (Molet *et al.,* 2001; Hizawa *et al.,* 2006).

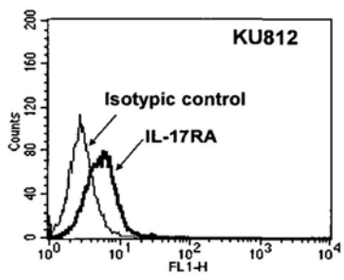
Transcription factor NF-κB and MAPKs were found to be involved in the IL-17A-mediated induction of inflammatory cytokines and chemokines of eosinophils during allergic inflammation (Cheung *et al.,* 2008). We found that NF-κB and p38 MAPK are important for synergistic induction of IL-6, growth factors, chemokines and adhesion molecules in the co-culture of eosinophils and bronchial epithelial cells activated by allergen house dust mite Der p1 protein (Wong *et al.,* 2006; Wang *et al.,* 2005). It has been shown that basophils must engage intercellular contact to exert the inhibitory effect on Th1 differentiation (Oh *et al.,* 2007). In the present study, we hypothesized that Th17 cytokine IL-17A might regulate allergic inflammation by the activation of human bronchial epithelial cells interacting with basophils and investigated the modulation of intracellular NF-κB and MAPK activities on regulating the expression of cytokines, chemokines and adhesion molecules upon the activation of IL-17A.

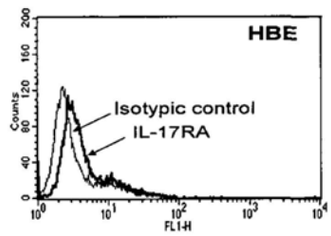
3.2 Results

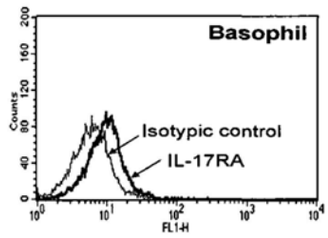
3.2.1 Protein expression of receptors for IL-17A and IL-17RA adaptor protein Act1 on primary bronchial epithelial cells/BEAS-2B cells and peripheral blood basophils/KU812 cells

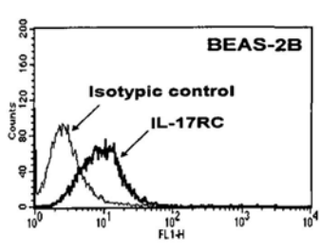
As shown by Figure 3.2.1, both human KU812 cells/basophils and BEAS-2B cells/primary bronchial epitheilial cells constitutively expressed IL-17RA and IL-17RC on their surfaces. Intracellular staining using flow cytometry showed that adaptor protein Act1 was constitutively expressed in KU812 cells/basophils and BEAS-2B cells/primary bronchial epitheilial cells and (Figure 3.2.1).

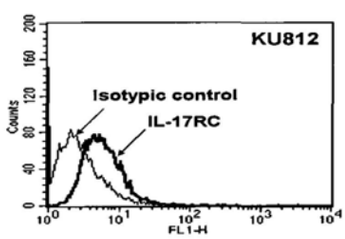

BEAS-2B
Isotypic control
IL-17RA
Counts
FL1-H

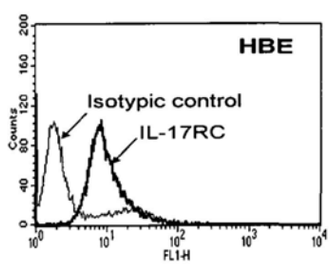

KU812
Isotypic control
IL-17RA
Counts
FL1-H

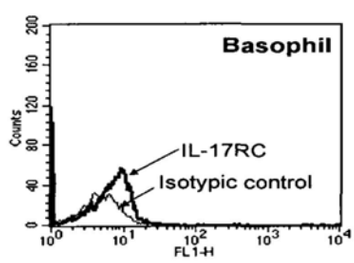

HBE
Isotypic control
IL-17RA
Counts
FL1-H

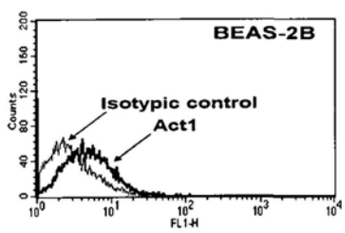

Basophil
Isotypic control
IL-17RA
Counts
FL1-H

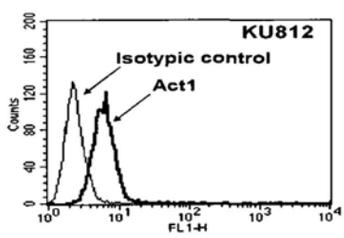

BEAS-2B
Isotypic control
IL-17RC
Counts
FL1-H

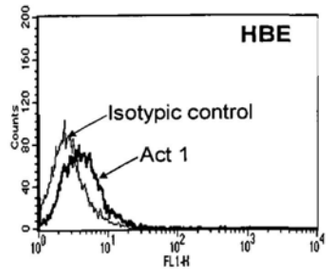

KU812
Isotypic control
IL-17RC
Counts
FL1-H


HBE
Isotypic control
IL-17RC
Counts
FL1-H


Basophil
IL-17RC
Isotypic control
Counts
FL1-H


BEAS-2B
Isotypic control
Act1
Counts
FL1-H


KU812
Isotypic control
Act1
Counts
FL1-H


HBE
Isotypic control
Act 1
Counts
FL1-H

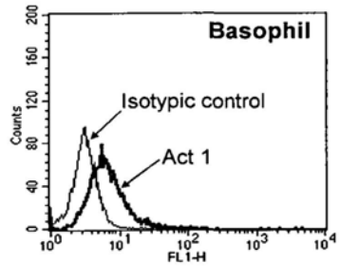

Basophil
Isotypic control
Act 1
Counts
FL1-H

Figure 3.2.1 Protein expression of IL-17RA, IL-17RC and IL-17RA adaptor protein Act1 on primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells. Cell surface expression of IL-17RA, IL-17RC, and intracellular expression of Act1 of BEAS-2B/primary bronchial epithelial cells and KU812 cells/basophils (5 x 10^5 cells) was determined by flow cytometry. The results are shown as mean fluorescence intensity (MFI) histograms. Experiments were performed in three independent replicates with essentially identical results and representative figures are shown.

HBE: human primary bronchial epithelial cells

3.2.2 Cytokine and chemokine release upon the interaction of primary bronchial epithelial cells/ BEAS-2B cells and basophils/ KU812 activated by IL-17A

Figure 3.2.2 shows the cytokine expression profile using antibody based human cytokine protein membrane array. KU812 and BEAS-2B cells were cultured either together or separately with or without IL-17A treatment. IL-17A (50 ng/ml) could promote the release of IL-6 (2h) and CCL2 (3e), CXCL8 (2j) and CXCL1 (1j) from BEAS-2B cells, but no prominent effect was observed on KU812 cells. Upon co-culture, only the levels of IL-6 (2h) and CCL2 (3e) were found to be higher than those of KU812 cells alone or BEAS-2B cells alone, with or without IL-17A activation. However, co-culture did not enhance the release of CXCL8 and CXCL1, with or without IL-17A activation.

Further investigation by ELISA confirmed that co-culture could synergistically induce the release of IL-6 and CCL2 while IL-17A could significantly enhance IL-6 and CCL2 release from both primary bronchial epithelial cells/BEAS-2B cells alone and co-culture with basophils/KU812 cells in a dose-dependent manner (Figure 3.2.3). However, in co-culture of primary cells, much more IL-6 was found to be induced by 17A (Figure 3.2.3C). Furthermore, both transcriptional inhibitor actinomycin D and protein synthesis inhibitor cycloheximide could significantly suppress the release of IL-6 and CCL2 in co-culture of BEAS-2B and KU812 cells with or without IL-17A stimulation (all $p < 0.001$, Figure 3.2.4)

G-CSF was usually produced by bronchial epithelial cells (Kawaguchi *et al.*, 2001; McAllister *et al.*, 2005). Using ELISA, we found that IL-17A (50 ng/ml) could significantly induce higher amount of G-CSF in BEAS-2B cells alone (data not shown, $p < 0.01$). However, BEAS-2B could not further induce G-CSF upon co-culture with KU812 cells, and IL-17A also could not further enhance G-CSF

release in co-culture (data not shown, both $p > 0.05$). Although the semi-quantitative cytokine array appeared to show no expression of G-CSF, ELISA method confirmed the expression of G-CSF from BEAS-2B cells with or without the treatment of IL-17A. We also observed human primary bronchial epithelial cells showed the expression of G-CSF that could be further significantly induced by IL-17A. In view of that, BEAS-2B cells can serve as the bronchial epithelial cell model in this study.

Together, in concordance with previous studies (Kawaguchi *et al.,* 2001; McAllister *et al.,* 2005; Laan *et al.*, 2003), IL-17A could actually induce the release of IL-6, IL-8, CCL2, CXCL1 and G-CSF from bronchial epithelial cells. However, we have observed co-culture with basophilic cells could only significantly enhance the release of CCL2 and IL-6 ($p < 0.05$). Other cytokines such as IL-4, IL-13 and TSLP were not increased in co-culture system. IL-17A could only further augment significantly the release of IL-6 and CCL2 from co-culture ($p < 0.05$).

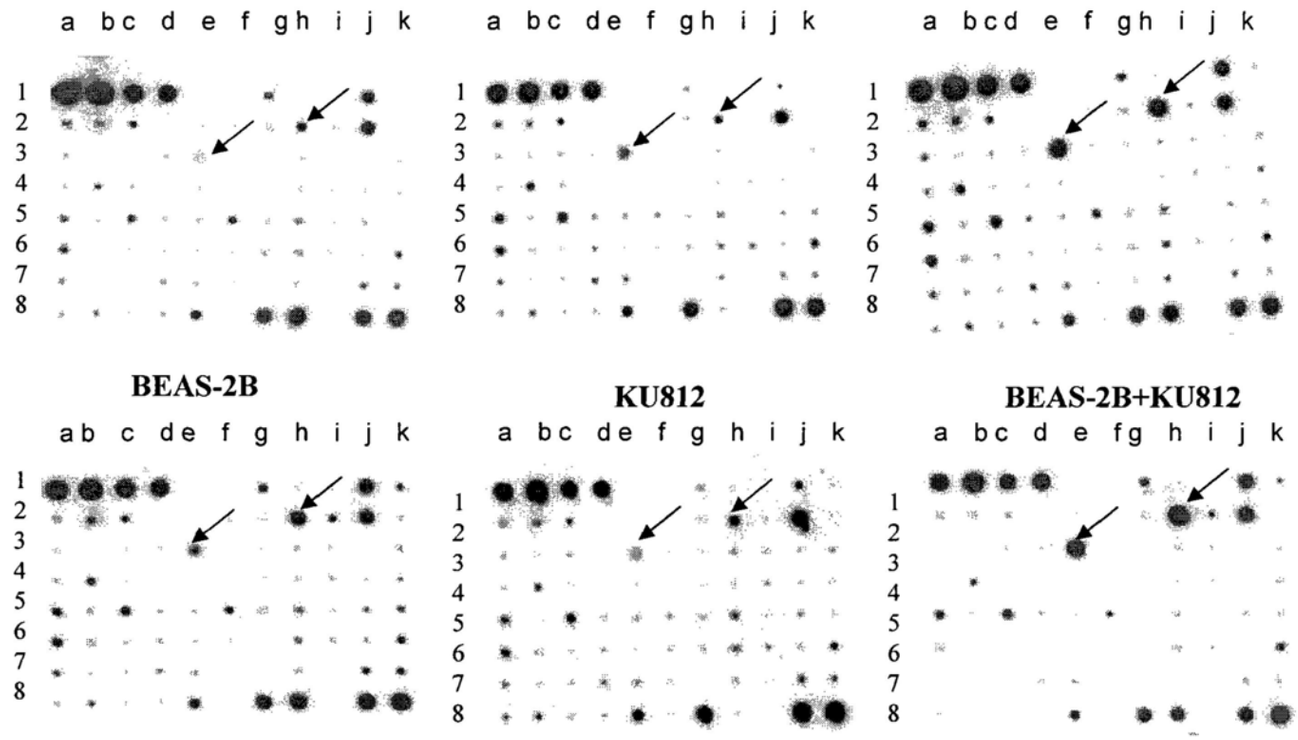
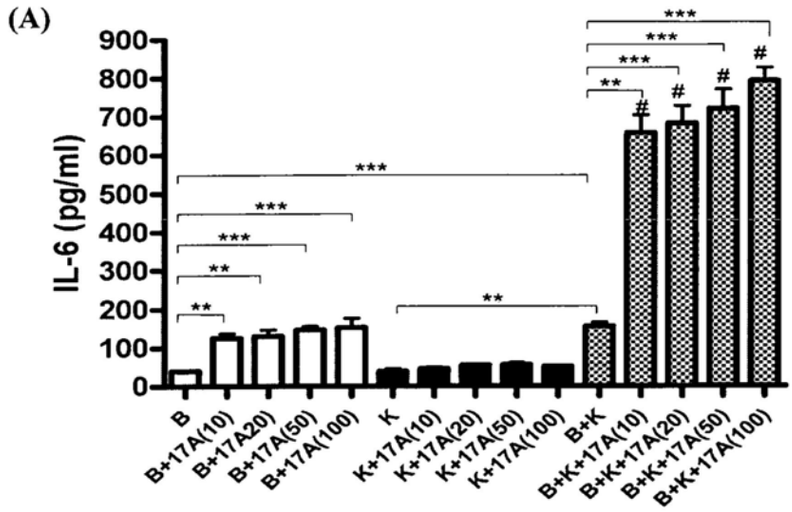
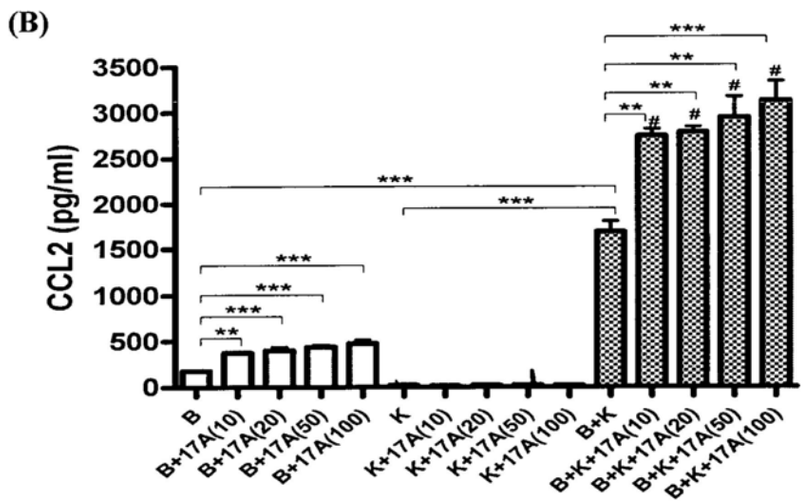


Figure 3.2.2 Representative profile of the release of cytokines from BEAS-2B cells and KU812 cells activated by IL-17A. KU812 cells (1.5 x 10^6 cells) and confluent BEAS-2B cells (3 x 10^5 cells) were cultured either together or separately with or without IL-17A (50 ng/ml) for 24 h. Culture supernatant was then harvested and 79 different cytokines in culture supernatant were semi-quantitated using antibody based RayBio^{TM} human cytokine array V. Positive and negative controls were designated at (1a, 1b, 1c, 1d, 8j, 8k) and (1e, 1f, 8i), respectively. The arrows indicated IL-6 (2h) and CCL2 (3e) released in culture supernatants. Triplicate experiments were performed with essentially identical results and representative figures are shown. Cytokine map of RayBio human cytokine array V is shown in Appendix (Page172-174).


(A)
IL-6 (pg/ml)
900
800
700
600
500
400
300
200
100
0
B
B+17A(10)
B+17A20)
B+17A(50)
B+17A(100)
K
K+17A(10)
K+17A(20)
K+17A(50)
K+17A(100)
B+K
B+K+17A(10)
B+K+17A(20)
B+K+17A(50)
B+K+17A(100)


(B)
CCL2 (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
B
B+17A(10)
B+17A(20)
B+17A(50)
B+17A(100)
K
K+17A(10)
K+17A(20)
K+17A(50)
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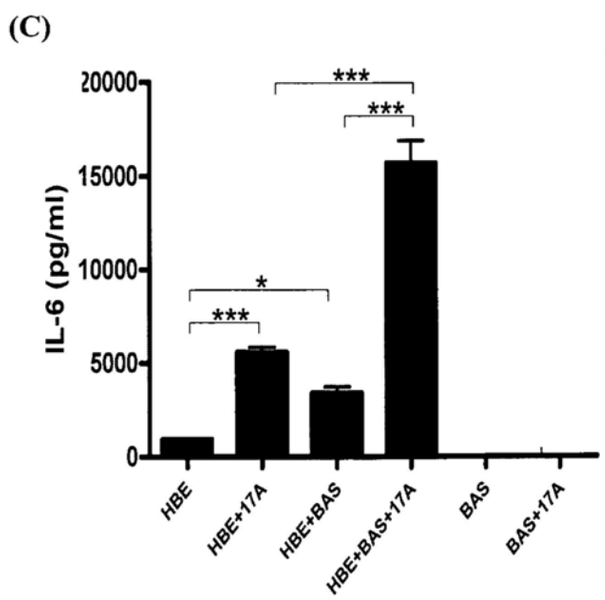
B+K+17A(50)
B+K+17A(100)


(C)
IL-6 (pg/ml)
20000
15000
10000
5000
0
HBE
HBE+17A
HBE+BAS
HBE+BAS+17A
BAS
BAS+17A

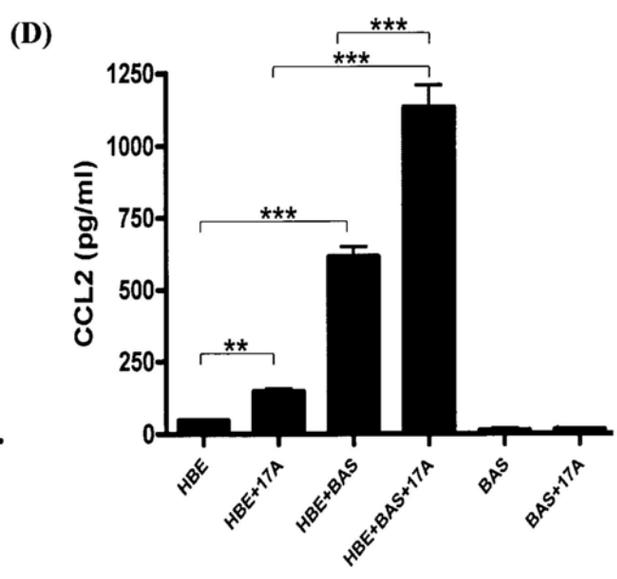
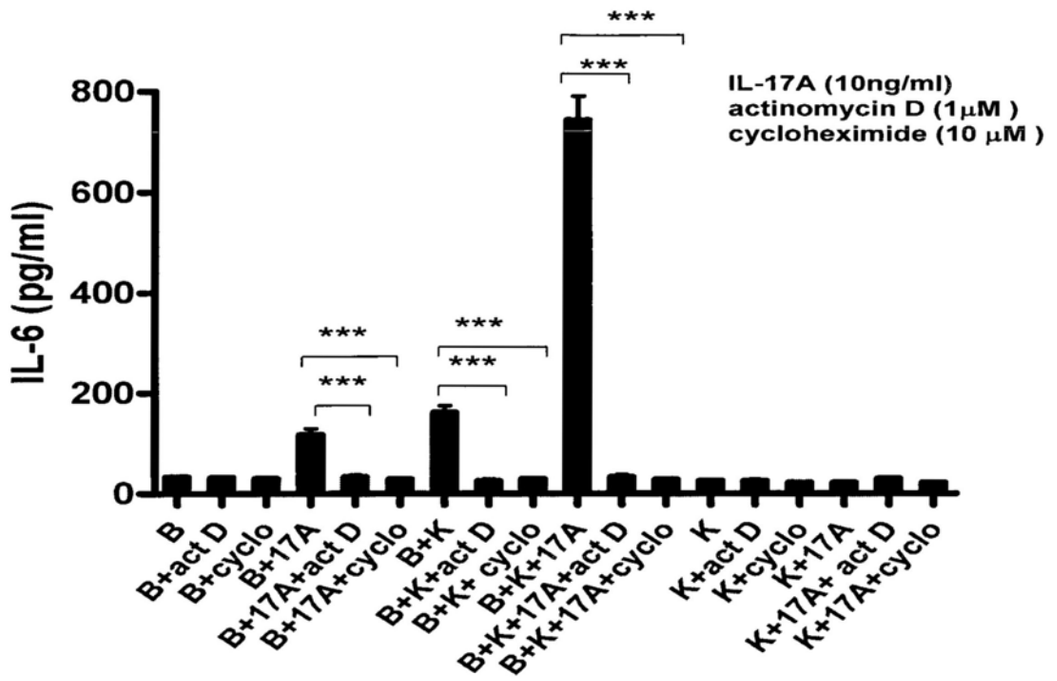

(D)
CCL2 (pg/ml)
1250
1000
750
500
250
0
HBE
HBE+17A
HBE+BAS
HBE+BAS+17A
BAS
BAS+17A

Figure 3.2.3 Effects of IL-17A on the induction of IL-6 and CCL2 upon the interaction of primary bronchial epithelial cells/BEAS-2B cells and basophils /KU812 cells. (A) KU812 cells (5 x 10^5 cells) and (B) confluent BEAS-2B cells (1 x 10^5 cells) were cultured either together or separately with or without IL-17A (0– 100 ng/ml) for 24 h. (C) Basophils (5 x 10^5 cells) and (D) confluent bronchial epithelial cells (1 x 10^5 cells) were cultured either together or separately with or without IL-17A (10 ng/ml) for 24 h. IL-6 and CCL2 released in culture supernatant were determined by ELISA. Results are expressed as the arithmetic mean plus SD of three independent experiments.

B: BEAS-2B cells; K: KU812 cells; HBE: human primary bronchial epithelial cells; BAS: basophils; 17A (10), (20), (50), (100): dose of IL-17A (10–100 ng/ml)

*$p<0.05$, **$p<0.01$, ***$p<0.001$ when compared between groups denoted by horizontal lines; #$p<0.05$ when compared with corresponding BEAS-2B alone treated with the same doses of IL-17A.

A


IL-6 (pg/ml)
800
600
400
200
0
***
***
***
***
***
***
IL-17A (10ng/ml)
actinomycin D (1μM)
cycloheximide (10 μM)
B
B+act D
B+cyclo
B+17A
B+17A+act D
B+17A+cyclo
B+K
B+K+act D
B+K+ cyclo
B+K+17A
B+K+17A+act D
B+K+17A+cyclo
K
K+act D
K+cyclo
K+17A
K+17A+ act D
K+17A+cyclo

B

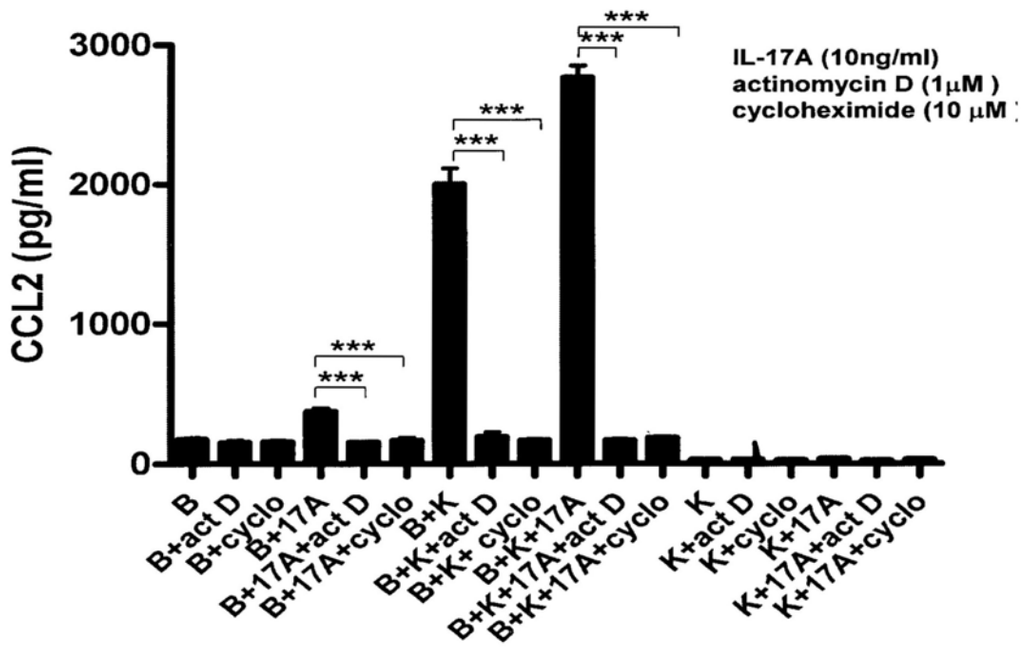

CCL2 (pg/ml)
3000
2000
1000
0
***
***
***
***
***
***
IL-17A (10ng/ml)
actinomycin D (1μM)
cycloheximide (10 μM
B
B+act D
B+cyclo
B+17A
B+17A+act D
B+17A+cyclo
B+K
B+K+act D
B+K+ cyclo
B+K+17A
B+K+17A+act D
B+K+17A+cyclo
K
K+act D
K+cyclo
K+17A
K+17A+act D
K+17A+cyclo

Figure 3.2.4 Effect of cycloheximide and actinomycin D on IL-17A induced release of (A) IL-6 and (B) CCL2 from BEAS-2B and KU812 cells alone or in co-culture with or without IL-17A activation. KU812 cells (5 x 10^5 cells) and confluent BEAS-2B cells (1 x 10^5 cells) were cultured either together or separately with or without IL-17A (10 ng/ml) and actinomycin D (1 μM) or cycloheximide (10 μM) for 24 h in a 24-well plate. Chemokines and cytokines released into the culture supernatant were determined by ELISA. Results are expressed as the arithmetic mean plus SD from three independent experiments. ***$p < 0.001$ when compared with the control.

B: BEAS-2B cells; K: KU812 cells; Act D: actinomycin D; Cyclo: cycloheximide

3.2.3 Source of IL-6 and CCL2 released in the co-culture system

To investigate the source of IL-6 and CCL2 released in the co-culture supernatant, 1 % paraformaldehyde was used to fix BEAS-2B/primary bronchial epithelial cells or KU812 cells/basophils to prevent cytokine and chemokine release while preserving the cell membrane integrity to maintain intercellular interaction. We compared the cytokine and chemokine levels in the co-culture of normal cells with the cells fixed with 1% paraformaldehyde. In the co-culture of fixed KU812/basophils and unfixed BEAS-2B/primary bronchial epithelial cells, the stimulatory effects of co-culture on the release of IL-6 and CCL2, and the IL-17A-induced stimulation were preserved. However, fixation of BEAS-2B/primary bronchial epithelial cells alone could almost completely abolish the secretion of IL-6 and CCL2 in co-culture with or without IL-17A stimulation. These indicated that primary bronchial epithelial cells/BEAS-2B cells were the main source for releasing IL-6 and CCL2 in co-culture upon IL-17A stimulation (Figure 3.2. 5A-D).

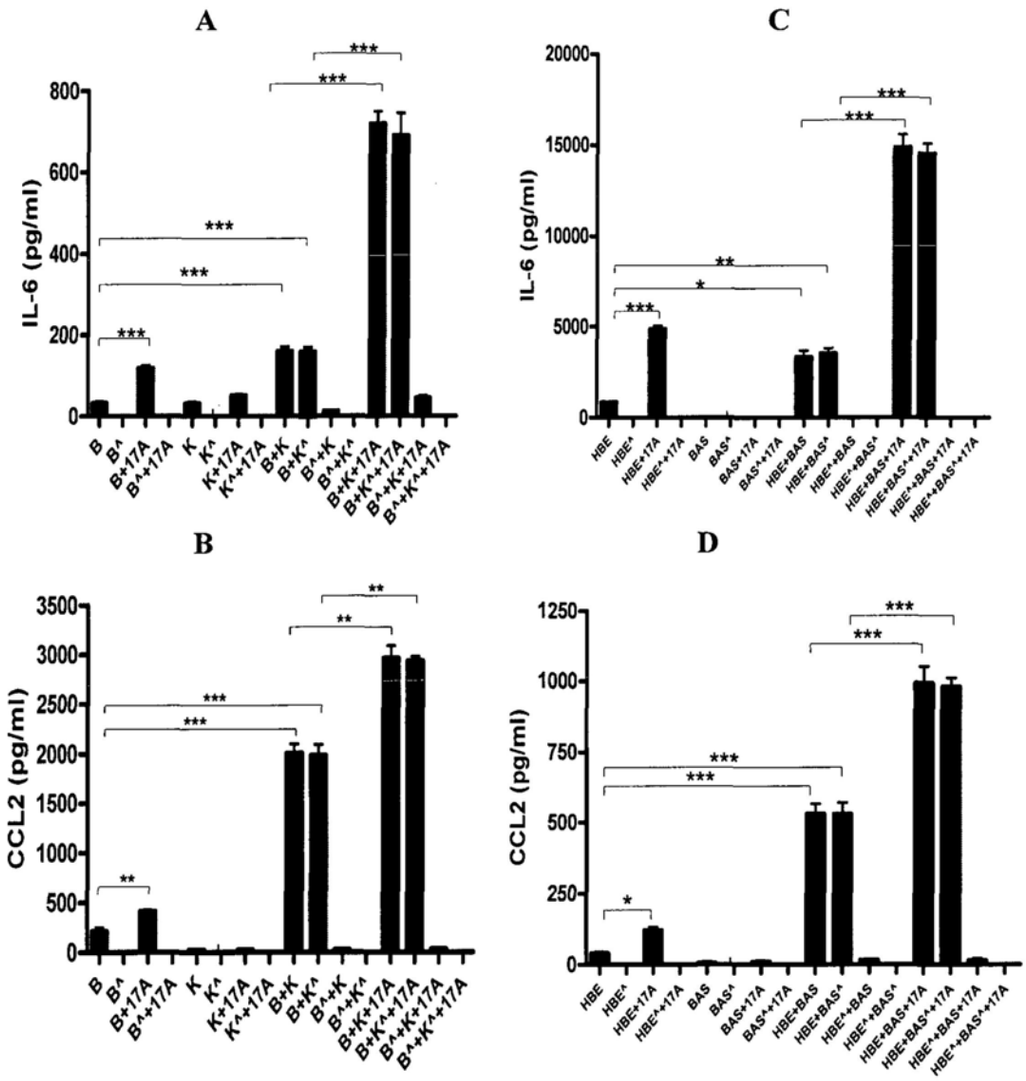


Figure 3.2.5 Source of IL-6 and CCL2 in co-culture of KU812 cells/basophils and BEAS-2B/primary bronchial epithelial cells under IL-17A stimulation. Confluent BEAS-2B/primary bronchial epithelial cells (1 x 10^5 cells) and KU812 cells/basophils (5 x 10^5 cells) were treated with or without 1% paraformaldehyde for 1 h on ice prior to being cultured together with or without IL-17A (10 ng/ml) for 24 h. IL-6 (A, C) and CCL2 (B, D) released in culture supernatant were determined by ELISA. Results are expressed as the arithmetic mean plus SD of three independent experiments.

B: unfixed BEAS-2B cells; B^: fixed BEAS-2B cells; K: unfixed KU812 cells; K^:

fixed KU812 cells; HBE: unfixed human primary bronchial epithelial cells; HBE^: fixed primary bronchial epithelial cells; BAS: unfixed basophils; BAS^: fixed basophils.

*$p<0.05$, **$p<0.01$, ***$p<0.001$ when compared between groups denoted by horizontal lines.

3.2.4 Direct interaction between primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells is required for IL-6 and CCL2 release in IL-17A-treated co-culture

To examine whether direct interaction was essential for IL-6 and CCL2 release in the co-culture upon IL-17A stimulation, transwell inserts (pore size: 0.4 μM) were used to separate BEAS-2B/primary bronchial epithelial cells and KU812 cells/basophils into two compartments in the co-culture system. Figure 3.2.6 show that the presence of transwell inserts could significantly suppress the IL-17A-induced secretion of IL-6 and CCL2 in co-culture. Without IL-17A stimulation, induction of IL-6 and CCL2 release in co-culture was also significantly abolished in the presence of transwell insert, implying that both IL-6 and CCL2 release in co-culture might depend on direct interaction between BEAS-2B cells/primary bronchial epithelial cells and KU812 cells/basophils.

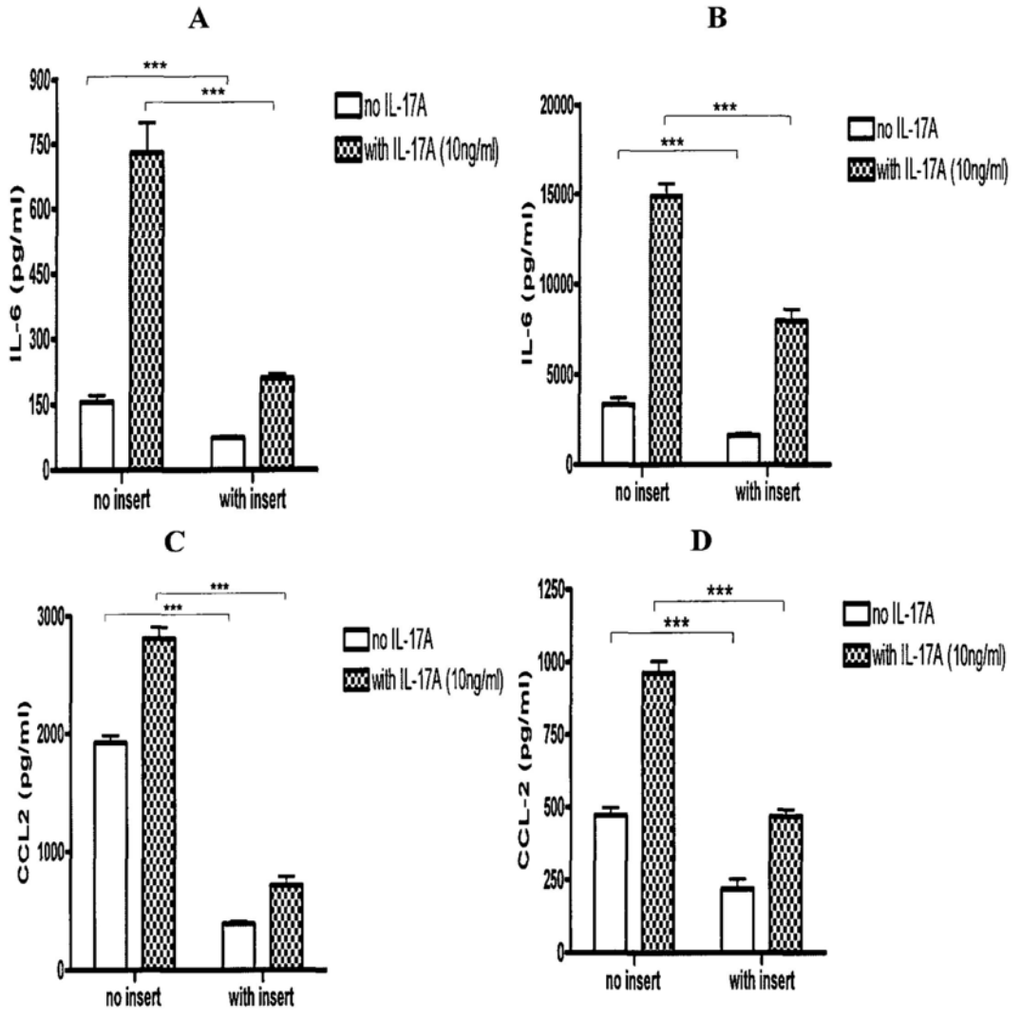


Figure 3.2.6 Effect of transwell inserts on the induction of IL-6 and CCL2 in co-culture of primary bronchial epithelial cells/BEAS-2B cells and basophils/KU812 cells under IL-17A stimulation. Confluent BEAS-2B/primary bronchial epithelial cells (1 x 10^5 cells) and KU812 cells/ basophils (5 x 10^5 cells) were cultured together with or without IL-17A (10 ng/ml) in the presence or absence of transwell inserts for 24 h. IL-6 and CCL2 released in culture supernatant of BEAS-2B and KU812 (A and C) or primary bronchial epithelial cells and basophils (B and D) were determined by ELISA. Results are expressed as the arithmetic mean plus SD of three independent experiments. ***$p<0.001$ when compared between groups denoted by horizontal lines.

3.2.5 Effects of IL-17A on adhesion molecule expression on primary bronchial epithelial cells/BEAS-2B cells in co-culture system

As shown in Figure 3.2.7, IL-17A could not significantly up-regulate the surface expression of ICAM-1 on BEAS-2B/primary bronchial epithelial cells alone or in co-culture of BEAS-2B/primary bronchial epithelial cells and KU812 cells/basophils. Upon co-culture with KU812 cells/basophils, ICAM-1 expression on BEAS-2B cells/primary bronchial epithelial cells was significantly increased. However, surface expression of ICAM-1 was not significantly up-regulated on KU812 cells/basophils by IL-17A, with or without the co-culture with BEAS-2B cells/primary bronchial epithelial cells (data not shown). Moreover, the expression of other adhesion molecules such as ICAM-3 and VCAM-1 on BEAS-2B cells/primary bronchial epithelial cells and ICAM-3, CD18 and L-selectin on KU812 cells/basophils remained unchanged upon co-culture and the addition of IL-17A.

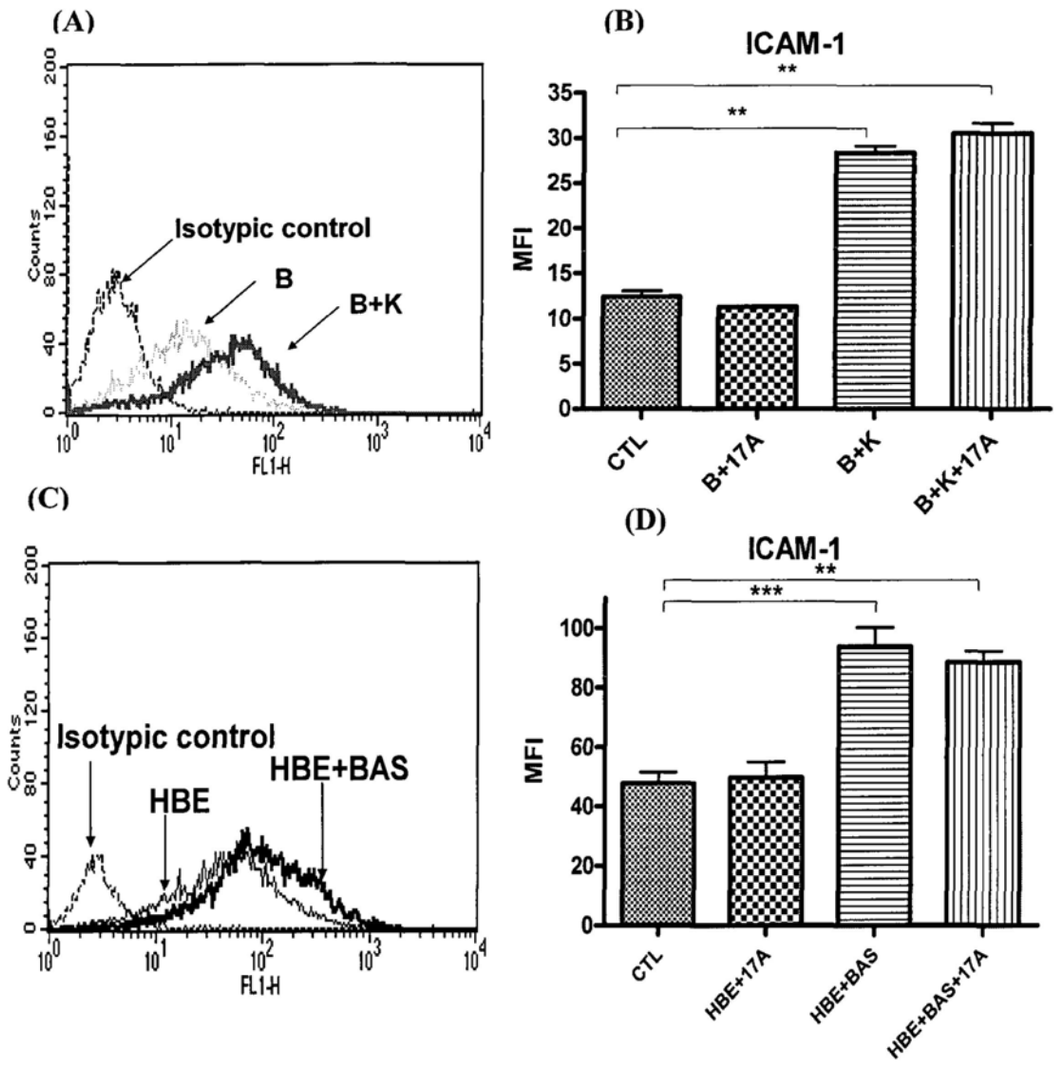


Figure 3.2.7 Effect of IL-17A on the surface expression of ICAM-1 on primary bronchial epithelial cells/BEAS-2B cells. (A, C) Representative MFI histogram of ICAM-1 expression on BEAS-2B/primary bronchial epithelial cells with or without co-culture with KU812 cells/basophils. (B, D) Bar chart of ICAM-1 expression on BEAS-2B/primary bronchial epithelial cells in co-culture with KU812 cells/basophils with or without treatment of IL-17A. Confluent BEAS-2B cells/primary bronchial epithelial cells (1 x 10^5 cells) and KU812 cells/basophils (5 x 10^5 cells) were cultured either together or separately with or without IL-17A (10 ng/ml) for 16 h. Surface expression of ICAM-1 on 5,000 BEAS-2B/primary

bronchial epithelial cells was analyzed by flow cytometry as MFI. Results have been normalized by subtracting appropriate isotypic control and are expressed as the arithmetic mean plus SD of three independent experiments.

** $p<0.01$, *** $p<0.001$ when compared between groups denoted by horizontal lines

B: BEAS-2B cells; K: KU812 cells; HBE: human primary bronchial epithelial cells; BAS: basophils;

3.2.6 Activation of ERK, JNK, p38 MAPK and NF-κB pathways in BEAS-2B cells and KU812 cells in co-culture upon IL-17A stimulation

For the subsequent signaling mechanistic study, we have used the representative BEAS-2B bronchial epithelial cells and KU812 basophilic cells. To investigate the underlying signaling mechanism(s), intracellular staining by multiparametric flow cytometry was applied. After fixation and permeabilization, BEAS-2B cells and KU812 cells were gated separately on the basis of ICAM-3 expression on KU812 cells only but not BEAS-2B cells (Figure 3.2.8A). Figure 3.2.8 B-E shows that IL-17A could activate ERK, p38 MAPK and NF-κB activity of BEAS-2B cells, and ERK activity of KU812 cells at 15 minutes. Upon co-culture, ERK, JNK, p38 MAPK and IκB-α in BEAS-2B cells, and ERK in KU812 cells were significantly phosphorylated upon stimulation. In the presence of IL-17A, the phosphorylation of ERK, JNK, p38 MAPK and IκB-α in BEAS-2B cells and ERK in KU812 cells were further enhanced in the co-culture.

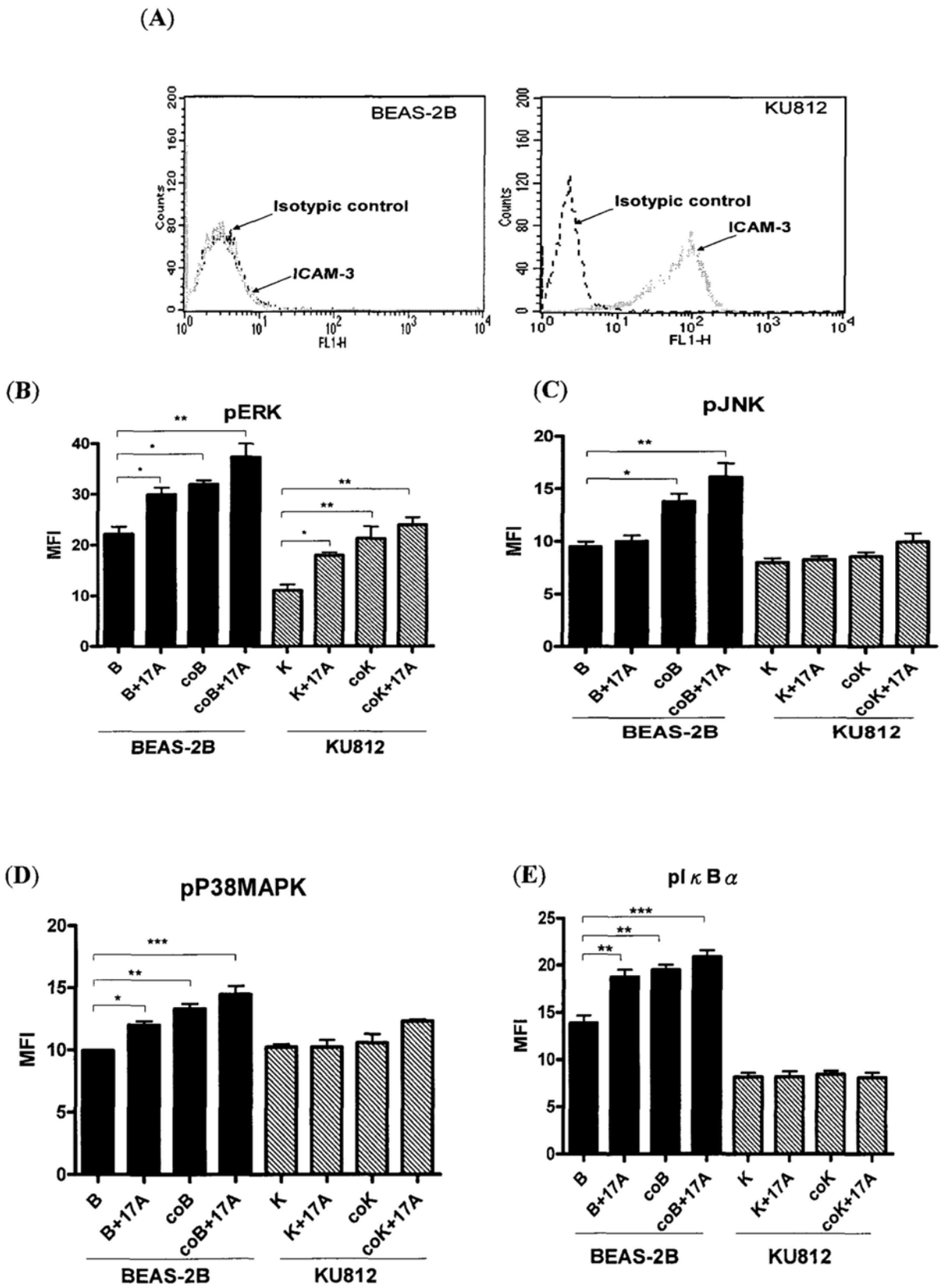

(A)
BEAS-2B
KU812
Isotypic control
ICAM-3
Counts
FL1-H
(B)
pERK
MFI
(C)
pJNK
MFI
(D)
pP38MAPK
MFI
(E)
pI κ B α
MFI
B
B+17A
coB
coB+17A
K
K+17A
coK
coK+17A
BEAS-2B
KU812

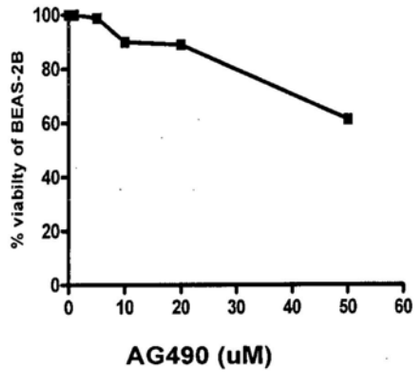
Figure 3.2.8 Activation of ERK, JNK, p38 MAPK and NF-κB in co-culture of BEAS-2B cells and KU812 cells under IL-17 stimulation. KU812 cells (5 x 10^5 cells) and confluent BEAS-2B cells (1 x 10^5 cells) were cultured either together or separately with or without IL-17A (10 ng/ml) stimulation for 15 min. (A) Representative MFI histogram of ICAM-3 expression on BEAS-2B cells and KU812 cells. After separation, fixation and permeabilization, KU812 cells (5 x 10^5 cells) and BEAS-2B cells (1 x 10^5 cells) were gated based on the cell expression of ICAM-3. Doted line: isotypic control, grey solid line: ICAM-3 expression. The intracellular contents of phosphorylated (B) ERK, (C) JNK, (D) p38 MAPK and (E) IκB-α of permeabilized KU812 and BEAS-2B cells were measured by intracellular immunofluorescence staining using flow cytometry. Results are shown in MFI subtracting corresponding isotypic control and are expressed as the arithmetic mean plus SD of three independent experiments.

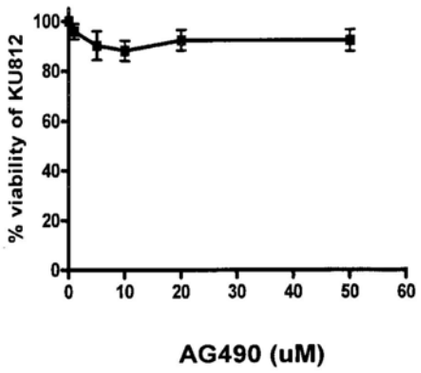
B: BEAS-2B cells only; coB: BEAS-2B cells in co-culture; K: KU812 cells only; coK: KU812 cells in co-culture

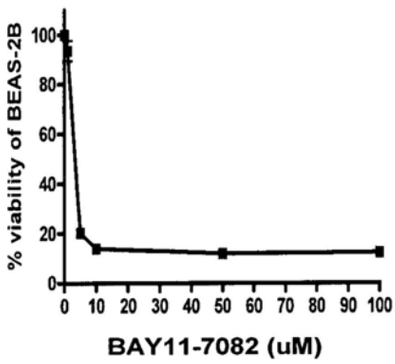
*$p<0.05$, **$p<0.01$, ***$p<0.001$ when compared between groups denoted by horizontal lines.

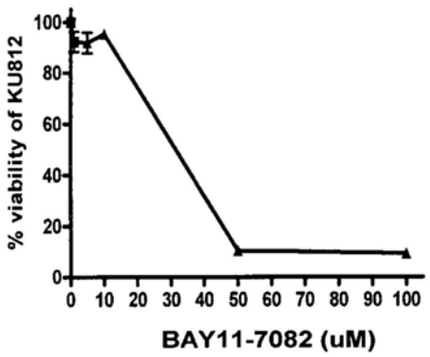
3.2.7 Effects of signaling molecule inhibitors on IL-6 and CCL2 release in co-culture upon IL-17A stimulation

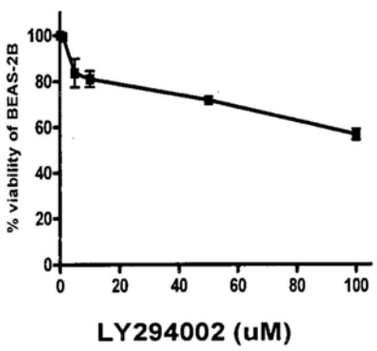
Based on the results of cytotoxicity assay using MTT assay (Figure 3.2.9), we used the optimal concentrations of JAK inhibitor AG490 (5 μM), NF-κB inhibitor BAY11-7082 (1 μM), PI3K inhibitor LY294002 (5 μM), ERK inhibitor U0126 (10 μM), p38 MAPK inhibitor SB203580 (20 μM) and JNK inhibitor SP600125 (5 μM) for significant inhibitory effects without any cell toxicity. As shown in Figure 3.2.10A and D, U0126, SB203580 and BAY11-7082 could suppress the induction of IL-6 and CCL2 by IL-17A in BEAS-2B alone. U0126, BAY11-7082 and SB203580 could significantly suppress the release of IL-6 in co-culture with or without IL-17A stimulation (Figure 3.2.10C), while U0126, BAY11-7082, SP600125 and SB203580 could significantly suppress the release of CCL2 release in co-culture with or without IL-17A stimulation (Figure 3.2.10F). However, all tested inhibitors did not exert any significant effect on IL-6 and CCL2 induction of KU812 cells alone with or without IL-17A stimulation (all $p > 0.05$, Figure 3.2.10B and E). DMSO solvent control did not have any significant effect in all inhibition experiments (all $p > 0.05$).

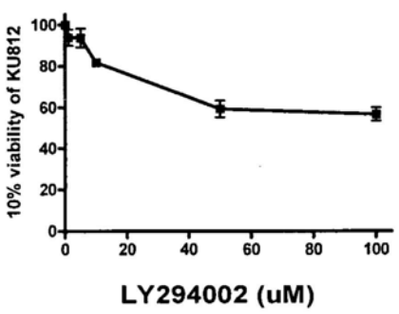

% viabilty of BEAS-2B
AG490 (uM)

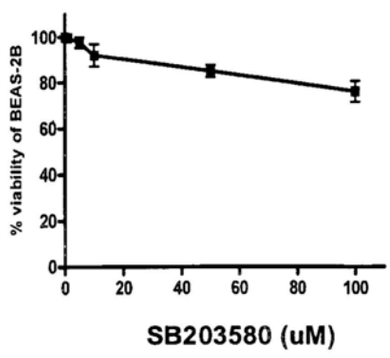

% viability of KU812
AG490 (uM)

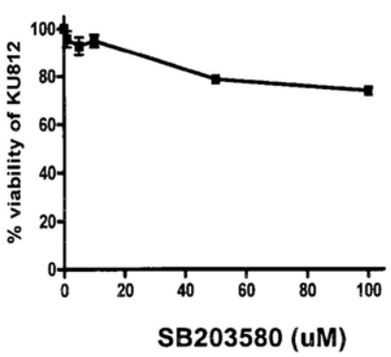

% viability of BEAS-2B
BAY11-7082 (uM)


% viability of KU812
BAY11-7082 (uM)


% viability of BEAS-2B
LY294002 (uM)


10% viability of KU812
LY294002 (uM)


% viability of BEAS-2B
SB203580 (uM)


% viability of KU812
SB203580 (uM)

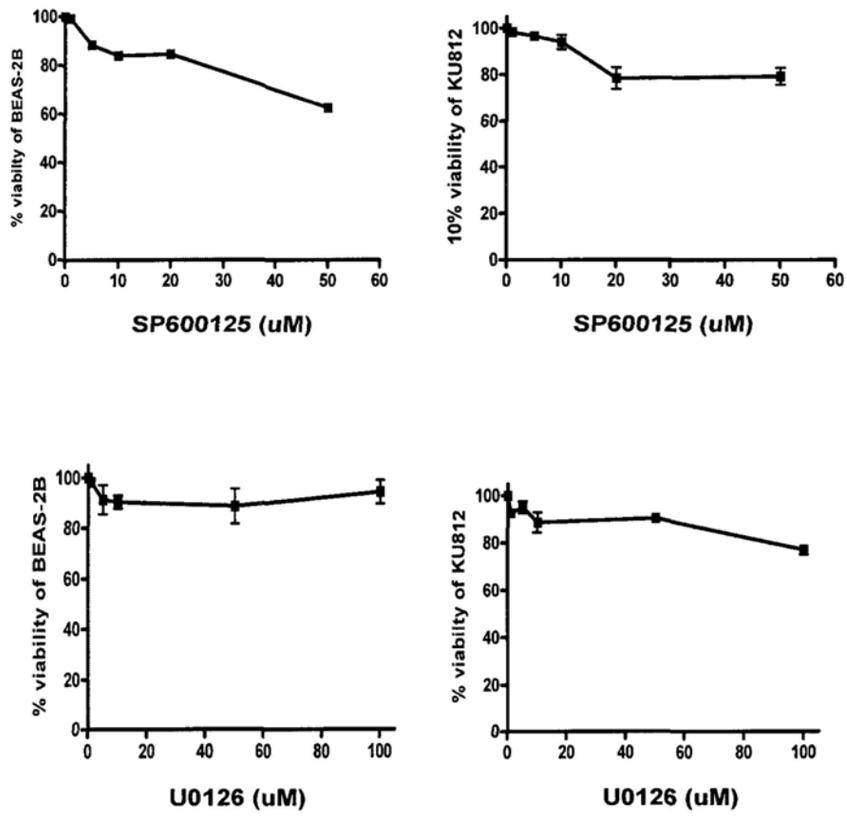


Figure 3.2.9 Effect of signaling molecule inhibitors on the viability of BEAS-2B or KU812. BEAS-2B (2 x 10^4 cells/0.2 ml) or KU812 (2 x 10^4 cells/0.2 ml) were incubated with serial concentrations of AG490, BAY11-7082, LY294002, SB203580 , SP600125 or U0126 for 24h, and the viability was assessed by MTT test. Results are expressed as arithmetic mean plus SD from triplicate experiments.

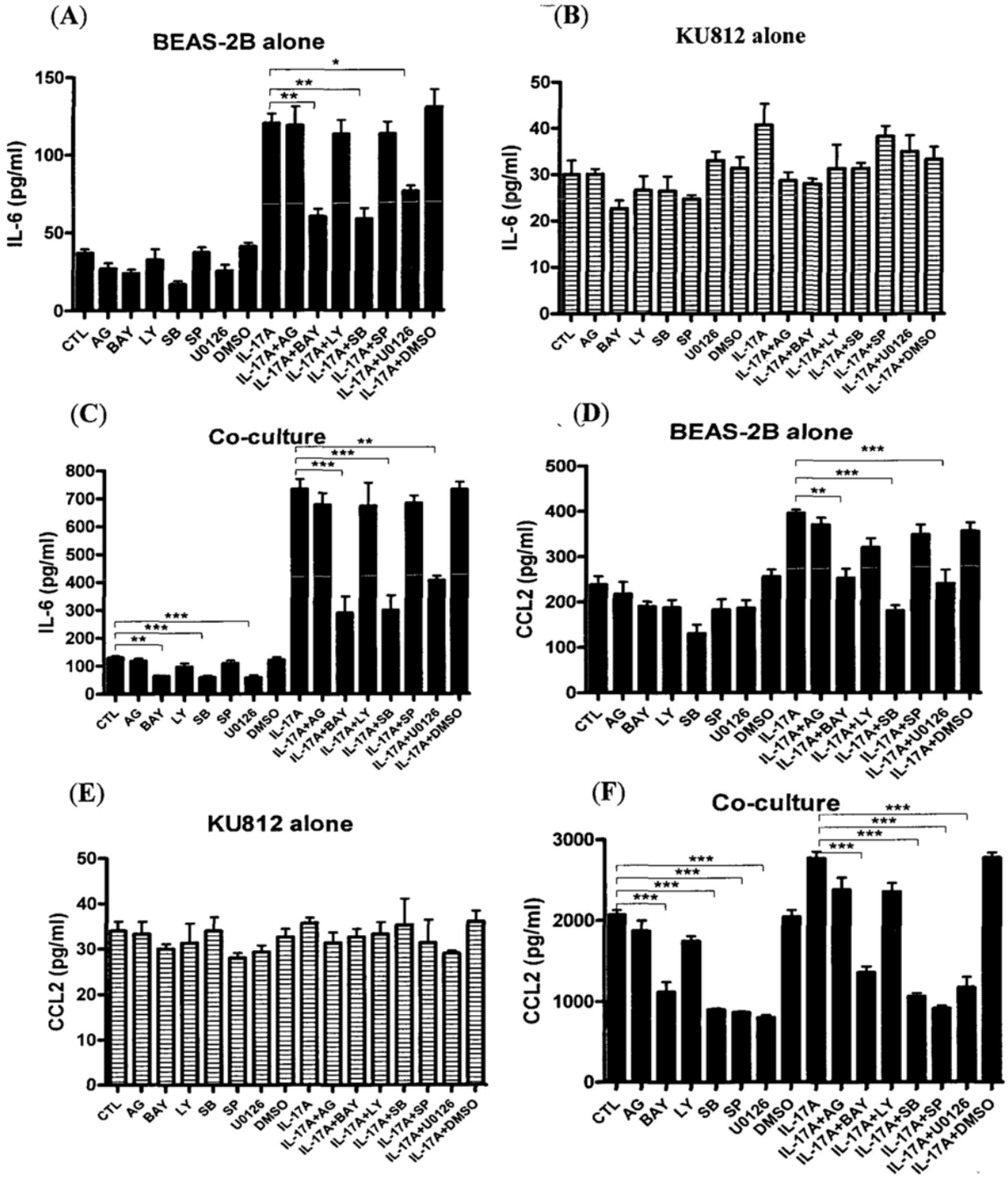

(A)
BEAS-2B alone
IL-6 (pg/ml)
(B)
KU812 alone
IL-6 (pg/ml)
(C)
Co-culture
IL-6 (pg/ml)
(D)
BEAS-2B alone
CCL2 (pg/ml)
(E)
KU812 alone
CCL2 (pg/ml)
(F)
Co-culture
CCL2 (pg/ml)
CTL
AG
BAY
LY
SB
SP
U0126
DMSO
IL-17A
IL-17A+AG
IL-17A+BAY
IL-17A+LY
IL-17A+SB
IL-17A+SP
IL-17A+U0126
IL-17A+DMSO

Figure 3.2.10 Effects of signaling molecule inhibitors on the release of IL-6 or CCL2. IL-6 released from (A) BEAS-2B cells, (B) KU812 cells with or without treatment of IL-17A (10 ng/ml) and (C) co-culture of BEAS-2B cells and KU812 cells with or without treatment with IL-17A (10 ng/ml); CCL2 released from (D) BEAS-2B cells, (E) KU812 cells with or without treatment of IL-17A (10 ng/ml) and (F) co-culture of BEAS-2B cells and KU812 cells with or without treatment with IL-17A (10 ng/ml). KU812 cells (5 x 10^5 cells) cultured alone or together with confluent BEAS-2B cells (1 x 10^5 cells) were pretreated with AG490 (5 μM), BAY11-7082 (1 μM), LY294002 (5 μM), SB203580 (20 μM), SP600125 (5 μM) or U0126 (10 μM) for 1 h, followed by incubation with or without IL-17A (10 ng/ml) in the presence of inhibitors for further 24h. Concentrations of IL-6 and CCL2 released in culture supernatant were determined by ELISA. Results are expressed as the arithmetic mean plus SD of three independent experiments. DMSO (0.1%) was used as the vehicle control.

*$p<0.05$, **$p<0.01$, ***$p<0.001$ when compared between groups denoted by horizontal lines

CTL: control, AG: AG490, BAY: BAY11-7082, LY: LY294002, SB: SB203580, SP: SP600125

3.2.8 Effects of signaling molecule inhibitors on the cell surface expression of ICAM-1 in co-culture upon IL-17A stimulation

Figure 3.2.11 shows that p38 MAPK inhibitor SB203580 (20 μM) and NF-κB inhibitor BAY11-7082 (1 μM) could suppress the up-regulation of ICAM-1 surface expression on BEAS-2B co-cultured with KU812 cells.

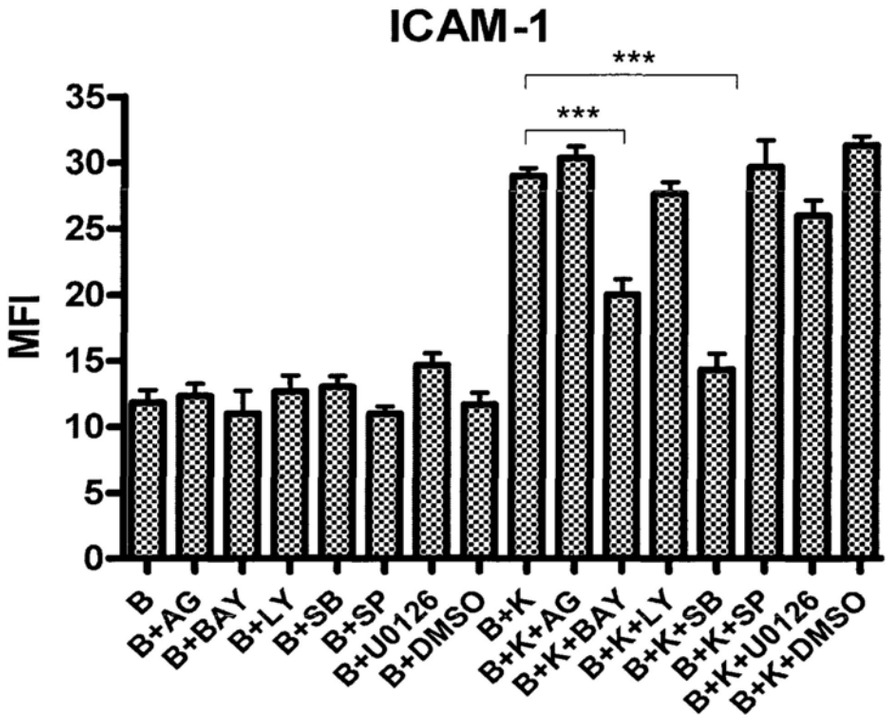


Figure 3.2.11 Effects of signaling molecule inhibitors on the cell surface expression of ICAM-1 on BEAS-2B cells in the co-culture. KU821 cells (5 x 10^5 cells) and confluent BEAS-2B cells (1 x 10^5 cells), either cultured separately or together, were pretreated with AG490 (5 μM), BAY11-7082 (1 μM), LY294002 (5 μM), SB203580 (20 μM), SP600125 (5 μM) or U0126 (10 μM) for 1 h, followed by incubation with inhibitors for further 24 h. Surface expression of ICAM-1 on 10,000 cells was analyzed by flow cytometry as MFI. Results have been normalized by subtracting appropriate isotypic control and are expressed as the arithmetic mean plus SD of three independent experiments. DMSO (0.1%) was used as the vehicle control.

***$p<0.01$ when compared between groups denoted by horizontal lines.

B: BEAS-2B cells only, K: KU812 cells only, AG: AG490, BAY: BAY11-7082, LY: LY294002, SB: SB203580, SP: SP600125

3.3 Discussion

Although basophils are crucial effector cells at the inflammatory sites of allergic asthma, their detailed immunopathogenic role for initiating local inflammation has not been well elucidated (Min, 2008). Following our previous studies of the interaction between eosinophils and BEAS-2B cells in allergic inflammation (Wong *et al.,* 2005; Wang *et al.,* 2005), we have developed an *in vitro* co-culture system to delineate the novel pathological mechanisms by the interactions among basophils, bronchial epithelial cells and Th17 cells in allergic asthma. In the present study, we have adopted BEAS-2B and KU812 cells which are widely used cell model. BEAS-2B cells maintain the characteristics of normal human primary bronchial epithelial cells including expression of adhesion molecule ICAM-1, eotaxin, IL-6 and CXCL8 (Matsukura *et al.,* 2003; Just *et al.,* 2003; Wang *et al.,* 2002) while KU812 cells constitute the phenotypes of blood basophils such as histamine release and FcgammaRII expression (Aichberger *et al.*, 2006; Brown *et al.*, 2009). Moreover, we also used primary bronchial epithelial cells and peripheral blood basophils to confirm our findings in coculture of BEAS-2B and KU812 cells under the stimulation of IL-17A.

We demonstrated that both BEAS-2B/primary bronchial epithelial cells and KU812 cells/basophils constitutively expressed IL-17RA, IL-17RC, and IL-17RA adaptor protein Act1 for IL-17 receptors by flow cytometric analysis. IL-17RA and IL-17RC form a heterodimer which could bind to IL-17A and IL-17F (Kuestner *et al.*, 2007). IL-17RC binds to both IL-17A and IL-17F with the same affinity, while IL-17RA binds to IL-17A with approximately ten-fold higher affinity than to IL-17F (Kuestner *et al.*, 2007; Hymowitz *et al.*, 2001). Previous studies have demonstrated that IL-17RA employed Act1 as its signal transducer to induce the expression of

cytokines and chemokines elicited by IL-17A and IL-17F (Chang *et al.,* 2006; Qian *et al.,* 2007). In accordance with the active roles of Th17 cytokines in allergic lung diseases (Wilson *et al.*, 2007; Molet *et al.,* 2001; Hizawa *et al.,* 2006; Kawaguchi *et al.,* 2001), we found that IL-17A could induce IL-6, CXCL8, CCL2, CXCL1 and G-CSF release from BEAS-2B cells/primary bronchial epithelial cells by quantitative ELISA. Interestingly, co-culture of BEAS-2B/primary bronchial cells and KU812 cells/basophils could increase the release of IL-6 and CCL2. In addition, IL-17A could dose-dependently and further induce the release of IL-6 and CCL2 from co-culture. In this study, the ratio of basophils/bronchial epithelial cells used was kept to be 5/1. Under this ratio, we found that the release of IL-6 and CCL2 was more significant than other ratios of 1:1, 2:1, or 4:1, and there was no significant difference for the release of IL-6 and CCL2 between 5:1 and 6:1 (data not shown). Since both transcriptional inhibitor actinomycin D and protein synthesis inhibitor cycloheximide could significantly suppress the release of IL-6 and CCL2 in co-culture with or without IL-17A stimulation, co-culture and IL-17A could induce the release of newly synthesized, instead of preformed IL-6 and CCL2. The induction of IL-6 indicated that IL-17A could be involved in mediating inflammatory and Th2 immune responses in allergic asthma (Rose-John *et al.,* 2006). Since IL-6 is able to induce the development of human Th17 cells in combination with other cytokines, such as IL-1β and IL-23 (Acosta-Rodriguez *et al.,* 2007; Wilson *et al.,* 2007; Brustle *et al.,* 2007), our results may suggest a novel immunopathological role of Th17 cells and basophils in allergic asthma through the IL-6 paracrine activation of Th17 cells initiated by the interaction between basophils and bronchial epithelial cells. CCL2 is a chemokine which could regulate the recruitment and activation of monocytes, mast cells, basophils, eosinophils and Th2 lymphocytes in allergic inflammation

(Romagnani, 2002). Therefore, our results further support the pathological role of Th17 cells, basophils and epithelial cells in local inflammatory sites of airways in allergic asthma. Basophils themselves act as an accessory cell type essential for Th17 paracrine activation, upon interacting with bronchial epithelial cells. We also found that IL-17A could not induce TSLP, an epithelial cell-derived proallergic Th2 cytokine, from BEAS-2B cells *in vitro*. It is reasonable because TSLP has been shown to be induced by inflammatory cytokine TNF-α and IL-1α, Th2 cytokine IL-4 and IL-13 and protease allergens but not IL-17A (Sokol *et al.*, 2008; Kato *et al.*, 2007; Bogiatzi *et al.*, 2007).

TNF-α has been shown to synergize with IL-17 for the release of cytokines and chemokines from epithelial cells by activating the NF-κB pathway (Awane *et al.*, 1999; McAllister *et al.*, 2005). However, we found that IL-17A or co-culture with KU812 basophilic cells could not significantly upregulate the expressions of p75TNFR and p55TNFR on the surface of BEAS-2B cells/primary bronchial cells using flow cytometry. Moreover, we could not detect any soluble TNF-α in the supernatants of co-culture of BEAS-2B and KU812 cells or primary bronchial epithelial cells and basophils using ELISA (detection limit < 8 pg/ml). Importantly, after pre-incubation and co-incubation with commercially available neutralizing/blocking monoclonal antibodies against p75TNFR and p55TNFR, we observed that anti-human p75TNFR or anti-human p55TNFR monoclonal antibodies could not significantly block the elevated induction of IL-6, CCL2 and ICAM-1 upon interaction between human basophilic and bronchial epithelial cells activated by IL-17A (data not shown). Taken together, the augmenting mechanism for IL-6 and CCL2 production in this study was not due to TNF-α induction. Apart from TNF-α, Th2 cytokine IL-4 and IL-13 in basophils also cannot account for this synergistic

effect in co-culture of human bronchial epithelial cells and basophils, since it has been demonstrated previously that the combination of IL-17 and IL-4 or IL-13 could not augment the expression of IL-6 in bronchial epithelial cells while comparing with bronchial epithelial cells stimulated with individual cytokine (Kawaguchi *et al.*, 2001). Nevertheless, other unidentified mediators derived from basophils for the activation of bronchial epithelial cells upon IL-17A stimulation cannot be excluded.

To investigate the source of IL-6 and CCL2 released in the co-culture system, we compared their levels in co-culture of normal cells with cells fixed with 1% paraformaldehyde. Our findings suggested that BEAS-2B/primary bronchial epithelial cells, instead of KU812/basophils, are the main source for releasing the IL-6 and CCL2 in co-culture upon IL-17A stimulation. Actually, IL-17 has been previously shown to induce IL-6 in human bronchial epithelial 16HBE cells (Laan *et al.*, 2001). To verify whether direct contact between bronchial epithelial cells and basophils was required for the induction of cytokines and chemokines in co-culture system, permeable transwell inserts barriers were applied. Results showed that the presence of transwell inserts could significantly suppress the release of IL-6 and CCL2 in co-culture, with or without IL-17A stimulation. Direct interaction between BEAS-2B/primary bronchial epithelial cells and KU812/basophils should at least partially contribute to the induction of IL-6 and CCL2. Since the direct interaction between their surface adhesion molecules on bronchial epithelial cells and basophils is the possible mechanism for the induction of IL-6 and CCL2 in co-culture, we further investigated the expression of adhesion molecules on the surface of BEAS-2B/primary bronchial epithelial cells and KU812/basophils in co-culture upon IL-17A stimulation. ICAM-1 is a crucial adhesion molecule present on bronchial epithelial cells and plays an essential role in cell adherence by interacting with the

highest affinity to the integrin family member LFA-1 (CD11a/CD18), a hallmark of allergic inflammation (Canonica *et al.,* 1995). We found that ICAM-1 expression on the surface of BEAS-2B/primary bronchial epithelial cells was significantly enhanced upon the interaction with KU812/basophils although IL-17A could not further up-regulate the expression of ICAM-1. Such ICAM-1/CD18 interaction might provide a potential stimulation on the release of IL-6 and CCL2 upon the interaction of human bronchial epithelial cells and basophils.

Regarding the signal transduction mechanisms, we applied multiparametric flow cytometry with cell surface ICAM-3 gating for differential analysis of the intracellular phosphorylation levels of different signaling molecules in permeabilized BEAS-2B and KU812 cells in co-culture under IL-17A stimulation by intracellular fluorescence staining (Cheung *et al.,* 2008). Intracellular staining assay using flow cytometry is a rapid and quantitative method which can determine the expression of phosphorylated signaling molecules of specified cells in mixed cell types by excluding the contamination of other cells. Our results showed that in the co-culture system, ERK, JNK, p38 MAPK and NF-κB pathways were activated in BEAS-2B cells, while only ERK was phosphorylated in KU812 cells, with or without IL-17A stimulation. We further elucidated the differential activation of signaling pathways above in the induction of IL-6, CCL2 and ICAM-1 expression using specific inhibitors. The effective doses of signaling molecule inhibitors with significant inhibitory effects on specific signaling pathways have been widely studied using different cell types including human bronchial epithelial cells (Cheung *et al.,* 2008; Bennett *et al.,* 2001; Ip WK *et al.,* 2007; Mori *et al.,* 2002; Wong *et al.,* 2010). Following previous publications and toxicity threshold values from the MTT assay, we used the optimal concentrations of JAK inhibitor AG490 (5 μM), NF-κB inhibitor

BAY11-7082 (1 μM), PI3K inhibitor LY294002 (5 μM), ERK inhibitor U0126 (10 μM), p38 MAPK inhibitor SB203580 (20 μM) and JNK inhibitor SP600125 (5 μM) for significant inhibitory effects without any cell toxicity. Results from inhibition experiments demonstrated that the production of IL-6 induced by IL-17A in BEAS-2B alone and in co-culture was mediated by ERK, p38 MAPK and NF-κB pathways. Results were therefore in line with previous reports suggesting that ERK, p38 MAPK and NF-κB pathways are differentially activated by IL-17A and IL-17F in eosinophils and IL-17A in bronchial epithelial cells (Cheung *et al.*, 2008; Kawaguchi *et al.*, 2001; Laan *et al.*, 2001). Regarding CCL2, in addition to ERK, p38 MAPK and NF-κB, JNK also played a role in the induction of CCL2 from co-culture. Moreover, the intracellular signaling mechanisms regulating ICAM-1 expression on the surface of BEAS-2B involved NK-κB and p38 MAPK. The results from inhibition experiments were therefore consistent with those of intracellular staining assays by flow cytometry. Since only ERK activity of KU812 cells was activated by IL-17A and co-culture, it may account for that KU812 cells were not the main source for releasing IL-6 and CCL2. Since AG490 and LY294002 did not exert any inhibitory effect, JAK-STAT and PI3K-Akt pathways did not play any regulatory roles in IL-6, CCL2 and ICAM-1 expression in the co-culture system.

Recent studies have successfully established a basophil-deficient state in mice using basophil-depleting antibodies (Ba103 and MAR-1), the generation and application of Ba103 and MAR-1 monoclonal antibodies can account for the recent considerable progress in basophil research (Obata *et al.*, 2007; Tsujimura *et al.*, 2008; Sokol *et al.*, 2008; Denzel *et al.*, 2008; Kojima *et al.*, 2007). Our further study will use basophil-deficient mice to further elucidate the immunopathological role of basophils in allergic asthma.

This is the first report elucidating the regulatory mechanisms for the increased release of IL-6, CCL2 and ICAM-1 expression upon the interaction of human bronchial epithelial cells and basophils activated by hallmark Th17 cytokine IL-17A (Figure 3.3.1). Together with previous findings on the active participation of IL-17A, IL-17E and IL-17F in Th2 responses and airway hyperresponsiveness (Tesmer *et al.*, 2008, Cheung *et al.,* 2008), our results therefore support the crucial immunopathological role of the IL-17 cytokine family and Th17 lymphocytes in the amplification of allergic diseases such as allergic asthma upon interacting with basophils and bronchial epithelial cells. Although basophils could not be activated to release IL-6 and CCL2, it can facilitate the IL-17A activation of bronchial epithelial cells by direct intercellular contact, probably through the up-regulation of adhesion molecule ICAM-1 expression and the activation of intracellular ERK, JNK, p38 MAPK and NK-κB pathway of bronchial epithelial cells. In view of recent advances in the application of ERK, p38 MAPK and NF-κB inhibitors as potential anti-inflammatory agents in asthma (O'Neill, 2006; Duan *et al.,* 2006), our present study may provide new clues for the development of new treatment for allergic diseases.

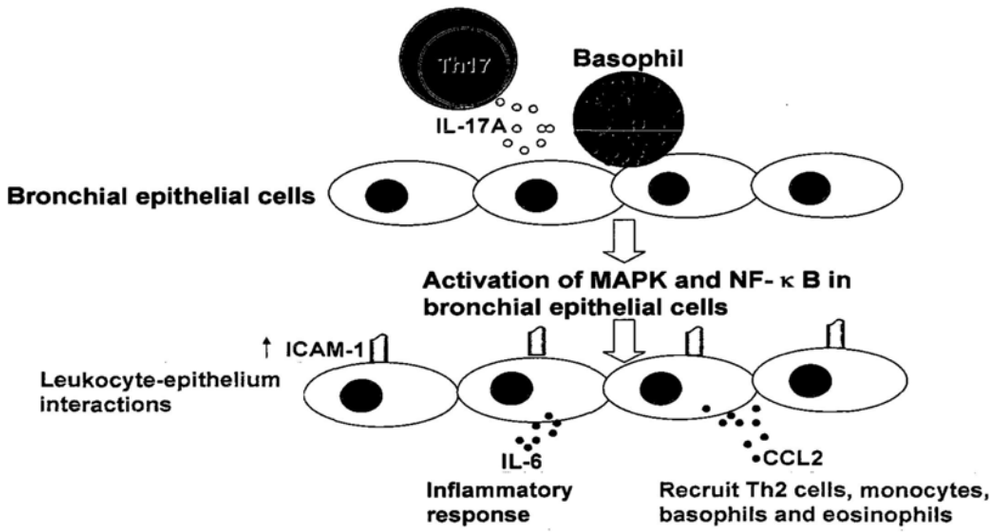


Figure 3.3.1 The novel mechanistic model of IL-17A activation on bronchial epithelial cells and basophils in allergic inflammation. Hyperproduction of IL-17A at local inflammatory sites such as airways can induce the release of IL-6, CXCL8, CCL2, CXCL1 and G-CSF from human bronchial epithelial cells. Number of basophils is increased in the asthmatic airways in response to allergen inhalation challenge. These increased basophils can then attach onto the bronchial epithelium. Interaction between human bronchial epithelial cells and basophils could enhance the release of CCL2 and IL-6, and upregulate the expression of ICAM-1 on the surface of bronchial epithelial cells. IL-17A could further augment the release of IL-6 and CCL2 from bronchial epithelial cells upon the direct contact to basophils. The elevated production of IL-6, CCL2 and ICAM-1 upon the interaction between human

bronchial epithelial cells and basophils activated by IL-17A is differentially regulated by intracellular ERK, JNK, p38 MAPK and NK-κB pathways. Synergistic induction of inflammatory cytokine IL-6 and chemokine CCL2 for the chemoattraction of neutrophils, monocytes, mast cells, basophils, eosinophils and Th2 lymphocytes can together mediate inflammatory and Th2 immune responses in allergic inflammation. Neutrophil chemokine CXCL8 and CXCL1 and neutrophil survival factor G-CSF released from human bronchial epithelial cells activated by IL-17A can also promote and sustain the airway inflammation.

Chapter 4

Activation of Human Bronchial Epithelial Cells by Inflammatory Cytokines IL-27 and TNF-α

4.1 Introduction

Cytokines are essentially involved in the development and regulation of innate and adaptive immunities. IL-27 is a novel member of the IL-6/IL-12 family cytokines that are produced by antigen-presenting cells (APC) such as dendritic cells (DC) and macrophages (Trinchieri, 2003; Kastelein *et al.,* 2007). IL-27 is predominantly produced early after stimulation of APC by pathogen-associated molecular patterns through Toll-like receptors (TLRs) (Hunter, 2005), and it is suggested that IL-27 could amplify early responses to weak infectious stimuli to achieve a robust response (Hölscher *et al.,* 2005; Hamano *et al.,* 2003). Similar to IL-12 and IL-23, IL-27 is another heterodimeric cytokine which is consisted of Epstein-Barr virus-induced gene 3 (EBI3) (an IL-12 p40 homologue originally described to be produced by Epstein–Barr-virus-transformed B cells) and p28, a p35 homologue (Pflanz *et al.,* 2002; Batten and Ghilardi, 2007). The heterodimeric receptor complex for IL-27 signaling is formed by T cell cytokine receptor (TCCR)/WSX-1 and glycoprotein 130 (gp130), the former confers ligand specificity

and the latter is shared by other IL-6 family cytokines (Pflanz *et al.*, 2004).

IL-27 mediates its various biological functions upon ligation with its receptor (Yoshida and Yoshiyuki, 2008). Although IL-12 is a key cytokine to drive naive T cells into the T helper type 1 (Th1) subset, IL-27 also plays an important role in commiting naïve T cells to differentiate into Th1 cells at the initial step of differentiation (Takeda *et al.*, 2003). IL-27 can directly act on naïve $CD8^+$ T cells and augment effector CD8+ T cell generation with enhanced granzyme B expression (Morishima *et al.*, 2005). Besides, IL-27 has the capacity to promote effector responses of natural killer (NK) cells and suppress the development of TGF-β-induced Forkhead box transcription factor p3-positive (Foxp3+) regulatory T cells (Treg) (Lucas *et al.*, 2003; Neufert *et al.*, 2007). In addition, IL-27 can activate human mast cells and monocytes to produce pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, IL-1β and IL-18 (Kalliolias and Ivashkiv, 2008). On the other hand, IL-27 is able to suppress inflammation by inhibiting the development of Th1, Th2 and Th17 cell subsets in some conditions (Hölscher *et al.*, 2005; Hamano *et al.*, 2003; Villarino *et al.*, 2003), and IL-27 could mediate the differentiation of naïve $CD4^+$ T cells into IL-10-producing anti-inflammatory T cells and IL-27 is capable of inducing Th1, Th2 and Th17 cells to produce IL-10(Stumhofer *et al.*, 2007; Awasthi *et al.*, 2007).

There is increasing evidence that airway epithelial cells could produce a variety of inflammatory mediators that modulate inflammation and immune responses in the airways (Schleimer *et al.*, 2007). Airway epithelial cells are the first barrier against external pathogens or concomitants, and they play an important role in innate immune responses by complex anti-microbial defense mechanisms, including the secretion of anti-microbial molecules such as defensins, as well as pro-inflammatory cytokines and chemokines (Mayer and Dalpke, 2007). In addition, airway epithelial cells contribute to the regulation of adaptive immune responses by the expression of soluble and cell-surface molecules, and they can modulate the development and biological functions of effector cells in the airways (Schleimer *et al.*, 2007; Kato and Schleimer, 2007). Therefore, airway epithelial cells not only activate and mediate innate immune responses but also regulate adaptive immune responses. Structural and functional abnormalities of airway epithelial cells could cause lung disorders, such as COPD, and asthma.

During airway inflammation, airway epithelial cells are capable of inducing migration of DC into the epithelium via chemokines CCL20 and β-defensin production (Stumbles *et al.*, 2001; Yang *et al.*, 1999). These accumulated DC are then activated by TLR ligands or infectious agents to produce IL-27 at the inflammatory sites (Hunter, 2005; Hammad and Lambrecht, 2008).It has been

demonstrated that there was an increased expression of IL-27 at the sites of inflammation during infection with *Mycobacteria tuberculosis* (Hölscher *et al.*, 2005). Since human bronchial epithelial cells were reported to express IL-27 receptor complex of WSX-1 and gp130 (Pflanz *et al.*, 2004), communication achieved through IL-27 between activated human APC and bronchial epithelial cells in the airway inflammation has to be elucidated. Therefore, we hypothesize that IL-27 may activate human bronchial epithelial cells to initiate the inflammatory cascades in airway infection. In an attempt to understand the role of IL-27 in the modulation of airway epithelium, we have investigated the *in vitro* effects of IL-27, alone or in combination with airway inflammation-related cytokine TNF-α on the pro-inflammatory activation of human primary bronchial epithelial cells (PBEC) or BEAS-2B cells, and the potential underlying intracellular mechanisms were also studied.

4.2 Results

4.2.1 IL-27, but not IL-12 and IL-23, up-regulated ICAM-1 expression on the cell surface of human bronchial epithelial cells

Figure 4.2.1A shows the kinetics and dose response of IL-27-inducing effects on expression of ICAM-1 on the surface of PBEC by flow cytometric analysis, we

found that IL-27 (10–100 ng/ml) could significantly up-regulate the surface expression of ICAM-1 at all the incubation times (12 h–72 h). Therefore, we chose the optimal incubation time (48 h) and concentration (50 ng/ml) of IL-27 in the following studies.

In contrast to the finding of a significant induction of ICAM-1 expression by IL-27, other members of IL-6/IL-12 family cytokines, IL-12 (10-100 ng/ml) and IL-23 (10-100 ng/ml) showed no significant effect on ICAM-1 expression on the surface of PBEC at all the incubation times (12 h–72 h) (Figure 4.2.1B and C, all $p > 0.05$). In the BEAS-2B cell line, IL-12 and IL-23 could not enhance ICAM-1 expression on the cell surface of BEAS-2B cells, but IL-27 could significantly up-regulate ICAM-1 expression on the surface of BEAS-2B cells (Figure 4.2.2).

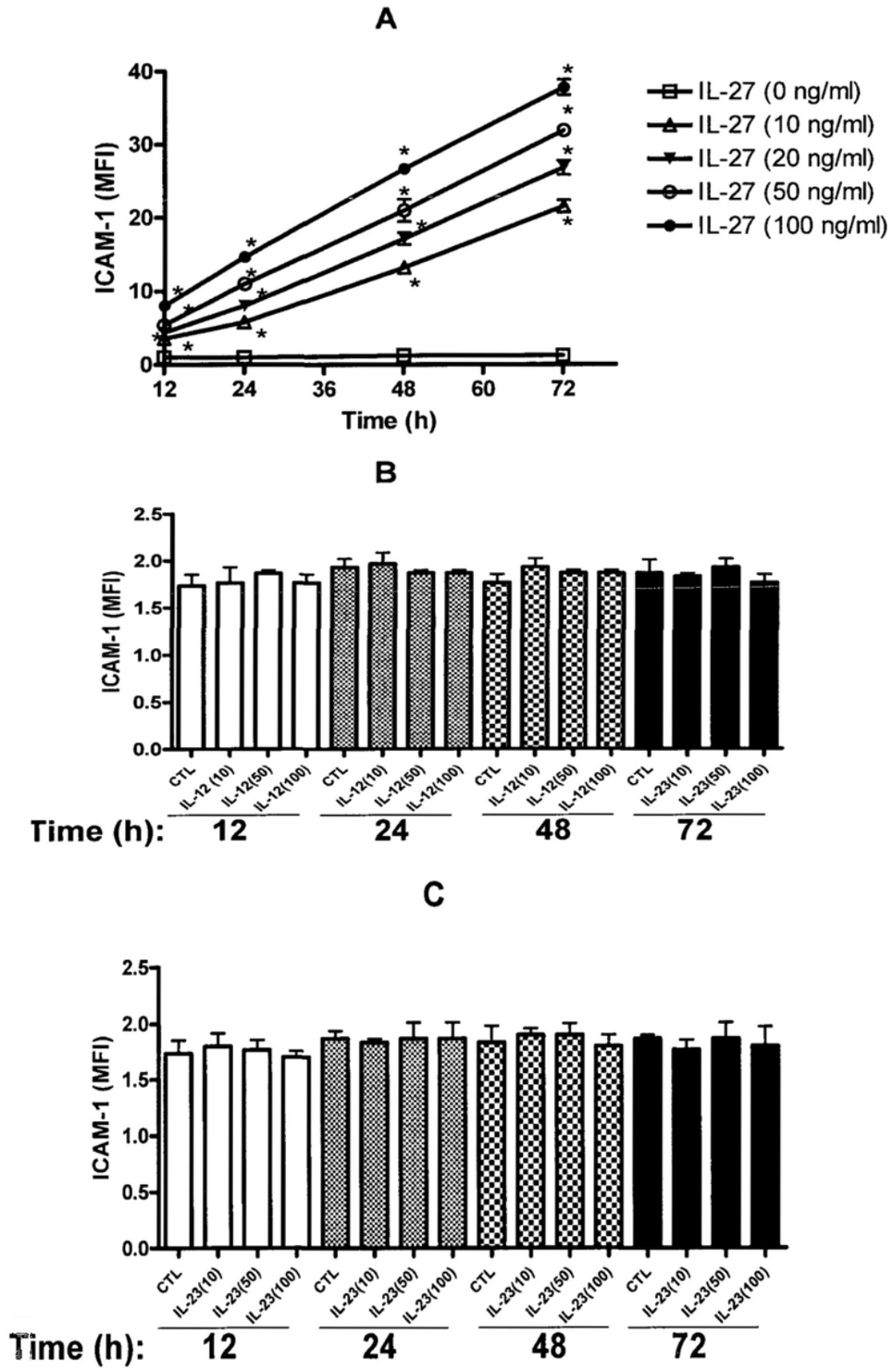

A
ICAM-1 (MFI)
Time (h)
IL-27 (0 ng/ml)
IL-27 (10 ng/ml)
IL-27 (20 ng/ml)
IL-27 (50 ng/ml)
IL-27 (100 ng/ml)
B
ICAM-1 (MFI)
CTL
IL-12 (10)
IL-12(50)
IL-12(100)
IL-23(10)
IL-23(50)
IL-23(100)
Time (h): 12 24 48 72
C
ICAM-1 (MFI)
Time (h): 12 24 48 72

Figure 4.2.1 Kinetic expression of ICAM-1 on the surface of PBEC activated by (A) IL-27, (B) IL-12 and (C) IL-23. PBEC were stimulated with or without IL-27 (10–100 ng/ml), IL-12 (10–100 ng/ml) or IL-23 (10–100 ng/ml) for 12, 24, 48 and 72 h. Surface expression of ICAM-1 of 5,000 cells was determined by flow cytometry as MFI. All results are expressed as the arithmetic mean plus SD from three independent experiments. *$p < 0.05$ when compared with the medium control (CTL, IL-27: 0 ng/ml) of the same point.

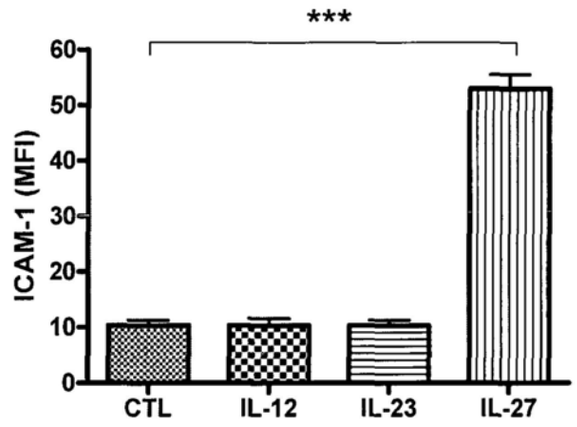


Figure 4.2.2 Effect of IL-27 on cell surface expression of ICAM-1 on BEAS-2B cells. BEAS-2B cells were incubated with either the medium alone or with IL-12 (50 ng/ml), IL-23 (50 ng/ml), or IL-27 (50 ng/ml). At 24 h, the expression of ICAM-1 was analyzed. Surface expression of ICAM-1 of 5, 000 cells was determined by flow cytometry as mean fluorescence intensity (MFI).All the results are expressed as the arithmetic mean plus SEM from five independent experiments. ***$p < 0.001$ when compared with medium control (CTL).

4.2.2 Synergistic up-regulation of ICAM-1 expression in combined treatment of IL-27 and TNF-α

Figure 4. 2. 3A shows that the combined treatment of IL-27 and TNF-α resulted in a synergistic up-regulation of ICAM-1 expression on the surface of PBEC by flow cytometric analysis. However, the combined treatment of IL-27 and Th1 cytokine IFN-γ did not have such a synergistic effect on ICAM-1 expression (Figure 4.2.3A). Besides, the combined treatments of IL-27 with other TLR ligands for TLR2, TLR3, TLR4 and TLR7 could not further enhance the surface expression of ICAM-1 (data not shown). To investigate whether the synergistic effect was dose-dependent on IL-27 and/or TNF-α, serial concentrations of IL-27 and TNF-α were used in the combined treatments. As shown in Figure 4.2.3B, a fixed concentration of TNF-α (20 ng/ml) with serial concentrations of IL-27 (10–100 ng/ml) showed that the synergistic effect could not be enhanced dose-dependently by IL-27. Conversely, the synergistic effect could be further enhanced when the TNF-α concentration increased from 5 to 50 ng/ml in combination with a fixed IL-27 concentration (50 ng/ml). Besides, similar dose effects of IL-27 and TNF-α on the surface expression of ICAM-1 was also observed in BEAS-2B cells (Figure 4.2.4).

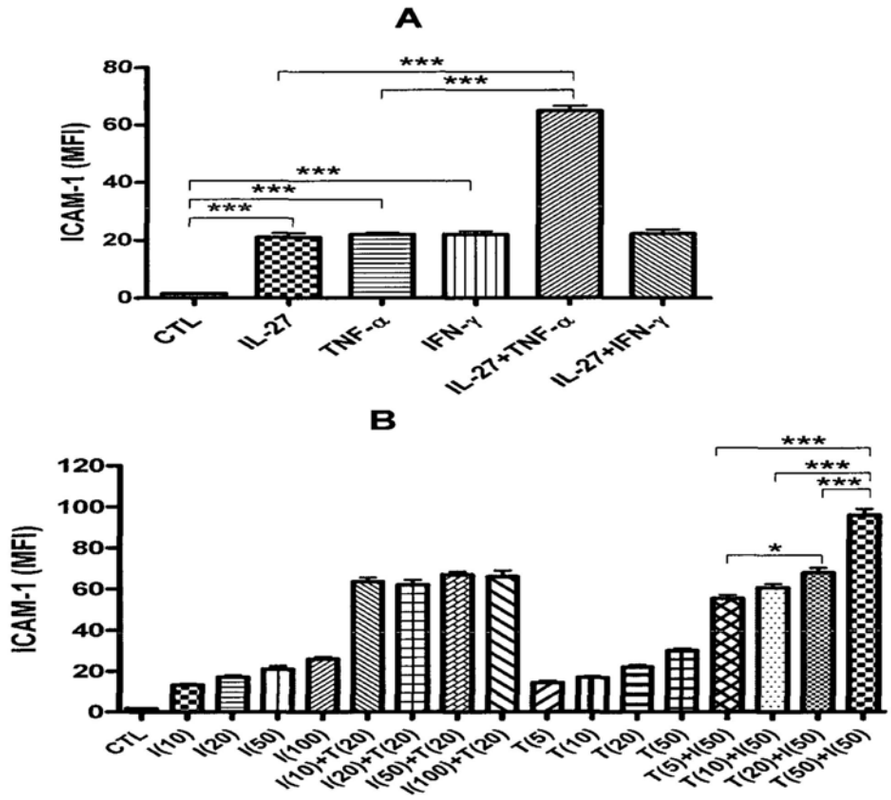


Figure 4.2.3 Effects of individual or combined cytokine stimulation on the surface expression of ICAM-1 on PBEC. (A) PBEC were incubated with or without IL-27 (50 ng/ml), TNF-α (20 ng/ml), and IFN-γ (20 ng/ml) alone or in combination for 48 h. (B) Synergistic effects of IL-27 and TNF-α on the expression of ICAM-1. PBEC were cultured with varied concentrations of IL-27 (I: 10-100 ng/ml) and TNF-α (T: 5-50 ng/ml) in combination for 48 h. The cell surface expression of ICAM-1 was determined by flow cytometry. Results are expressed as the arithmetic mean plus SD from three independent experiments. *$p < 0.05$, ***$p < 0.001$ when compared between groups denoted by horizontal lines. Medium control: CTL.

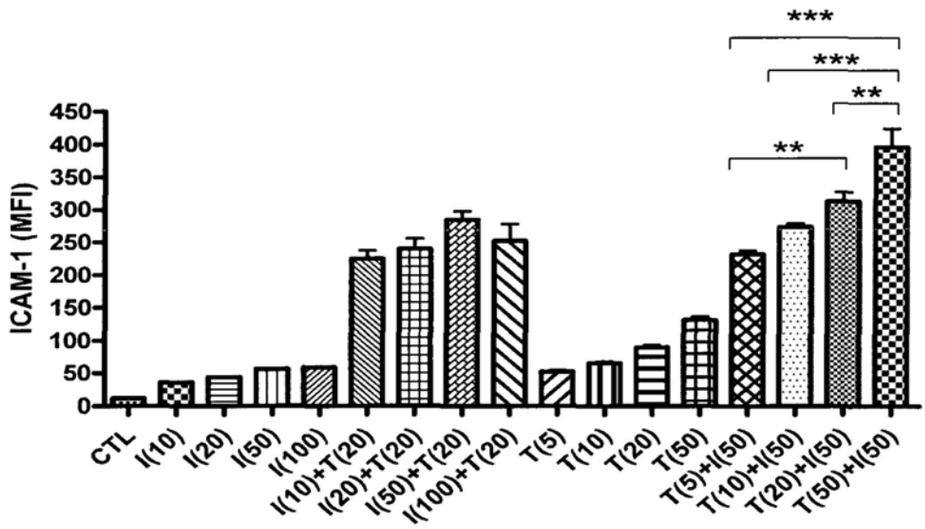


Figure 4.2.4 Synergistic effects of IL-27 and TNF-α on the expression of ICAM-1 on the surface of BEAS-2B cells. BEAS-2B cells (1 x 10^5 cells) were cultured with varied concentrations of IL-27 (I: 10-100 ng/ml) and TNF-α (T: 5-50 ng/ml) in combination for 24 h. The surface expression of ICAM-1 was determined by flow cytometry. Results are expressed as the arithmetric mean plus SD of five independent experiments. **$p < 0.01$, ***$p < 0.001$ when compared between groups denoted by horizontal lines.

4.2.3 IL-27 could enhance TNF-α-induced IL-6 release from human bronchial epithelial cells

Figure 4.2.5 illustrates the cytokine expression profile using semi-quantitative antibody based human cytokine protein membrane array. IL-27 could not stimulate PBEC or BEAS-2B cells to produce pro-inflammatory cytokines and chemokines among the 79 different cytokines being screened after 48 h incubation compared with that of medium control. We next examined the effect of IL-27 on basal and TNF-α-induced IL-6 release from PBEC by quantitative ELISA. Interestingly, although IL-27 did not induce IL-6 release from PBEC, the combination of IL-27 and TNF-α significantly augmented the release of IL-6 in comparison with that stimulated with individual cytokines (Figure 4.2.6A). To investigate whether the augmenting effect on IL-6 release was dose dependent on IL-27 and/or TNF-α, serial concentrations of IL-27 and TNF-α were used in the combined treatments. As shown in Figure 4.2.6B, the synergistic effect on IL-6 release could be further enhanced when the TNF-α concentration increased from 5 to 50 ng/ml in combination with a fixed IL-27 concentration (50 ng/ml). Moreover, a fixed concentration of TNF-α (20 ng/ml) with serial concentrations of IL-27 (10–100 ng/ml) showed that the augmenting effect could also be enhanced dose-dependently by IL-27, although it was less potent than that of TNF-α (Figure 4.2.6B). Since the addition of IL-27 and

TNF-α did not significantly increase the number of PBEC (Figure 4.2.7, all $p > 0.05$), the augmenting IL-6 release was not due to the increased number of PBEC. In BEAS-2B cells, we also found that IL-27 could significantly enhance TNF-α-induced IL-6 production with similar dose effects (Figure 4.2.8).

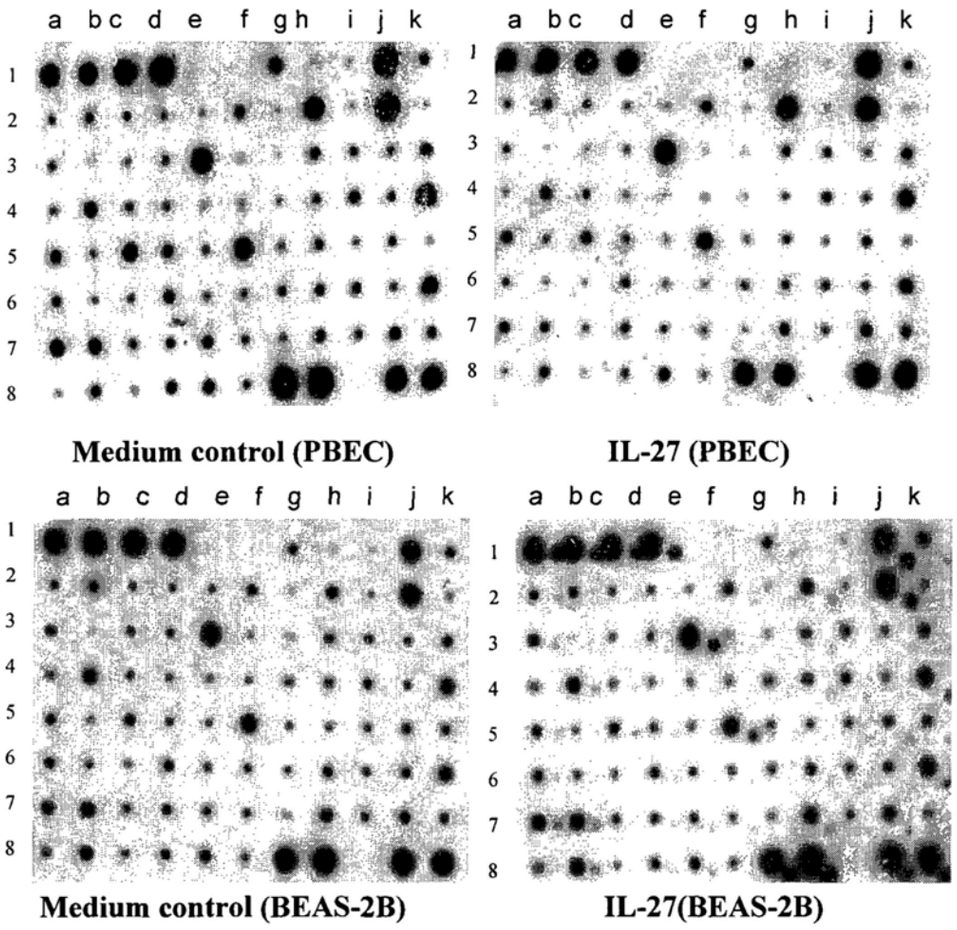


Figure 4.2.5 Representive profile of the release of cytokines from IL-27 activated PBEC or BEAS-2B. PBEC (1 x 10^6) or BEAS-2B cells (1 x 10^6) were treated with or without IL-27 (50 ng/ml) for 48 h. Cell free culture supernatant was then harvested and 79 different cytokines in culture supernatant were semi-quantitated using antibody based RayBio^{TM} human cytokine array V. Positive and negative controls were designated at (1a, 1b, 1c, 1d, 8j, 8k) and (1e, 1f, 8i) respectively. Triplicate experiments were performed with essentially identical results and representative figures are shown. Cytokine map of RayBio human cytokine array V is shown in Appendix (Page172-174).

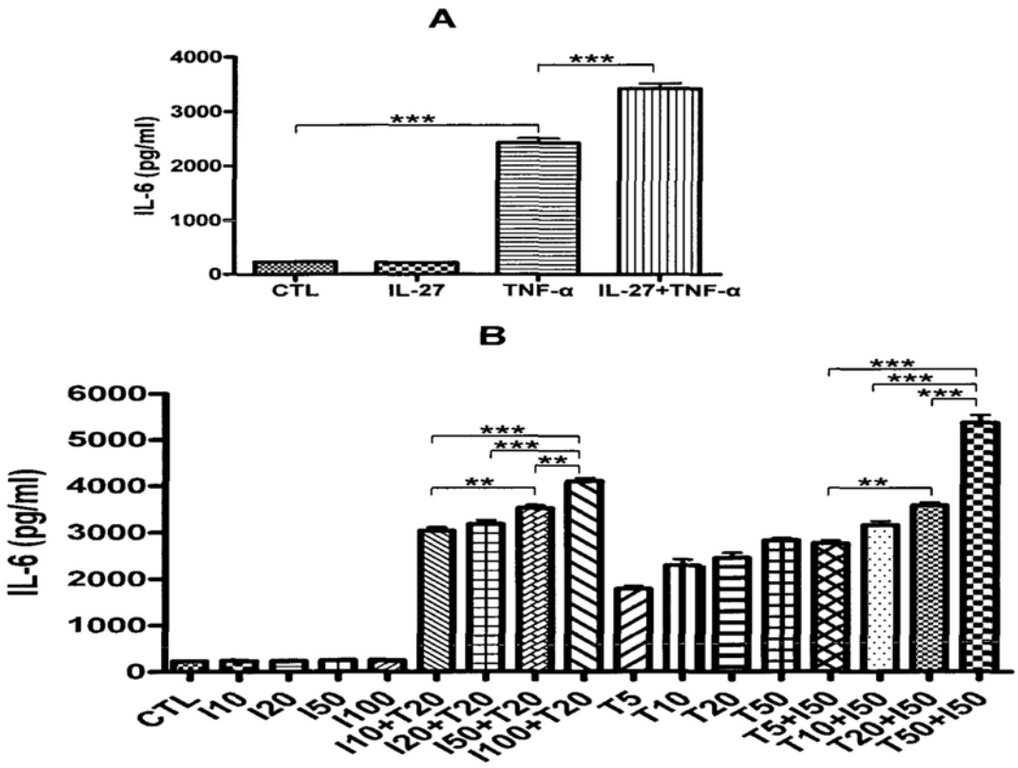


Figure 4.2.6 The enhanced effects of IL-27 on TNF-α-induced IL-6 release in PBEC. (A) PBEC were cultured with or without IL-27 (50 ng/ml), and TNF-α (20 ng/ml) alone or in combination for 48 h. IL-6 released from PBEC in culture supernatant was determined by ELISA. (B) The dose-dependent effects of IL-27 (I: 10-100 ng/ml) and TNF-α (T: 5-50 ng/ml) on the release of IL-6 from PBEC. Results are expressed as the arithmetic mean plus SD from three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ when compared between groups denoted by horizontal lines. Medium control: CTL.

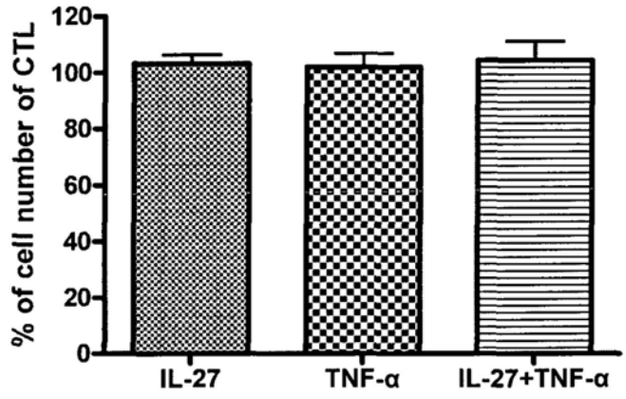


Figure 4.2.7 Effects of IL-27 and TNF-α on the cell number of PBEC. PBEC were cultured with or without IL-27 (50 ng/ml), and TNF-α (20 ng/ml) alone or in combination for 48 h, and PBEC were then counted with haemacytometer. Results are expressed as relative cell number % of that of medium control (CTL) from three independent experiments.

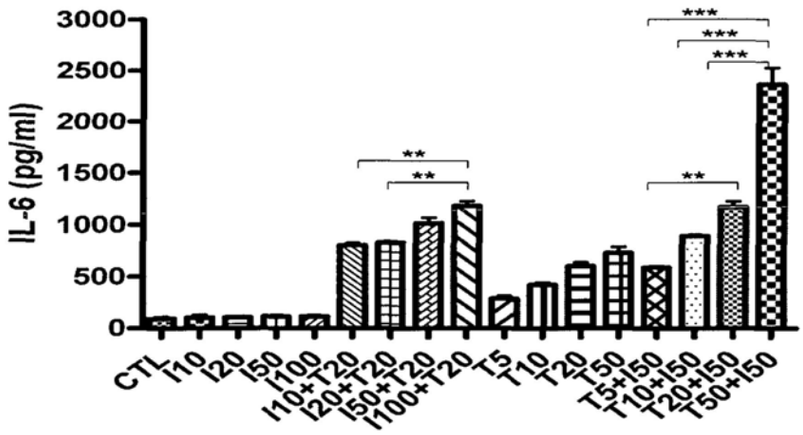


Figure 4.2.8 The dose-dependent effects of IL-27 (I: 10-100 ng/ml) and TNF-α (T: 5-50 ng/ml) on the release of IL-6 from BEAS-2B cells. BEAS-2B cells (1 x 10^5 cells) were cultured with or without IL-27 (50 ng/ml), and TNF-α (20 ng/ml) alone or in combination for 24 h. IL-6 released into the culture supernatants were determined by ELISA. Results are expressed as the arithmetric mean plus SD of three independent experiments. **$p < 0.01$, ***$p < 0.001$ when compared between groups denoted by horizontal lines.

4.2.4 IL-27 could enhance the protein expression of p55TNFR, but not P75TNFR on the surface of human bronchial epithelial cells

In order to explore the potential mechanism by which IL-27 enhanced TNF-α-induced ICAM-1 expression and IL-6 release in PBEC, we analyzed the modulating effect of IL-27 on the expression of TNF-α receptors. Using flow cytometry, IL-27 was found to significantly enhance the protein expression of p55TNFR (Figure 4.2.9A), but not p75TNFR (Figure 4.2.9B) on the cell surface of PBEC. However, the expression of IL-27 receptor complex of gp130 and WSX-1 was not up-regulated by the treatment TNF-α.

Using commercially available neutralizing/blocking monoclonal antibodies against TNF-α receptors, we further found that the augmenting effects of the combination of IL-27 and TNF-α on the up-regulation of ICAM-1 (Figure 4.2.10A) and IL-6 (Figure 4.2.10B) were significantly attenuated after preincubation and coincubation with neutralizing/blocking monoclonal antibodies against the p55TNFR but not p75TNFR.

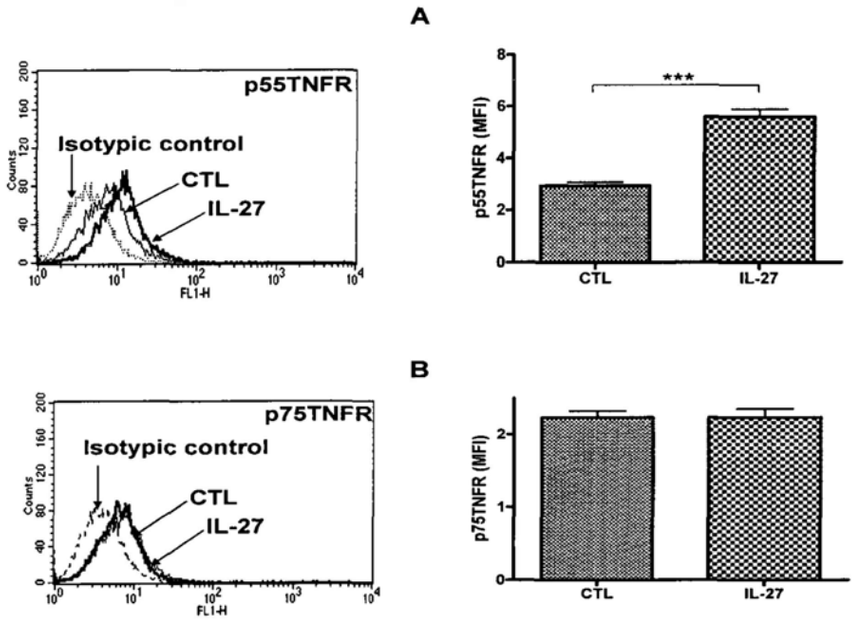


Figure 4.2.9 Effects of IL-27 on the protein expression of (A) p55TNFR and (B) p75TNFR on the cell surface of PBEC. PBEC were incubated with or without IL-27 for 48 h. Representative histograms illustrate the expression of p55TNFR and p75TNFR in 5,000 cells assessed by flow cytometry. Results of the expression of p55TNFR and p75TNFR are expressed as the arithmetic mean plus SD from three independent experiments. ***$p < 0.001$ when compared between groups denoted by horizontal lines. Medium control: CTL.

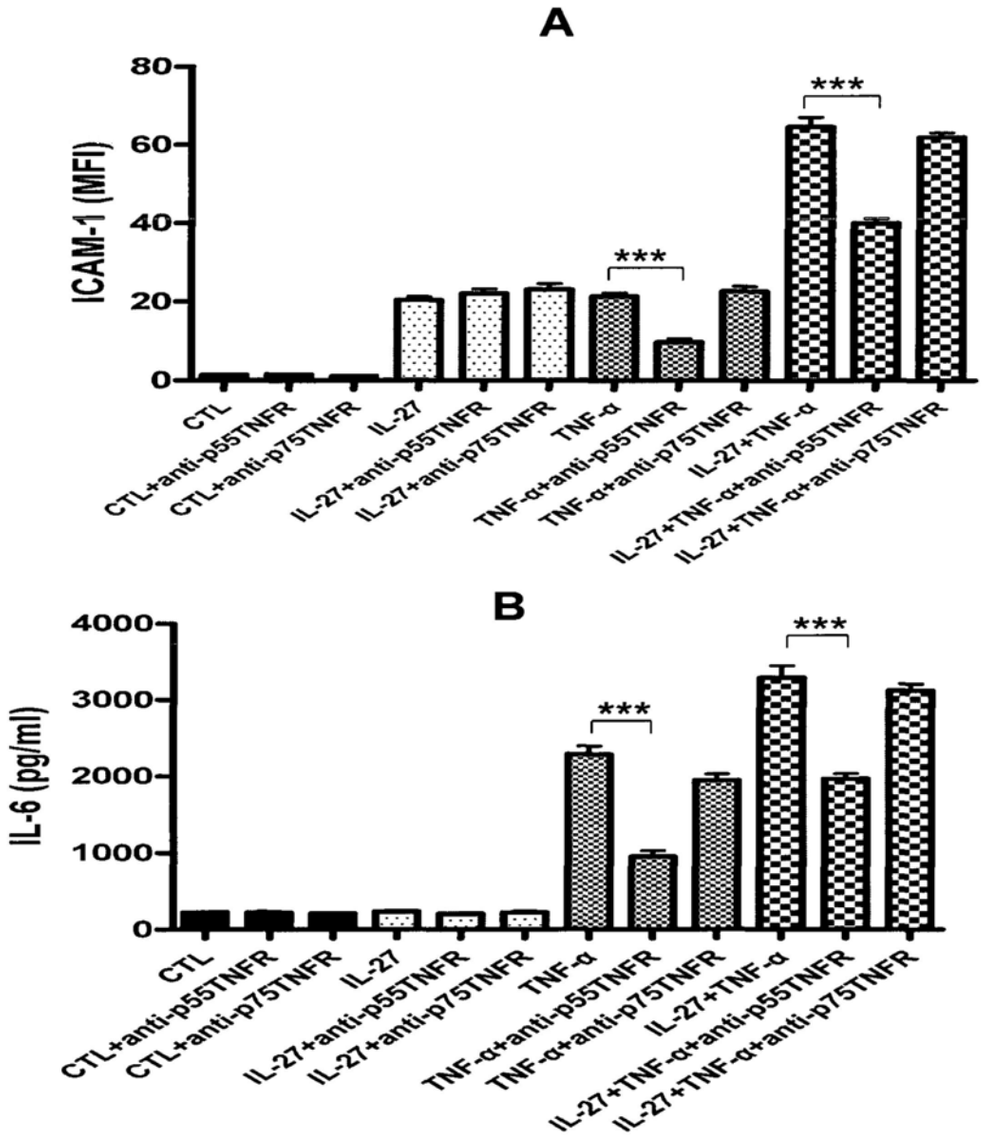


Figure 4.2.10 Effects of neutralizing/blocking monoclonal antibodies against TNF-α receptors on ICAM-1 expression and IL-6 release in PBEC. PBEC were pretreated with neutralizing anti-human p55TNFR (1 μg/ml) or p75TNFR (1 μg/ml) monoclonal antibodies for 2h followed by incubation with or without IL-27 (50 ng/mL) or TNF-α (20 ng/ml) for further 48h. The surface expression of (A) ICAM-1 was determined by flow cytometry and the release of (B) IL-6 was determined by

ELISA. Results are expressed as the arithmetic mean plus SD from three independent experiments. ***$p < 0.001$ when compared between groups denoted by horizontal lines. Medium control: CTL.

4.2.5 Effects of IL-27 and TNF-α on the activation of PI3K-Akt, p38 MAPK and nuclear factor (NF)-κB signaling pathways in human bronchial epithelial cells

Regarding the signal transduction mechanisms, we adopted a rapid and quantitative method established previously (Cheung *et al.*, 2008). Using intracellular fluorescence staining by flow cytometry, we measured the MFI of phosphorylated Akt, p38 MAPK, and IκB-α in fixed and permeabilized PBEC at 5 min after the stimulation by IL-27 and TNF-α, alone or in combination. As shown in Figure 4.2.11A-C, IL-27 could induce significant phosphorylation of Akt and IκB-α in PBEC. For TNF-α, it could induce the activation of Akt, p38 MAPK and IκB-α. However, combined treatment of IL-27 and TNF-α could not further increase the phosphorylation of Akt, p38 MAPK, and IκB-α. Representative histograms are also shown in Figure 4.2.12. In BEAS-2B cells, similarly, IL-27 could induce significant phosphorylation of Akt and IκB-α and TNF-α could activate Akt, p38 MAPK and IκB-α signaling pathways (Figure 4.2.13).

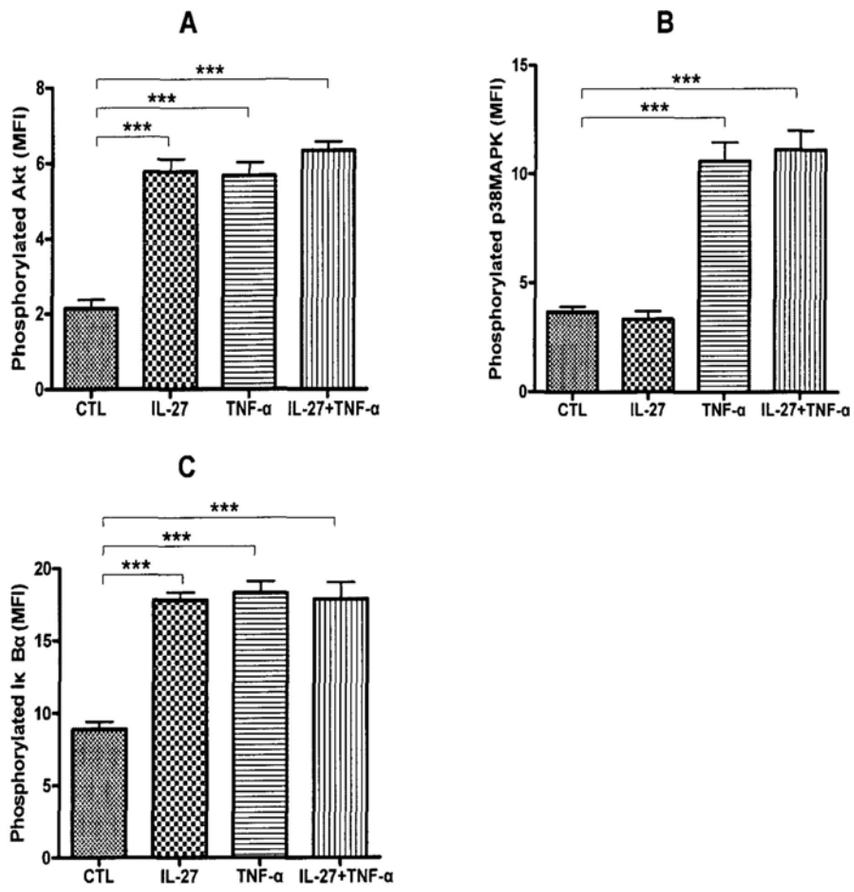
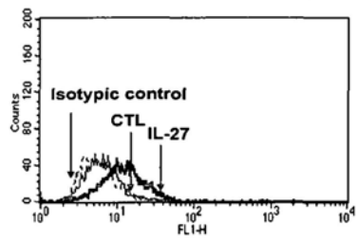
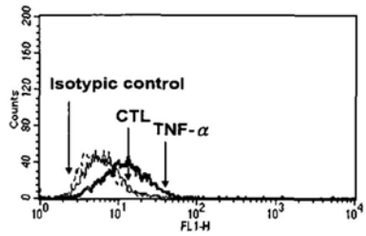
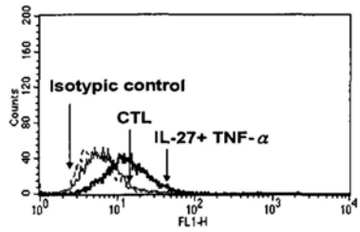


Figure 4.2.11 Effects of IL-27 and TNF-α on intracellular (A) Akt, (B) p38 MAPK and (C) NF-κB activities in PBEC. PBEC were incubated with or without IL-27 (50 ng/ml) and TNF-α (20 ng/ml), alone or in combination for 5 min. The amounts of intracellular phosphorylated signaling molecules in 5,000 permeabilized cells were measured by flow cytometry. Results of phosphorylated Akt, phosphorylated p38 MAPK, and phosphorylated IκB-α are shown in MFI subtracting corresponding isotypic control and are expressed as the arithmetic mean plus SD from three independent experiments. ***$p < 0.001$ when compared between groups denoted by horizontal lines. Medium control: CTL.

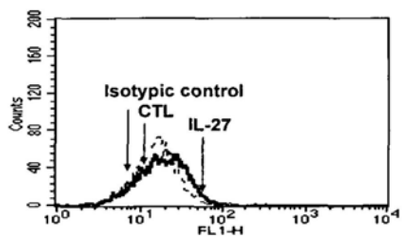
Phosphorylated Akt

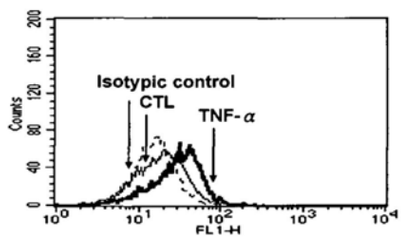


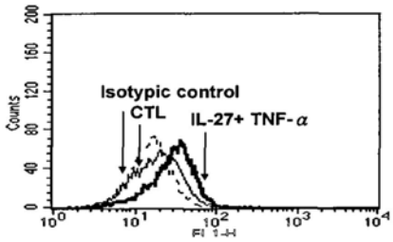




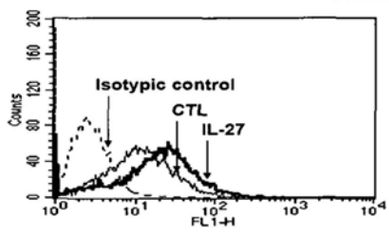
Phosphorylated p38MAPK

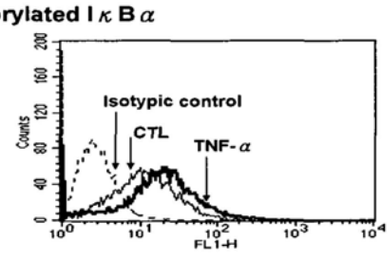






Phosphorylated I κ B α





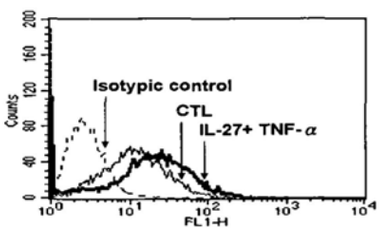
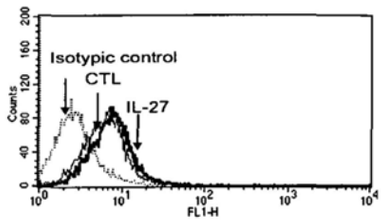
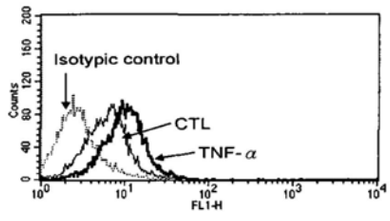
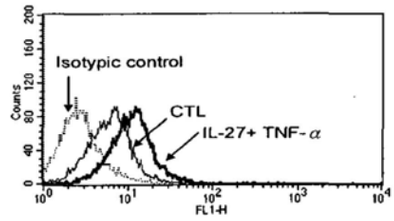


Figure 4.2.12 Effects of IL-27 and TNF-α on intracellular Akt, p38 MAPK and NF-κB activities in PBEC. PBEC were incubated with or without IL-27 (50 ng/ml) and TNF-α (20 ng/ml), alone or in combination for 5 min. After fixation and permeabilization, the amounts of intracellular phosphorylated signaling molecules in 5,000 permeabilized cells were measured by flow cytometry. Representative histograms illustrate the intracellular expression of phosphorylated Akt, phosphorylated p38 MAPK and phosphorylated IκB-α. The isotypic control represents the cell populations stained with anti-mouse IgG1 isotype control. Medium control: CTL.

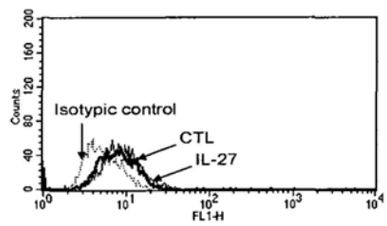
Phosphorylated Akt

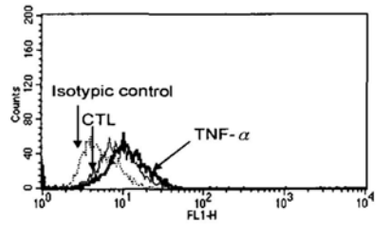


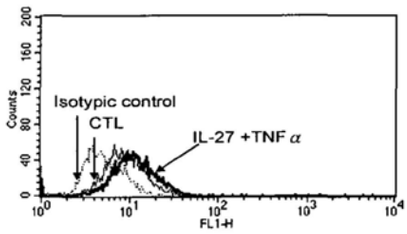




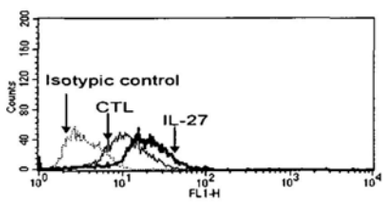
Phosphorylated p38MAPK

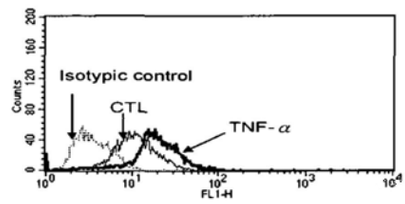






Phosphorylated I κ B α





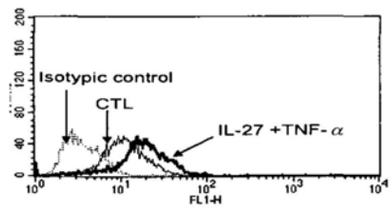


Figure 4.2.13 Effects of IL-27 and TNF-α on intracellular Akt, p38 MAPK, and NF-κB activities in BEAS-2B cells. BEAS-2B cells were incubated with or without IL-27 (50 ng/ml) and TNF-α (20 ng/ml), alone or in combination for 5 min followed by fixation, permibilization and intracellular staining. Representative histograms indicated by the arrows illustrate the intracellular expression of phosphorylated Akt, phosphorylated p38 MAPK, and phosphorylated IκB-α. The isotypic control represents the cell populations stained with anti-mouse IgG1 isotype control. The amounts of intracellular phosphorylated signaling molecules in 5,000 permeabilized cells were measured by flow cytometry.

4.2.6 IL-27 but not TNF-α could activate transcriptional factor Stat1 in human bronchial epithelial cells

Since IL-27 has been reported to activate Stat1 (Kalliolias and Ivashkiv, 2008), which has been shown to regulate ICAM-1 expression on human tracheobronchial epithelial cells upon the stimulation of IFN-γ (Walter *et al.*, 1997). We therefore examined the potential effects of IL-27 and TNF-α on the activation of Stat1 by intracellular fluorescence staining. Figure 4.2.14 shows that the phosphorylated Stat1 was highly expressed in PBEC upon IL-27 stimulation at 15 min. However, TNF-α could not activate Stat1, and the combined treatment of IL-27 and TNF-α could not enhance the phosphorylation of Stat1. Representative histograms are also shown in Figure 4.2.15.

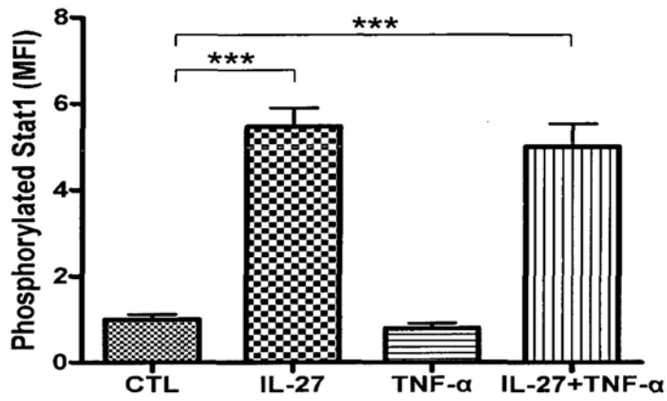


Figure 4.2.14 Effects of IL-27 and TNF-α on intracellular Stat1 activities in PBEC. PBEC were incubated with or without IL-27 (50 ng/ml) and TNF-α (20 ng/ml), alone or in combination for 15 min. The phosphorylated Stat1 was determined by intracellular fluorescence staining. Results of phosphorylated Stat1 are shown in MFI subtracting corresponding isotypic control and are expressed as the arithmetic mean plus SD from three independent experiments. ***$p < 0.001$ when compared between groups denoted by horizontal lines. Medium control: CTL.

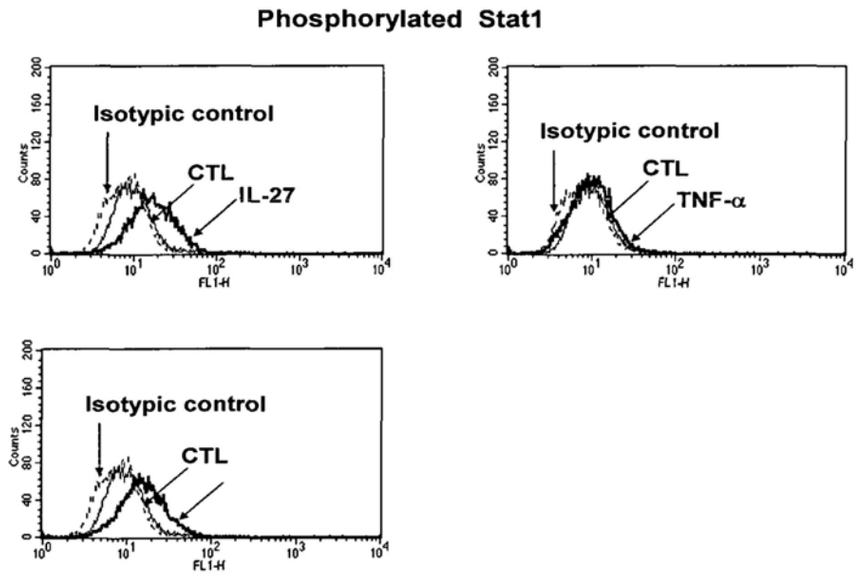


Figure 4.2.15 Effects of IL-27 and TNF-α on intracellular Stat1 activities in PBEC. PBEC were incubated with or without IL-27 (50 ng/ml) and TNF-α (20 ng/ml), alone or in combination for 15 min. Representative histograms illustrate the intracellular expression of phosphorylated Stat1 and isotypic control. Medium control: CTL.

4.2.7 Effects of signaling molecule inhibitors on ICAM-1 expression on the surface of human bronchial epithelial cells activated by IL-27 and TNF-α

The cytotoxicities of different signaling molecule inhibitors on PBEC were first determined by MTT assay (Figure 4. 2. 16), and the effective doses of these inhibitors with significant inhibitory effects on specific signaling pathways have been widely studied using different cell types including human bronchial epithelial cells (Cheung *et al.,* 2008; Bennett *et al.,* 2001; Ip WK *et al.,* 2007; Mori *et al.,* 2002; Wong *et al., 2010*). Following previous publications and toxicity threshold values from the MTT assay, we therefore used the optimal concentrations of JAK inhibitor AG490 (5 μM), NF-κB inhibitor BAY11-7082 (0.8 μM), PI3K inhibitor LY294002 (10 μM), ERK inhibitor U0126 (10 μM), p38 MAPK inhibitor SB203580 (20 μM) and JNK inhibitor SP600125 (5 μM) with significant inhibitory effects without any cell toxicity. As shown in Figure 4.2.17, LY294002 could significantly suppress IL-27-induced ICAM-1 expression on the surface of PBEC, while BAY11-7082 could significantly suppress TNF-α-induced up-regulation of ICAM-1. In addition, both LY294002 and BAY117082 could partially but significantly suppress the synergistic effect of IL-27 and TNF-α on ICAM-1 expression on the surface of PBEC. However, AG490, SB203580, SP600125 and U0126 did not exert any significant effect on the expression of ICAM-1 induced by IL-27 and TNF-α.

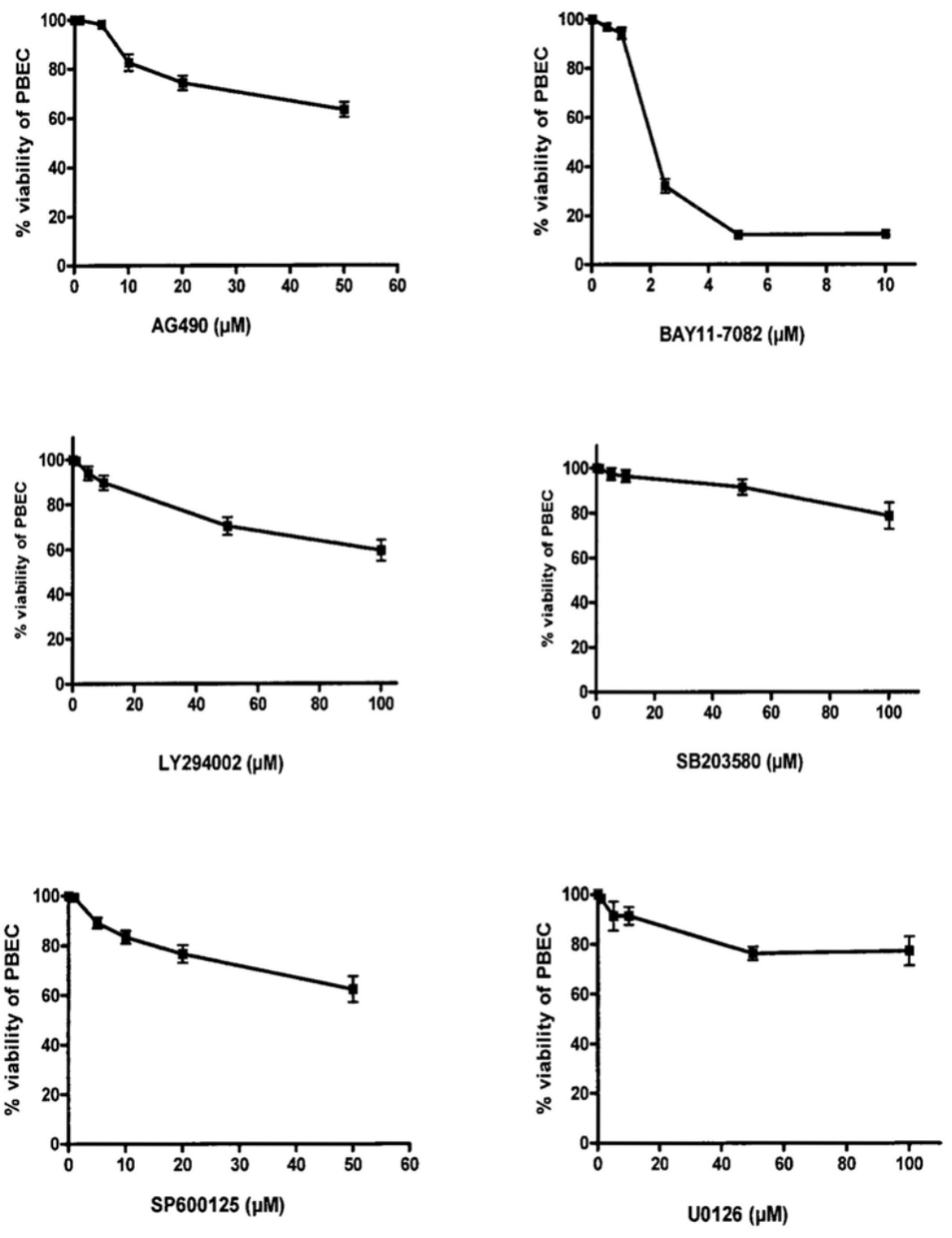


Figure 4.2.16 Effects of signaling molecule inhibitors on the viability of PBEC. PBEC (2 x 10^4/0.2 ml) were incubated with serial concentrations of AG490, BAY11-7082, LY294002, SB203580, SP600125 or U0126 for 48h, and the viability was assessed by MTT test. Results are expressed as arithmetic mean plus SD from triplicate experiments.

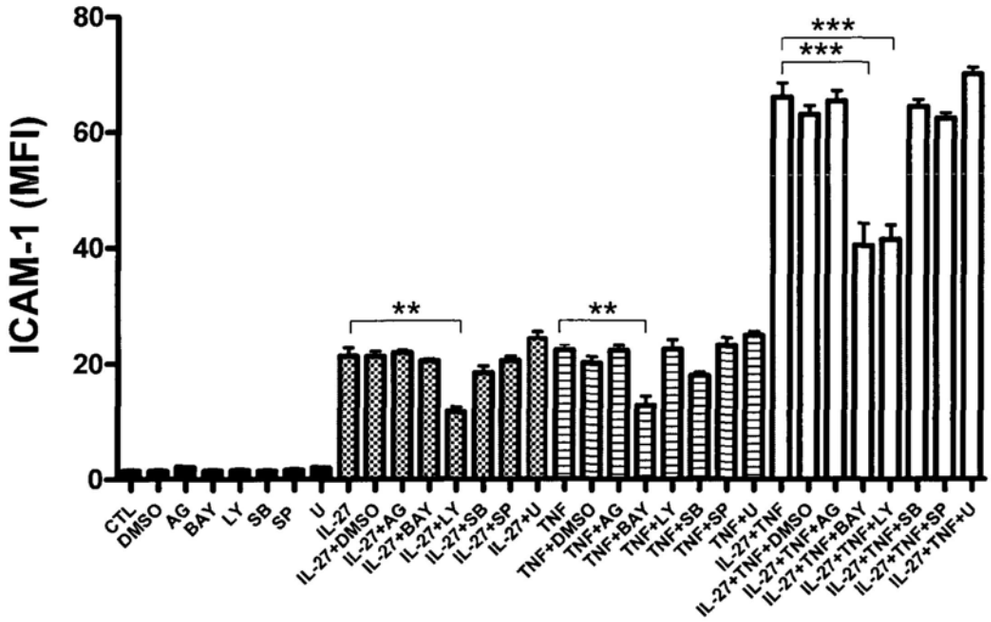


Figure 4.2.17 Effects of signaling molecule inhibitors on IL-27- and TNF-α-induced synergistic effect on ICAM-1 expression of PBEC. PBEC were pretreated with AG490 (AG, 5 μM), BAY11–7082 (BAY, 0.8 μM), LY294002 (LY, 10 μM), SB203580 (SB, 20 μM), SP600125 (SP, 5 μM) or U0126 (U, 10 μM) for 1 h followed by incubation with or without IL-27 (50 ng/mL) or TNF-α (20 ng/ml, TNF) in the presence of inhibitors for further 48 h. Surface expression of ICAM-1 of 5,000 cells was assessed by flow cytometry as MFI. Results are expressed as the arithmetic mean plus SD from three independent experiments. DMSO (0.1 %) was used as the vehicle control. **$p < 0.01$, ***$p < 0.001$ when compared between groups denoted by horizontal lines. Medium control: CTL.

4.2.8 Effects of signaling molecule inhibitors on the augmenting release of IL-6 in human bronchial epithelial cells activated by IL-27 and TNF-α

As shown in Figure 4.2.18, the release of IL-6 induced by TNF-α in PBEC could be significantly suppressed by BAY11–7082 and SB203580, and they could significantly suppress the augmenting effect of IL-27 on TNF-α-induced IL-6 release. Other inhibitors AG490, LY294002, SP600125 and U10126 did not exert any significant effect on this augmenting release of IL-6, and they also did not exert any significant effect on IL-6 induction in PBEC with or without IL-27 stimulation.

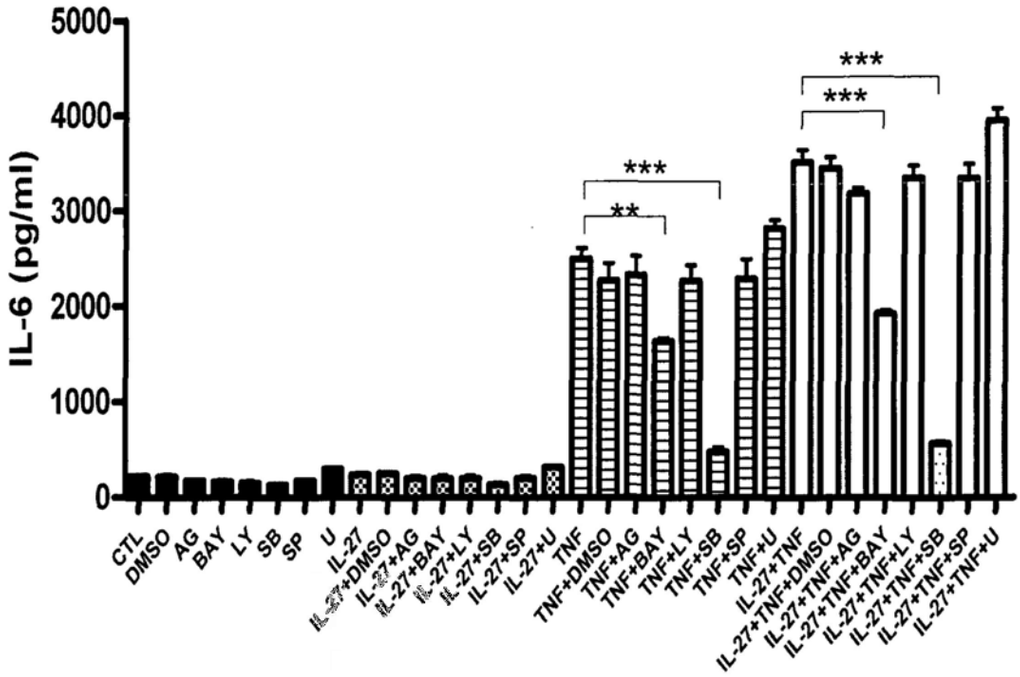


Figure 4.2.18 Effects of signaling molecule inhibitors on enhanced TNF-α-induced IL-6 production in PBEC by IL-27. PBEC were pretreated with AG490 (5 μM; AG), BAY11–7082 (0.8 μM; BAY), LY294002 (10 μM; LY), SB203580 (20 μM; LY), SP600125 (5 μM; SP) or U0126 (10 μM; U) for 1 h followed by incubation with or without IL-27 (50 ng/mL) or TNF-α (20 ng/ml, TNF) in the presence of inhibitors for further 48 h. The release of IL-6 was determined by ELISA. Results are expressed as the arithmetic mean plus SD from three independent experiments. DMSO (0.1 %) was used as the vehicle control. **$p < 0.01$, ***$p < 0.001$ when compared between groups denoted by horizontal lines. Medium control: CTL.

4.2.9 Effects of signaling molecule inhibitors on the phosphorylation of different signaling molecules

In order to verify the specificities of the inhibitors (BAY11-7082, SB203580, or LY294002) that could suppress the increase of ICAM-1 or IL-6 at previous used concentrations, we further examined the effects of these inhibitors on the phosphorylation of Akt, p38 MAPK, and IκB-α induced by IL-27 and TNF-α. As shown in Figure 4.2.19A-C, preincubation of PBEC with LY294002 could only significantly repress the phosphorylation of Akt but not IκB-α and p38MAPK (Figure 4.2.19A), and preincubation of PBEC with SB203580 could only significantly repress the phosphorylation of p38MAPK but not Akt and IκB-α (Figure 4.2.19B). Similarly, preincubation of PBEC with BAY11-7082 could only significantly inhibit the phosphorylation of IκB-α but not Akt and p38MAPK (Figure 4.2.19C).

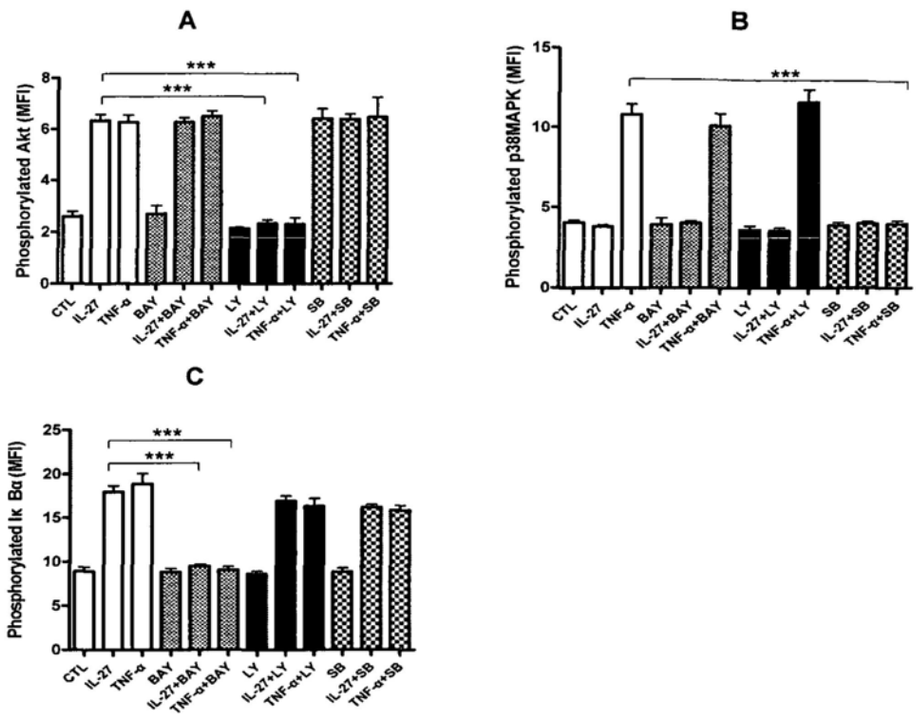


Figure 4.2.19 Effects of signaling molecule inhibitors on intracellular (A) Akt, (B) p38 MAPK and (C) NF-κB activities in well-differentiated PBEC stimulated by IL-27 and TNF-α. PBEC were pretreated with BAY11–7082 (0.8 μM; BAY), LY294002 (10 μM; LY), or SB203580 (20 μM; LY) for 1 h and then incubated with or without IL-27 (50 ng/mL) or TNF-α (20 ng/ml) for 5 min. After fixation and permibilization, the amounts of intracellular phosphorylated signaling molecules in 5,000 permeabilized cells were analyzed by flow cytometry. Results of phosphorylated Akt, phosphorylated p38 MAPK, and phosphorylated IκB-α are shown in MFI subtracting corresponding isotypic control and are expressed as the arithmetic mean plus SD from three independent experiments. ***$p < 0.001$ when compared between groups denoted by horizontal lines. Medium control: CTL.

4.3 Discussion

Human bronchial epithelial cells are essentially involved in innate and adaptive immunities by expressing a variety of inflammatory mediators beyond their roles in forming the first physical barrier. However, molecular mechanisms regulating the production of inflammatory mediators from human bronchial epithelial cells remain to be fully elucidated. Previous studies have shown that IL-27 could play an active role in regulating the biological functions of different cell types including T cells, NK cells, monocytes, mast cells and keratinocytes (Takeda *et al.,* 2003; Neufert *et al.*, 2007; Kalliolias and Ivashkiv, 2008; Wittmann *et al.,* 2009). In this study, using flow cytometric analysis and ELISA, we further found that IL-27 was able to induce the expression of ICAM-1 and to augment TNF-α-induced production of ICAM-1 and IL-6 in human bronchial epithelial cells via the differential activation of PI3K-Akt, p38 MAPK and NF-κB pathways, thereby suggesting a potential role of IL-27 in the pathogenesis of airway infection or inflammatory diseases.

It is well known that ICAM-1 is a transmembrane glycoprotein of the immunoglobulin supergene family expressed by a variety of cell types, and the expression of ICAM-1 could facilitate the progress of inflammatory reactions. As a ligand for integrins, the interaction of ICAM-1 and integrins is essentially involved in leukocyte recruitment and transendothelial migration (Wegner *et al.,* 1990).

ICAM-1 is low-expressed by bronchial epithelial cells in normal conditions, but its level of expression can be up-regulated by inflammatory cytokines such as TNF-α, IFN-γ or IL-1β (Krunkosky *et al.,* 2000; Dustin and Springer, 1998; Look *et al.,* 1992). Moreover, increased expression of ICAM-1 on airway epithelial cells has been observed in inflammatory lung diseases such as asthma and chronic bronchitis, as well as in hyperoxic lung injury and airway hyperresponsiveness, indicating its active role in the pathogenesis of airway inflammatory diseases (Wegner *et al.,* 1990; Welty *et al.,* 1993; Broide and Sriramarao, 2001). Blockade of ICAM-1 functions by antisense oligonucleotides or monoclonal antibodies is a potential way for treating inflammatory diseases (Xu and Li, 2009; Mackay, 2008). Here, we firstly reported that IL-27 is another potent inducer of ICAM-1, while other IL-6/IL-12 family cytokines IL-12 and IL-23 could not increase the expression of ICAM-1 on the surface of human bronchial epithelial cells. The up-regulation of ICAM-1 by IL-27 secreted by APCs provides a functional linkage between APC and bronchial epithelial cells and may be important for understanding the inflammatory responses in the airways.

There are a variety of cytokines contributing to the progress of airway inflammation and they can interact with each other to regulate inflammation in the airways. TNF-α and IFN-γ have been shown to have multiple biological effects and

have been involved in the pathogenesis of airway inflammatory diseases including asthma and pulmonary fibrosis, and they could enhance the expression of ICAM-1 and IL-6 in human bronchial epithelial cells (Li *et al.,* 1995; Kawaguchi *et al.,* 2001; Reisinger *et al.,* 2005). To examine whether IL-27 modulates the production of ICAM-1 and IL-6 in human bronchial epithelial cells activated by either TNF-α or IFN-γ, we conducted a comparative study in cells after stimulation with individual cytokines. We found that these cytokines IL-27, TNF-α, and IFN-γ could potently increase the cell-surface expression of ICAM-1, which concurs with findings of previous studies (Krunkosky *et al.,* 2000; Kawaguchi *et al.,* 2001). Synergistic effect was observed in the combined treatment of IL-27 and TNF-α but not IFN-γ on the expression of ICAM-1. Besides, we also found that this synergistic effect was dose-dependent on TNF-α but not IL-27, suggesting that it was mainly due to the effects of TNF-α. IL-27 actually augments the expression of IL-6 in bronchial epithelial cells when they are stimulated with TNF-α, although IL-27 alone did not induce IL-6 expression, which is in contrast to the finding of a significant induction of IL-6 expression by TNF-α. Further study showed that the augmenting effect on IL-6 release was dose-dependent on IL-27 and TNF-α. While the synergistic release of IL-6 could be enhanced dose-dependently by either TNF-α or IL-27, the synergistic surface expression of ICAM-1 was dose-dependent on TNF-α but not

IL-27. These results indicate that the effective concentration of TNF-α is crucial for the synergistic surface expression of ICAM-1, which may be the reason for that the increase in the concentration of IL-27 (10-100 ng/ml) could not further enhance the synergistic expression of ICAM-1 at a constant concentration of TNF-α, at least at the concentration of 20 ng/ml. Although the IL-27 and TNF-α doses in the present study are similar to previous doses adopted in *in vitro* study (Kanda and Watanabe, 2008; Wittmann *et al.,* 2009; Krunkosky *et al.,* 2000), we cannot exclude the possibility that the doses of IL-27 and TNF-α in this experiment were still not well optimized, and TNF-α (20 ng/ml) may require other different doses of IL-27 to further enhance the synergistic surface expression of ICAM-1.

Our findings that IL-27 could augment TNF-α-induced up-regulation of ICAM-1 and IL-6 in human bronchial epithelial cells are somewhat consistent with that of IL-27 promoting IL-1β-induced β-defensin-2 expression in human keratinocytes (Kanda and Watanabe, 2008). Besides, the augmenting effect on IL-6 release by IL-27 may be partially related to the reduced level of IL-6 in EBI3−/− mice as compared with wild-type mice (Wirtz *et al.,* 2006). IL-6 and TNF-α are important effector cytokines that mediate damage in airway tissues (Martin *et al.,* 1997; Khair *et al.,* 1996). The potential involvement of IL-27 in airway inflammation is therefore, at least in part, through the induction of ICAM-1 and IL-6 interacting

with TNF-α. Although we found that IL-27 could not increase the production of TNF-α in human bronchial epithelial cells (data not shown), a recent study has demonstrated that IL-27 could activate human primary monocytes and mast cells to express an increased level of TNF-α (Pflanz *et al.,* 2004). Human monocytes and mast cells are also well recognized effector cells essentially involved in airway inflammation, the elevated production of ICAM-1 and IL-6 in human bronchial epithelial cells activated by IL-27 interacting with TNF-α may thus contribute both to the development of airway remodeling and inflammation. Besides, due to the fact that IL-27 production from APC is induced upon microbial stimuli, the ability of IL-27 to enhance TNF-α activation on bronchial epithelial cells might also imply a potential link between microbial infection and airway diseases. Our results provide new insight into the molecular mechanisms involved in airway remodeling and inflammation.

The mechanism by which IL-27 interacts with TNF-α in augmenting ICAM-1 expression and IL-6 production is currently unclear. We therefore analyzed the expression of their receptors. We found that IL-27 could enhance the protein expression of p55TNFR (TNFR1), but not P75TNFR (TNFR2) on the surface of human bronchial epithelial cells. In accordance with a previous report that TNF-α–induced ICAM-1 surface expression was attenuated after preincubation and

coincubation with a commercially available neutralizing/blocking monoclonal antibody against the TNFR1 but not TNFR2 (Krunkosky *et al.,* 2000), we further found that neutralizing/blocking monoclonal antibodies against the p55TNFR could partially but significantly suppress the synergistic effect of IL-27 and TNF-α on ICAM-1 surface expression of human bronchial epithelial cells, the synergistic effects on the production of ICAM-1 and IL-6 might be due to the up-regulation of TNFR1 expression induced by IL-27. However, there is still the possibility that additional post-transcriptional and/or translational regulation could account for the regulation of ICAM-1 and IL-6 expression by IL-27 and TNF-α, and further work is needed to address this issue.

JAK/STAT signaling pathway has been involved in IL-27-mediated immune responses in T cells (Hunter, 2005). However, little information has been available regarding the intracellular signaling pathway of IL-27 in bronchial epithelial cells. Our previous studies suggested the important roles of NF-κB, ERK, JNK, JAK and p38 MAPK pathways in the expression of adhesion molecules and release of cytokines and chemokines from activated bronchial epithelial cells upon exposure to diverse stimuli such as TNF-α, IL-4, IL-13 and IL-31 (Ip *et al.,* 2006; Wong *et al.,* 2006; Ip *et al.,* 2007). The present intracellular mechanistic study indicated that IL-27 could activate PI3K-Akt, NF-κB and Stat1 pathways, while PI3K-Akt, p38

MAPK and NF-κB pathways were activated in response to TNF-α. Following the results of MTT cytotoxicity assays for the signaling molecule inhibitors, we used the optimal concentrations of JAK inhibitor AG490, NF-κB inhibitor BAY11-7082, PI3K inhibitor LY294002, ERK inhibitor U0126, p38 MAPK inhibitor SB203580 and JNK inhibitor SP600125 for significant inhibitory effects without any cell toxicity, we further elucidated the involvement of different signaling pathways in regulating ICAM-1 expression. It was shown that IL-27-induced up-regulation of ICAM-1 was regulated by the activation of intracellular PI3K-Akt but not NF-κB or JAK/Stat, while TNF-α-induced up-regulation of ICAM-1 was regulated by NF-κB but not PI3K-Akt or JAK/Stat. Besides, JAK/Stat pathway has been shown to regulate ICAM-1 expression in human tracheobronchial epithelial cells upon the stimulation of IFN-γ in another study (Walter *et al.*, 1997). The above discrepancy of the intracellular signaling mechanisms of different cytokines for the induction of ICAM-1 expression suggests that different signaling pathways selectively regulate ICAM-1 expression in a cytokine-specific manner during immune and/or inflammatory responses. Since the induction of ICAM-1 by IL-27 and TNF-α was not completely inhibited by LY294002 and SB203580, respectively, other unidentified signaling pathways might also contribute to the expression of ICAM-1. Anyway, here we identified that PI3K-Akt is a novel signaling pathway of IL-27 in

regulating the expression of ICAM-1 on the surface of human bronchial epithelial cells.

Regarding IL-6 release, the enhanced TNF-α-induced IL-6 production by IL-27 was mediated by both p38 MAPK and NF-κB pathways. It is reasonable that p38 MAPK and NF-κB pathways are commonly involved in the release of IL-6 from human bronchial epithelial cells, since they are highly active at airway inflammatory sites in various diseases, including airway infectious diseases (Pomerantz and Baltimore, 2002; Underwood *et al.,* 2000). On the other hand, inhibition of p38 MAPK suppressed the augmenting production of IL-6 to nearly the basal level, while inhibition of NF-κB could partially suppress the augmenting release of IL-6. These results suggest that the augmenting production of IL-6 is regulated by p38 MAPK pathway dominantly and at least in part via NF-κB pathway. In fact, previous reports have demonstrated that p38 MAPK is required for NF-κB–dependent cytokine gene expression by the modulation of DNA binding of TATA-binding protein to the TATA box (Bergmann *et al.*, 1998; Carter *et al.,* 1999), and there may be a cross-talk between p38 MAPK and other unknown signaling pathways which mediates the release of IL-6 in bronchial epithelial cells. Besides, IL-27 could also activate JAK/Stat signaling pathway in bronchial epithelial cells, but we found that activation of JAK/Stat was not involved in the IL-27-enhanced ICAM-1 expression and IL-6

release because JAK inhibitor AG490 could not suppress the elevated production of ICAM-1 and IL-6. Therefore, the functional role of activated JAK pathway in bronchial epithelial cells requires further investigation.

In conclusion, this study firstly demonstrates that IL-27 alone or in combination with TNF-α could modulate ICAM-1 and IL-6 production in human bronchial epithelial cells via, at least in part, the activation of PI3K-Akt, p38 MAPK and NF-κB pathways, suggesting a novel role of IL-27 in linking airway innate and adaptive immunities. In airway inflammatory responses, APC are activated and in turn produce IL-27. Our present results thus suggest that IL-27 is a new stimulator for ICAM-1 and IL-6 production and may promote and potentiate airway inflammation. Moreover, since TNF-α is a well recognized crucial cytokine in the initiation of airway inflammation, the combined effects of IL-27 and TNF-α on human bronchial epithelial cells would further strengthen their roles in the pathogenesis of airway inflammation.

Chapter 5

Conclusions and Future Perspectives

5.1 General conclusions

Airway inflammatory diseases are common diseases with an increasing prevalence in the world, but current therapies such as steroids could not provide clinical benefit to all suffered patients, and broad-spectrum anti-inflammatory approaches are suggested to treat airway inflammatory diseases (Desai and Brightling, 2009; Barnes, 2009). There have been increased evidences demonstrating that the activation of bronchial epithelial cells plays an important role in the orchestration of inflammatory responses in the airway inflammatory diseases (Martin *et al.*, 1997; Schleimer *et al.*, 2007). However, the detailed molecular mechanisms regulating the activation of bronchial epithelial cells in response to different stimuli remain to be elucidated.

Airway inflammation is a multi-cellular process involving the interactions among bronchial epithelial cells and other inflammatory cells, or cytokines, chemokines and growth factors. Our group has previously indicated the pivotal roles of Th2 cytokines IL-4, IL-13, IL-31 and allergen Derp1 protein in mediating the expression of adhesion molecules and release of cytokines and chemokines from human bronchial

epithelial cells (Ip *et al.*, 2006; Wong *et al.*, 2006; Ip *et al.*, 2007). Besides, we also found that interaction of human bronchial epithelial cells and eosinophils could induce the release of cytokines and chemokines and expression of adhesion molecules (Wong *et al.*, 2005; Wang, *et al.*, 2005). These above effects were regulated by the activation of different signaling pathways including MAPKs and NF-κB. In this thesis, we provide new insight into the molecular mechanisms by which human bronchial epithelial cells become activated in airway inflammation. We found that human bronchial epithelial cells could be activated by different stimuli including basophils, IL-17, IL-27 and TNF-α through the activation of different signaling pathways, thereby leading to the increased production of various inflammatory mediators.

Basophils are the rare circulating granulocytes that can infiltrate and accumulate at the airways of asthmatics as a result of allergic stimuli and an over-exuberant Th2 cell immune response, thereby contributing to airway inflammation and hyperresponsiveness (Marone *et al.*, 2002; Galli *et al.*, 2005). Previous studies have indicated that there were increased numbers of basophils in bronchial biopsy specimens from asthmatic as compared with normal subjects, which could be further enhanced after allergen challenge (Gauvreau *et al.*, 2000; Nouri-Aria *et al.*, 2001). IL-17A is the hallmark cytokine of Th17 cells, which has been shown to play

important roles in airway inflammatory and other diseases ((Iwakura *et al.*, 2008; Yagi *et al.*, 2007). Here we found that interaction between human bronchial epithelial cells and basophils could increase the release of inflammatory cytokine IL-6 and chemokine CCL2 from human bronchial epithelial cells, while IL-17A could further augment the release of IL-6 and CCL2 in this co-culture system. Besides, surface expression of adhesion molecule ICAM-1 on bronchial epithelial cells was also up-regulated. The increased production of IL-6, CCL2 and ICAM-1 would exaggerate the inflammatory reactions in the airways of allergic inflammation. For the signaling transduction mechanisms, we found that intracellular ERK, JNK, p38 MAPK and NK-κB pathways differentially regulated the elevated production of these inflammatory mediators upon the interaction between human bronchial epithelial cells and basophils activated by IL-17A. Taken together, our findings firstly demonstrated that basophils could facilitate the activation of bronchial epithelial cells by Th17 cell cytokine IL-17A, thereby suggesting a novel immunopathological role of Th17 cells and basophils in allergic asthma. In fact, both bronchial epithelial cells and basophils are involved in initiating allergic inflammation in the airways. Cross-talk among them and other inflammatory cells participating in allergic inflammation through the production of Th17 cytokines including IL-17A and IL-17F or Th2 cytokines including IL-4, IL-13, IL-33 and

TSLP would result in feedback loops contributing to dysregulated allergic inflammation in the airways (Paul and Zhu 2010). Elucidating the cross-regulation among different cell types will be helpful for controlling allergic inflammation in asthma patients.

Apart from basophils and cytokine IL-17A produced by Th17 cells, IL-27, a novel member of the IL-6/IL-12 family cytokines produced by APC, was also found to activate human bronchial epithelial cells. We demonstrated firstly that IL-27 was an inducer of ICAM-1 on the surface of human bronchial epithelial cells, and the combined treatment of IL-27 and TNF-α resulted in a synergistic effect on ICAM-1 expression. Besides, IL-27 could augment TNF-α-induced IL-6 production. The synergistic effects of IL-27 and TNF-α on ICAM-1 and IL-6 production were partially due to the up-regulation of TNF-α receptor p55TNFR expression induced by IL-27. Furthermore, these effects on the enhanced production of ICAM-1 and IL-6 induced by IL-27 and TNF-α were differentially regulated by the activation of PI3K-Akt, p38 MAPK and NF-κB pathways. Our study therefore suggests a novel role of IL-27 and TNF-α in the pathogenesis of airway inflammatory diseases. IL-27 is a pleiotropic cytokine that exerts multiple biologic effects on different cell types. Augmentation of IL-27 effects could suppress the progress of some allergic diseases, autoimmune diseases and tumors *in vivo* (Yoshida *et al.,* 2009). However, all these

findings are observed in animal models. In humans, the precise role of IL-27 has not been established. The activation effects of IL-27 on human bronchial epithelial cells may have important therapeutic implications, and monoclonal antibodies or specific small molecule inhibitors targeting against IL-27 or its receptor WSX-1 may be useful in treating airway inflammatory diseases.

In summary, all of findings in this thesis demonstrated that human bronchial epithelial cells could be activated by a variety of stimuli including IL-17A, IL-27, TNF-α and the interaction with basophils. Besides, we also investigated the underlying intercellular signaling mechanisms regulating the activation of bronchial epithelial cells by these stimuli. Our results unveil some previously unknown molecular mechanisms involved in airway inflammation, and may contribute to treat airway inflammatory diseases.

5.2 Future perspectives

Bronchial epithelial cells are crucial participants in airway inflammation, and improved clarification of the role of bronchial epithelial cells in immune and inflammatory responses would provide new insight for developing new therapeutic approaches. The activation of human bronchial epithelial cells by IL-17A, IL-27, TNF-α and basophils would promote us to develop anti-IL-17A, anti-IL-27,

anti-TNF-α and anti-basophil therapeutic monoclonal antibodies or small molecule inhibitors for treating airway inflammatory diseases by specifically inhibiting their biological functions.

The role of basophils in the pathogenesis of allergic airway inflammation has been clarified step-by-step in recent years. Since respiratory infection can cause allergen sensitization and subsequently amplify and sustain airway inflammation in allergic asthma, we will continue to elucidate NLR and RIG-I-like receptors (RLR)-mediated activation of human basophils upon interacting with bronchial epithelial cells. Since genetic factors including gene polymorphisms also play an important role in airway inflammatory diseases (Leung, 1998), we will continue to study the association between airway inflammatory diseases and the gene polymorphisms of IL-17A, IL-27 and TNF-α. Besides, microRNA (miRNA) has emerged to play a pivotal role in regulating immune responses (Bi *et al.,* 2009), the study on the function and mechanism of miRNA in the activation of human bronchial epithelial cells and basophils would further our understanding about the pathogenesis of airway inflammatory diseases.

Infiltration of neutrophils, mast cells, DC, T cells, B cells and other inflammatory cells into bronchial epithelium has been observed in airway inflammatory diseases (Kato and Schleimer, 2007), and the detailed immunopathological effects of these

cells interacting with bronchial epithelium on the inflammatory responses in airway inflammatory diseases are still unknown. In our unpublished data, we found that co-culture of human mast cell line LAD-2 with BEAS-2B could induce the release of IL-6 and CXCL1 and CXCL8, suggesting that interaction with human mast cells could also activate bronchial epithelial cells. In addition to bronchial epithelial cells, other tissue structural cells including smooth muscle cells and fibroblasts also participate in the development and progress of airway inflammation (Meurs *et al.*, 2008), how they induce the local inflammation in the airways is an important topic which requires further studies. Besides, there are some newly identified epithelial cytokines, including TSLP, IL-33 and BAFF (Kato and Schleimer, 2007), the effects of them on the activation of basophils will also help us to understand more about the molecular pathogenesis of airway inflammatory diseases. Notably, a recent study has suggested that a nuclear protein, high-mobility group box 1 (HMGB1) was increased in COPD patients compared with healthy individuals, and receptor for advanced glycation end products (RAGE) co-localized with HMGB1 was overexpressed in the airway epithelium and smooth muscle of COPD patients (Ferhani *et al.*, 2010),. Therefore, we shall also investigate the effects of HMGB1 on the biological functions of human bronchial epithelial cells and basophils.

Although *in vitro* model systems using human primary cells and cell lines are

particularly useful in unraveling potential mechanisms of airway inflammation at the cellular and molecular levels, it is also necessary for us to establish excellent animal models in order to investigate airway pathophysiology systemically *in vivo* due to the complex variety of interactive processes involved in airway inflammation. Until now, animal models of airway inflammation have been successfully established using mouse, rats and guinea pig (Meurs *et al.,* 2008). Besides, with the availability of transgenic and gene-targeted animals, as well as the generation of cell-function-deficient animals by using depleting antibodies (Obata *et al.,* 2007; Meurs *et al.,* 2008), we will move our next step from the understandings of the activation of human bronchial epithelial cells by IL-17A, IL-27, TNF-α and basophils in *in vitro* cell models to *in vivo* animal models, which will definitely provide supplementary information about the pathogenesis of airway inflammation.

Although corticosteroids are effective in suppressing airway inflammation in the majority of asthma cases, about 10% asthma patients remain poorly treated by these drugs and there is a marked corticosteroid resistance in COPD patients (Desai and Brightling, 2009; Meurs *et al.,* 2008). The attempts to inhibit cytokines including TNF-α and IL-6 using specific blocking antibodies have been done in some patients, but the results were not satisfactory, while anti-TSLP and anti-IL-1β therapeutic drugs are under development (Desai and Brightling, 2009). Signaling molecule

inhibitors are other potential therapeutic drugs, as they can suppress gene expression of multiple cytokines and chemokines, however, systemic treatment using signaling molecule inhibitors may cause severe side effects, and further studies are required to confirm the clinical safety of signaling molecule inhibitor drugs (O'Neill, 2006). Besides, investigating the effects of Traditional Chinese Medicine (TCM) on inhibiting the activation of human bronchial epithelial cells by different stimuli is our ongoing work and will contribute to drug development for treating airway inflammatory diseases.

Appendix

Cytokine map of RayBio human cytokine array V

Location	Abbreviation	Name of cytokine
a1	Pos	Positive control
a2	I-309	CC Chemokine I-309
a3	IL-12p40p70	IL-12
a4	MIP-1δ	Macrophage inflammatory protein-1δ
a5	Oncostain M	Oncostain M
a6	FGF-4	Fibroblast growth factor-4
a7	IGFBP-3	Insulin-like growth factor binding protein-3
a8	NT-4	Neurotrophin-4
b1	Pos	Positive control
b2	IL-1α	IL-1α
b3	IL-13	IL-13
b4	RANTES	Regulated upon activation, normal T-cell expressed, and secreted
b5	Thromopoietin	Thromopoietin
b6	FDF-6	Fiber differentiation factor-6
b7	IGFBP-4	Insulin-like growth factor binding protein-4
b8	Osteoprotegerin	Osteoprotegerin
c1	Pos	Positive control
c2	IL-1β	IL-1β
c3	IL-15	IL-15
c4	SCF	Stem cell factor
c5	VEGF	Vascular endothelial growth factor
c6	FGF-7	Fibroblast growth factor-7
c7	IL-16	IL-16
c8	PARC	Pulmonary and activation-regulated chemokine
d1	Pos	Positive control
d2	IL-2	IL-2
d3	IFN-γ	IFN-γ
d4	SDF-1	Stromal cell-derived factor-1
d5	PDGF-BB	Platelet-derived growth factor-BB
d6	FGF-9	Fibroblast growth factor-9

d7	IP-10	Interferon γ-inducible protein 10
d8	PIGF	Placenta growth factor
e1	Neg	Negative control
e2	IL-3	IL-3
e3	MCP-1	Monocyte chemotactic protein-1
e4	TARC	Thymus and activation-regulated chemokine
e5	Leptin	Leptin
e6	FIT-3 Ligand	FIT-3 Ligand
e7	LIF	Leukemia inhibitory factor
e8	TGF-β2	Transforming growth factor- β2
f1	Neg	Negative control
f2	IL-4	IL-4
f3	MCP-2	Monocyte chemotactic protein-2
f4	TGF-β1	Transforming growth factor- β1
f5	BDNF	Brain-derived neurotrophic factor
f6	Fractalkine	Fractalkine
f7	LIGHT	Lymphotoxins, inducible expression, competes with HSV glycoprotein D for HVEM, a receptor expressed on T-lymphocytes
f8	TGF-β3	Transforming growth factor- β3
g1	ENA-78	Epithelial neutrophil activating peptide
g2	IL-5	IL-5
g3	MCP-3	Monocyte chemotactic protein-3
g4	TNF-α	Tumor necrosis factor-α
g5	BLC	B lymphocyte chemokine
g6	GCP-2	Granulocyte chemotactic peptide-2
g7	MCP-4	Monocyte chemotactic protein-4
g8	TIMP-1	Tissue inhibitor of metalloproteinase-1
h1	GCSF	Granulocyte-colony stimulating factor
h2	IL-6	IL-6
h3	MCSF	Macrophage colony stimulating factor
h4	TNF-β	Tumor necrosis factor-β
h5	Ck beta8-1	CC chemokine CK beta8-1
h6	GDNF	Glial cell line-derived neurotrophic factor
h7	MIF	Macrophage migration inhibitory factor
h8	TIMP-2	Tissue inhibitor of metalloproteinase-2
i1	GM-CSF	Granulocyte-macrophage

		colony-stimulatingfactor
i2	IL-7	IL-7
i3	MDC	Macrophage Derived Chemokine
i4	EGF	Epidermal growth factor
i5	Eotaxin	Eotaxin
i6	HGF	Hepatocyte Growth Factor
i7	MIP-3α	Macrophage inflammatory protein-3α
i8	Neg	Negative control
j1	GRO	Growth regulated oncogene
j2	IL-8	IL-8
j3	MIG	mMonokine induced by interferon-γ
j4	IGF-1	Insulin-like growth factor-1
j5	Eotaxin-2	Eotaxin-2
j6	IGFBP-1	Insulin-like growth factor binding protein-1
j7	NAP-2	Neural antiproliferative protein-2
j8	Pos	Positive control
k1	GRO-α	Growth regulated oncogene-α
k2	IL-10	IL-10
k3	MIP-1β	Macrophage inflammatory protein-1β
k4	Angiogenin	Angiogenin
k5	Eotaxin-3	Eotaxin-3
k6	IGFBP-2	Insulin-like growth factor binding protein-2
k7	NT-3	Neurotrophin-3
k8	Pos	Positive control

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