Intracellular Regulatory Mechanisms of the Activation of Human Eosinophils by TSLP, IL-27 and Ligands of NOD-like Receptors in Allergic Inflammation

HU, Shuiqing

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Abbreviations

AD	Atopic dermatitis
AHR	Airway hyperresponsiveness
ANOVA	Analysis of variance
AP-1	Activator protein-1
APC	Antigen-presenting cell
APS	Ammonium persulfate
ATF-2	Activating transcription factor-2
BAL	Bronchoalveolar lavage
BCP	Bromochloropropane
CARD	Caspase-recruitment domain
CBA	Cytometric beads array
CCR	CC chemokine receptor
CFU	Colony-forming unit
CLC	Charcot-Leyden crystal
CO_2	carbon dioxide
DC	Dendritic cell
ddH_2O	Double distilled water
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
dNTP	2'-Deoxyribonucleoside 5'-Triphosphate
DTT	Dithiothreitol
EBD	Effector-binding domain
ECL	Enhanced chemiluminescent
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
EDN	Eosinophil derived neurotoxin
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic Mobility Shift Assay
EPO	Eosinophil peroxidase
ERK	Extracellular signal regulated kinase
FACS	Fluorescence-activated cell sorting

FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FMLP	Formyl peptide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRO	Growth related oncogene
GSK	Glycogen synthase kinase
HBEpiC	Human Bronchial Epithelial Cells
HEK	Human Epidermal Keratinocytes
HEPES	N-2-hydroxy-ethyl-piperazine-N'-2-ethane-sulfonic acid
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cells
ICAM	Intercellular cell adhesion molecule
iE-DAP	γ-D-glutamyl-meso-diaminopimelic acid
iE-Lys	γ-D-glutamyl-lysine
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRAK	IL-1 receptor-associated kinase
IRF	IFN regulatory factors
IκB	Inhibitor kappa B
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LFA-1	Leukocyte function-association antigen-1
LPS	Lipopolysaccharide
LRD	Ligand-recognition domain
LRR	Leucine-rich repeat
Mac-1	Macrophage-antigen 1
MAdCAM	Mucosal addressin cell adhesion molecule
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
MBP	Major basic protein
MCP	Monocyte chemotactic protein
MDP	N-acetylmuramyl-L-alanyl-D-isoglutamine (Muramyl dipeptide)
MEP	Mucosa-associated epithelial chemokine
MFI	Mean fluorescent intensity

MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MSGA-α	Melanoma growth stimulating activity, alpha
MyD88	Myeloid differentiation factor 88
NaCl	Sodium Chloride
NAP-3	Neutrophil-activating protein 3
NF-κB	Nuclear factor kappa B
NLR	Nucleotide-binding oligomerization domain (NOD) like receptor
NOD	Nucleotide-binding oligomerization domain
ODN	Oligodeoxynuleotides
OVA	Ovalbumin
PAF	Platelet activation factor
PAGE	Polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PG	Prostaglandin
PGN	Peptidoglycan
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PMA	Phorbol-12-myristate-13-acetate
poly(I:C)	Polyinosine:polycytidylic acid
PRR	Pattern-recognition receptor
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
RANTES	Regulated upon activation normal T-cell expressed and secreted
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
RTK	Tyrosine kinase receptors
RT-PCR	Reverse transcription-polymerase chain reaction
SAPK	Stress-activated protein kinases
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SH2	Src-homology 2
SOCS	Suppressor of cytokine signaling
STAT	Signal transducers and activators of transcription

TAE	Tris-acetate-buffer
TARC	Thymus and activation-regulated chemokine
TBE	Tris-Borate-EDTA
TBST	Tris-buffered saline Tween 20
TE	Tris EDTA
TEMED	N, N, N', N'-Tetra-methylethylenediamine
TF	Transcription factor
TGF	Transforming growth factor
Th	T helper lymphocyte
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLA	Very late activated antigen

Abstract

Allergic diseases are prevalent and their incidences have been increasing worldwide. Eosinophils are the principal effector cells for the late phase response in allergic inflammation. The infiltration of eosinophils together with other inflammatory cells at the local inflammatory sites is the major characteristic in allergic inflammation. However, the detailed immunopathological responses and mechanisms of the activation of eosinophils in allergic inflammation are not well defined. In the present study, we investigated and attempted to elucidate the mechanisms of eosinophil activation induced by various stimuli, including thymic stromal lymphopoietin (TSLP), the novel interleukin (IL)-12 family cytokine IL-27, and ligands of nucleotide-binding oligomerization domain (NOD) like receptor (NLR) protein NOD1 and NOD2 upon interaction with bronchial epithelial cells.

TSLP is a novel IL-7-like cytokine highly expressed by bronchial epithelial cells and skin keratinocytes in allergic diseases. TSLP acts as a master switch for allergic inflammation through the activation of dendritic cells and mast cells for initiating inflammatory Th2 responses. To elucidate the immunological cascades of epithelium/keratinocyte-eosinophil mediated allergic inflammation, we examined the modulating effects of TSLP on human eosinophils. We observed that human eosinophils constitutively expressed TSLP receptor complex comprising TSLP-binding chain TSLPR and IL-7Rα chain. TSLP could significantly delay eosinophil apoptosis, up-regulate the cell surface expression of adhesion molecule CD18 and intercellular adhesion molecule-1 (ICAM-1) but down-regulate L-selectin, enhance eosinophil adhesion to fibronectin, and induce the release of inflammatory cytokine IL-6 and chemokines CXCL8, CXCL1 and CCL2. All these effects were concentration-dependent and TSLP-specific. TSLP regulated the above effects through the

activation of extracellular signal-regulated protein kinase (ERK), p38 mitogen activated protein kinase (MAPK) and nuclear factor (NF)-κB signaling pathway, but not signal transducer and activator of transcription (STAT)-5 and STAT-3 which were usually activated in other effector cells upon TSLP stimulation. Collectively, the above findings elucidated the pro-allergic mechanisms of TSLP via the activation of distinct intracellular signaling pathways in eosinophils.

Recently, the novel IL-12 family member IL-27 was found to regulate immune responses, exerting either stimulation or suppression effects. We found that eosinophils constitutively expressed IL-27 receptor heterodimer, gp130 and WSX-1. IL-27 could prolong eosinophil survival by reducing apoptosis, modulate the expression of adhesion molecules to facilitate eosinophil adhesion and accumulation, and induce the release of proinflammatory cytokines IL-6, tumor necrosis factor (TNF)-α, IL-1β and chemokines CCL2, CXCL8 and CXCL1. The stimulatory effects of IL-27 on eosinophils could not be abrogated by IL-25, hematopoietic cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) and toll-like receptor (TLR) 4 ligand lipopolysaccharide (LPS). These findings were different from the effects of IL-27 and LPS on monocytes. Intracellular signaling mechanistic studies showed that IL-27-mediated eosinophil activation was differentially regulated by MAPKs and NF-κB. Based on the above results, IL-27 could play crucial roles in allergic diseases by the activation of eosinophils via differential intracellular signaling cascades. However, IL-27 has been shown to suppress allergic diseases in mouse models. According to our findings of its activating effects on human eosinophils, IL-27 may play pleiotropic roles in human allergic responses.

Accumulating evidence has indicated that microbial infection could intensify allergic responses. Previous findings demonstrated that eosinophil activation could be elicited by bacterial and viral conserved molecular pattern through TLR. Recently, two cytoplasmic pattern recognition receptors, NLR protein NOD1 and NOD2, have been discovered and the important roles in innate immunity have been elucidated. Eosinophils alone have little

responses upon the stimulation with ligands of NOD1 and NOD2. Since airway eosinophils increase in more numbers of asthmatic patients compared to control subjects, we investigated the co-culture system of eosinophils and human bronchial epithelial cells to illustrate the potential immunopathological roles of NOD1 and NOD2 in asthma processes. In the co-culture system, NOD1 ligand γ-D-Glu-mDAP (iE-DAP) and NOD2 ligand muramyl dipeptide (MDP) could upregulate cell surface expression of CD18 and ICAM-1 on eosinophils and ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) on bronchial epithelial cells, as well as induce chemokines CCL2 and CXCL8 release. These findings therefore imply the direct interaction and activation between the two cells upon NOD1 and NOD2 ligand stimulation.

In conclusion, the above findings demonstrated that eosinophils could be potently activated by diverse stimuli and regulated by multiple intracellular regulatory mechanisms. The elucidation of eosinophil activation may offer new therapeutic strategies and clues for the treatment of allergic diseases.

摘要

過敏性疾病近年來在全球的流行趨勢和發病率逐漸上升。嗜酸性粒細胞是過敏炎症反應遲發相中重要的效應細胞。嗜酸性粒細胞和其他炎症細胞浸潤入局部炎症反應部位是過敏反應的重要特點。但是，關於嗜酸性粒細胞在過敏病理炎症中的詳細反應機制仍然沒有闡明。在本研究中，我們著重探討了嗜酸性粒細胞被多種介質激活的機制，這些刺激物包括胸腺基質淋巴細胞生成素（TSLP），白介素（IL）-12 家族新成員 IL-27，以及含有核苷酸結合寡聚域（NOD）樣受體（NLR）的蛋白 NOD1 和 NOD2 的配體。

TSLP 是一種新型的、與 IL-7 類似的細胞因子，並廣泛的在過敏性疾病中的呼吸道上皮細胞和皮膚表皮角化細胞表達。TSLP 在過敏炎癥反應中起著重要開關的作用，因為其能夠激活樹突狀細胞和肥大細胞並啟動 Th2 反應。為了進一步闡明呼吸道上皮細胞、皮膚表皮角化細胞與嗜酸性粒細胞之間相互作用介導的過敏反應，我們研究了 TSLP 對人嗜酸性粒細胞的調節作用。我們發現嗜酸性粒細胞表達 TSLP 受體復合物，由 TSLP 結合亞單位 TSLPR 和 IL-7Rα 鏈組成。TSLP 可以顯著地減少嗜酸性粒細胞的凋亡，上調粘附分子白細胞分化抗原 18（CD18）和細胞間粘附分子-1（ICAM-1），下調 L-選擇素（L-selectin），增強其粘附與纖維連接蛋白，並誘導炎症因子 IL-6 和趨化因子 CXCL8、CXCL1、CCL2 的釋放。所有這些反應都是濃度梯度依賴並是 TSLP

特異性介導的。TSLP 激活嗜酸性粒細胞胞外調節激酶（ERK），p38 絲裂原活化蛋白激酶（p38MAPK）和胞核因子-kappaB（NF-κB），而非其他細胞中 TSLP 激活的細胞轉導與轉錄激活因子（STAT）-3 和 STAT-5。總之，以上結果表明 TSLP 可以通過不同的信號途徑激活嗜酸性粒細胞。

新近發現的 IL-12 家族成員 IL-27 可以刺激或抑制免疫反應。我們研究發現嗜酸性粒細胞表達 IL-27 異二聚體受體，糖蛋白（gp）-130 和 WSX-1。IL-27 可以通過減少嗜酸性粒細胞的凋亡而維持其存活，調節粘附分子的表達以增強其粘附與聚集功能，並誘導炎症因子 IL-6、IL-1β、腫瘤壞死因子（TNF）-α 和趨化因子 CXCL8、CXCL1、CCL2 的釋放。IL-27 的這些作用不能夠被 IL-25、粒細胞-巨噬細胞集落刺激因子（GM-CSF） 和 Toll 樣受體（TLR）4 的配體脂多糖（LPS）所逆轉，而在單核細胞中 IL-27 的刺激作用可以被 LPS 所逆轉。胞內信號機制研究發現 IL-27 通過激活 MAPK 和 NF-κB 而介導上述反應。這些結果表明 IL-27 可以通過激活嗜酸性粒細胞内不同信號通路在過敏反應中發揮作用。然而在小鼠模型中發現，IL-27 可以抑制過敏反應發生。綜合現在的發現結果，我們認為 IL-27 在人類的過敏反應中起著多種重要的調節作用。

有證據表明微生物感染可以加重過敏反應。以前研究發現嗜酸性粒細胞可以通過其表達的保守的 TLR 識別細菌和病毒而被激活。近年來新發現的定位於胞漿的病原體模式識別受體 NOD1 和 NOD2 在固有免疫中發揮著重要的作用。NOD 蛋白的配體刺激單獨培養的嗜酸性粒細胞幾乎沒有任何反應。由於大量嗜酸性粒細胞在哮喘發生中

浸潤呼吸道上皮，因此我們研究了 NOD 配體刺激共培養的嗜酸性粒細胞與呼吸道上皮細胞，試圖闡明 NOD 蛋白在哮喘發生中的免疫病理作用。在共培養體系中，NOD1 配體 γ-D-谷氨酸-meso-二氨基庚二酸（iE-DAP）和 NOD2 配體胞壁酸二肽（MDP）能夠顯著上調嗜酸性粒細胞表明粘附分子 CD18 和 ICAM-1 的表達而下調 L-selectin 的表達，同時上調呼吸道上皮細胞 ICAM-1 和血管細胞粘附分子-1（VCAM-1）的表達，並誘導趨化因子 CCL2 和 CXCL8 的釋放。這些結果表明 NOD1 和 NOD2 配體刺激共培養系統，兩種細胞之間存在相互作用並且被激活。

總之，上述發現表明嗜酸性粒細胞能夠被各種不同的刺激和胞內調控機制所激活。對嗜酸性粒細胞激活的深入理解能夠給予治療過敏性疾病更新的策略和線索。

Publications

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1.1 Allergic diseases and allergic inflammation

1.1.1 Prevalence of allergic diseases

The word "Allergy" was derived from the ancient Greek *allos* meaning "other" and *ergon* meaning "reaction" and originally introduced in 1906 by Austrian physician Clemens von Pirquet to describe the hypersensitivity reaction of patients who had quicker, more severe reactions to a second injections of horse serum or smallpox vaccine if these patients had previously received these injections before (Kay, 2000). An allergy is a disorder of the immune system often also referred to as atopy. Allergens are antigens that trigger specific immunologic mechanisms to cause allergic reactions, resulting in the development of allergic diseases, which include allergic asthma, atopic dermatitis (AD), allergic rhinitis, eczema, allergic conjunctivitis, urticaria, food hypersensitivity, drug hypersensitivity, insect sting or bite hypersensitivity, and/or anaphylaxis (Johansson *et al.*, 2004).

According to published results until now, the prevalence of atopic diseases has increased abruptly in past decades. By 1998, a worldwide survey (ISAAC Phase I) found amongst children of 13 - 14 years of age that one in three suffered from asthma, one in four suffered from hay fever and one in five suffered from atopic eczema (Asher and Weiland, 1998; Pearce *et al.*, 2000). In addition, the prevalence atopic rate with symptoms of current wheeze, speech limiting wheeze, rhinoconjunctivitis and flexural dermatitis was high in Chinese school children though there are differences among Hong Kong (41.2%), Beijing (23.9%) and Guangzhou (30.8%) (Wong *et al.*, 2001). Dramatic increases in the prevalence of atopy and

asthma have occurred over the past few decades in Westernized countries, including United Kingdom, America, Australia, Canada and Hong Kong, and more recently in less-developed nations, such as China (Eder *et al.*, 2006). It is estimated that as many as 300 million people worldwide currently suffer from allergic asthma, a total that is expected to rise by an additional 100 million, mainly in children, over the next 15 - 20 years (Braman, 2006; Fanta, 2009; Warner *et al.*, 2006). Anticipated increase in the prevalence of allergic diseases may become a severe challenge of clinical practice and public health planning.

1.1.2 Causes of allergic diseases

Multiple risk factors are responsible for allergic diseases. Generally, these factors could be placed in two categories, host factors and environmental factors (Grammatikos, 2008).

Host factors, including heredity, sex, race and age, to some extent, predispose humans to develop allergies in the first place. The most significant and essential pre-condition is the genetic background, which is coded in our genes and inherited from our parents with a tendency for allergy. It has been well-established that genetic factors strongly affect susceptibility to allergy and its associated traits. Allergic diseases strongly tend to occur in families: parents with allergy are more likely to have atopic children; identical twins are more likely to have the same allergic diseases than non-identical twins (De Swert, 1999; Galli, 2000). The risk of allergic sensitization and the development of allergies are different among ages. The young children are most at risk for allergy and show the highest IgE levels, which fall rapidly between the ages of 10 and 30 years (Croner, 1992; De Swert, 1999). Besides, boys show a higher risk for atopy and developing allergy than girls, although for some diseases, such as asthma in adulthood, women are more likely to be affected (Anderson *et al.*, 1992; De Swert, 1999). Recent studies have also suggested that different genetic loci are associated with asthma for people from European, Hispanic, Asian, and African (Barnes *et al.*, 2007).

Though genetic and other host reasons are most important factors accounting for allergy, however, genetic changes in populations would be too slow to account for the rapid change in the increasing prevalence of allergy in recent years. Most information on the effects of environmental exposures on the risk of allergic diseases has been reported. Environmental factors, including tobacco, air pollution, allergens, infection, microbial substances, dietary changes and etc., are also important for allergic disorders. A number of studies have shown that active smoking is associated with the onset of asthma in adolescents and adults (Eder *et al.*, 2006). The level of exposure to allergens such as house-dust mites and cat dander affects a person's risk for the development of IgE antibodies against these allergens. However, exposure to environments rich in these substances, such as stables and barns of traditional dairy farms, has consistently been shown to significantly reduce the risk of asthma and atopy (Eder *et al.*, 2006). Changing environmental exposures may not affect disease prevalence immediately. However, robust data have revealed the linking changes in the environment to changes in the prevalence and incidence of allergy over time. In addition, respiratory infections, especially respiratory virus infections, are also associated with attacks of bronchial asthma and they also have been associated with the onset of allergic sensitization (Busse and Gern, 1997).

Besides the contribution of host factors in the mix of environmental factors, other elements including social, cultural, or economic factors are also important and involved in allergy. The true causes of the increase in allergy are probably a combination of several factors.

1.1.3 Immunopathology of allergic inflammation

The immune system normally responds to a variety of microbial invaders with little or no damage to host tissues. However, in some situations, immune responses can lead to more severe tissue damaging reactions (immunopathology). This "overreactivity" by the immune

system to antigens is often referred to as hypersensitivity to antigens of both microbial origin and self antigens (autoimmunity). Hypersensitivity reactions are antigen specific and occur after the immune system has already responded to an antigen, which means the immune system has been primed. Hypersensitivity reactions have long been classified into four types by Gell and Coombs (Gell and Coombs, 1963). Allergy is one of four forms of hypersensitivity and is called type I (or immediate) hypersensitivity. The pathophysiology of allergic responses can be divided into two phases (Larché *et al.*, 2006). The first is an early/immediate phase of the allergic reaction that occurs immediately, usually within one hour, after exposure to an allergen. The early phase reaction can then drive and progress into the late phase reaction which can prolong the symptoms of allergic responses, and even lead to tissue damage (Figure 1.1).

High-affinity receptor for IgE (FcεRI) is highly expressed on the surface of mast cells and basophils. Upon allergen challenge, the crosslinking of the IgE-FcεRI complexes leads to mast cells and basophils degranulation, releasing vasoactive amines (such as histamine), lipid mediators [such as prostaglandin D, platelet-activating factor (PAF), and cysteinyl leukotriene C4 (LTC4), LTD4 LTE4], chemokines [such as CXC-chemokine ligand 8 (CXCL8), CXCL10, CC-chemokine ligand 2 (CCL2), CCL4 and CCL5] and other cytokines (such as Interleukin (IL)-4, IL-5 and IL-13), all of which characterize the early phase of the allergic reaction. Under the influence of chemokines and cytokines, other leukocytes such as lymphocytes and eosinophils migrate to the initial site of allergen stimulation. Allergen-specific CD4+ T cells are activated and clonally expanded, and eosinophils are also infiltrated in the allergen exposure site, leading to the development of late phase of the allergic reaction (Kay, 2001; Larché *et al.*, 2006; Pearlman, 1999). Activated CD4+ T cells are induced towards Th2 immune responses, leading to Th2 cytokines release, including IL-4, IL-5 and IL-13, which could promote IgE production, mast cell differentiation, and eosinophil growth, migration and activation which then lead to the pathologic abnormalities in allergic

diseases. Eosinophils could release cytotoxic proteins and inflammatory mediators, contributing to the late phase responses of allergic diseases. Moreover, the impaired function of regulatory T cells has also been involved in allergic diseases (Larché *et al.*, 2006).

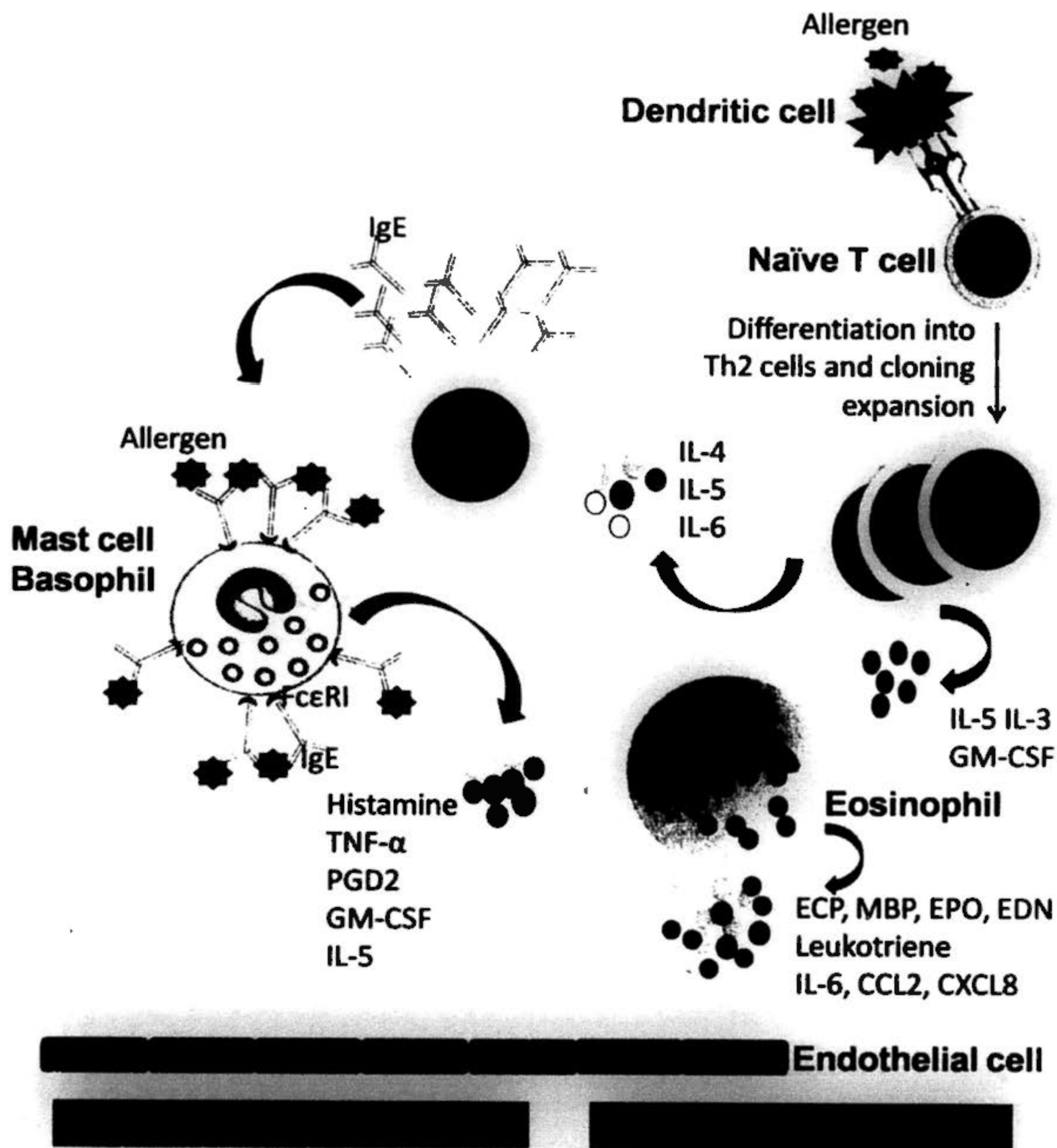


Figure 1.1 Pathophysiology of immediate phase and late phase allergic reaction (modified from Larché *et al.*, 2006).

1.2 Biology of eosinophils

Eosinophils are specialized multifunctional white blood cells involved in initiation and propagation of diverse inflammatory responses, as well as modulators of innate and adaptive immunity. Eosinophils are granulocytes that develop and mature during haematopoiesis in the bone marrow before migrating into blood and residing in tissues. Human eosinophils were first identified as a distinct type of peripheral blood leukocyte over a century ago by Paul Ehrlich (Wenzel, 2009). Eosinophils now have been implicated in the pathogenesis of numerous inflammatory processes including allergic diseases, parasitic helminth, bacterial and viral infection, tissue injury and tumor immunity.

1.2.1 Development and distribution of eosinophils

Eosinophils are produced in the bone marrow from pluripotential hematopoietic stem cells (HSC), which have the capacity for long-term self-renewal and differentiation along multiple lineage pathways (Bedi and Sharkis, 1995). The development of eosinophils from HSC is a complicated progress regulated through lineage commitment, terminal differentiation and growth arrest (Marone, 2000). Hematopoietic progenitors for various lineages were originally identified and defined using colony-forming assays, that the cell populations containing lineage-specific hematopoietic progenitors were cultured in semisolid hematopoietic support media, which allows commitment to a given differentiation pathways (Bradley and Metcalf, 1966; Pluznik and Sachs, 1965). The lineage-specific colonies for a given number of cultured cells are colony-forming units (CFU), and each CFU represents the various progeny of a single progenitor cell under standard plating conditions. The identified CFU include erythroid (burst-forming unit-erythroid, BFU-E), megakaryocytic (Meg-CFU), granulocyte/macrophage (GM-CFU), eosinophil/basophil (Eo/B-CFU) and lymphocyte-CFU (pre-B and Pre-T) (Figure 1.2).

Granulocytes are derived from a common myeloid progenitor known as the

colony-forming unit-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM), which can further differentiate to GM-CFU and Eo/B-CFU. Eo/B-CFU is a common late stage progenitor and could further differentiate to eosinophils and basophils. Hematopoietic cytokines IL-3, IL-5, and granulocyte-macrophage colony-stimulatory factor (GM-CSF) are critical cytokines responsible for regulating eosinophil and basophil lineage development. These cytokines are encoded by closely linked genes on chromosome 5q31 and bind to their specific receptors that share a common β chain and have unique α chains (De Swert, 1999; Ober and Hoffjan, 2006; Walley *et al.*, 2001). Of these three cytokines, IL-5, also known as eosinophil differentiation factor, is the most specific to the eosinophil lineage and is responsible for selective differentiation of eosinophils. IL-5 also stimulates the release of eosinophils from the bone marrow into the peripheral circulation. The crucial role of IL-5 in the production and development of eosinophils is further demonstrated by genetic manipulation of mice. Transgenic mice overexpressing IL-5 developed profound eosinophilia (Dent *et al.*, 1990; Mishra *et al.*, 2002; Tominaga *et al.*, 1991), while IL-5 gene deficient mice could cause a significant reduction of eosinophils in the blood and lungs after allergen challenge (Foster *et al.*, 1996; Kopf *et al.*, 1996). The overproduction of IL-5 or a combination of IL-5, GM-CSF and IL-3 also occurs in humans with eosinophilia diseases (Owen *et al.*, 1989; Rothenberg and Hogan, 2006).

Under homeostatic conditions, terminally differentiated mature eosinophils migrate into thymus, mammary gland, ovary, uterus, gastrointestinal tract, spleen and lymph nodes, though circulating in peripheral blood at low concentrations, but not in the lung, skin, esophagus, or some other internal organs. The presence of eosinophils in the latter organs is associated with diseases. Eosinophils persist in the circulation for only a short time, usually within one day, and can survive in tissues for an additional several days in the absence of stimulation (Rothenberg and Hogan, 2006). Eosinophils eventually undergo apoptosis and are subsequently recognized and ingested by macrophages (Rothenberg and Hogan, 2006).

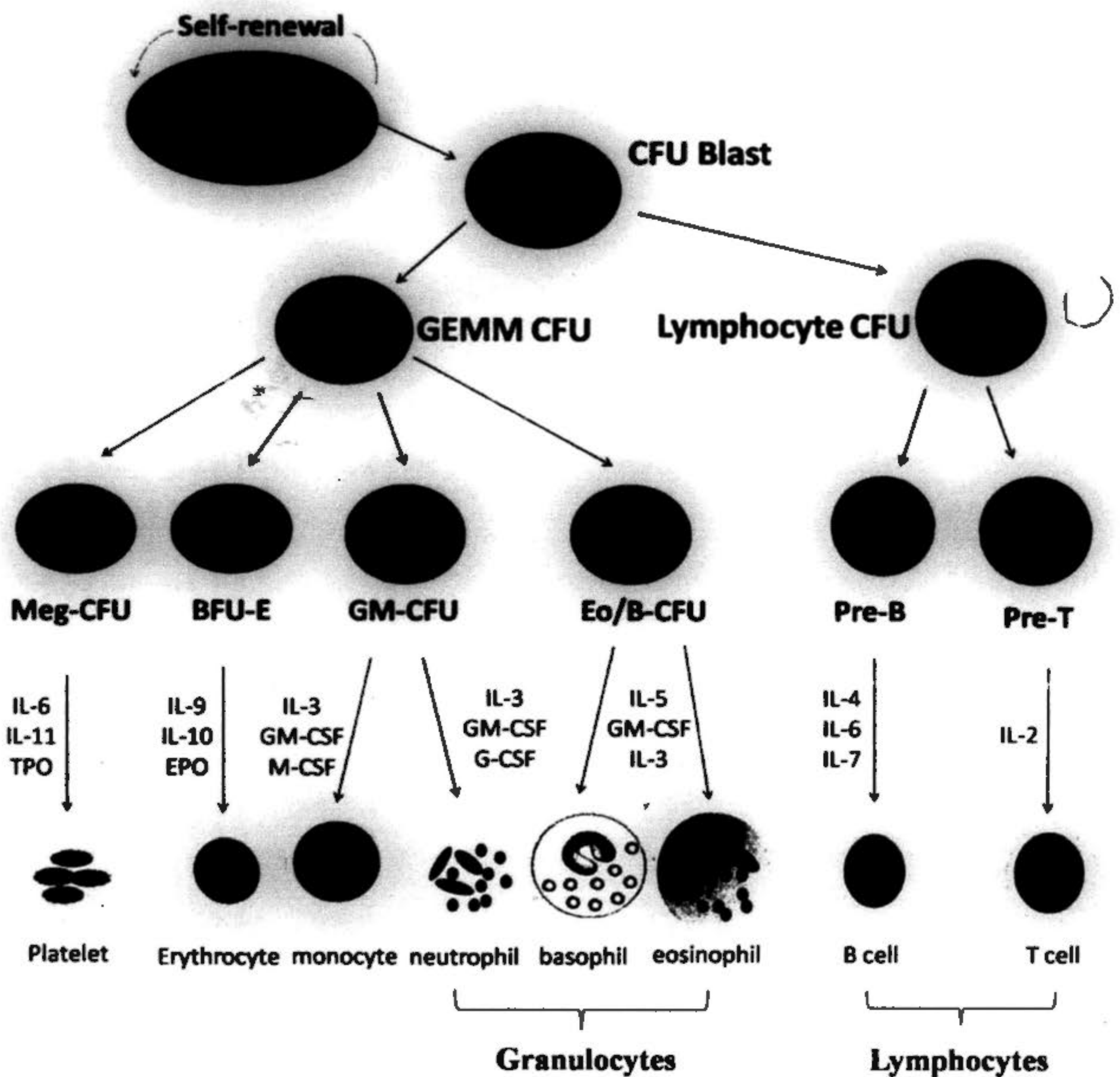


Figure 1.2 Commitment and differentiation of hematopoietic cell lineage. Pluripotential hematopoietic stem cells (HSC) in the bone marrow give rise to blood cells of all lineages under the influence of different local cytokines (modified from Robb, 2007).

1.2.2 Morphology of eosinophils

Eosinophils are approximately 8 μm in diameter and each of them has a bilobed nucleus (Figure 1.3). The distinguishing feature of eosinophils was its affinity for staining with the acid aniline dye eosin. The eosin binds to cationic proteins, such as major basic protein (MBP) and eosinophil peroxidase (EPO). In contrast, the other two granulocytes, neutrophils and basophils, do not bind eosin because of their different unique granule populations. Until now, there still has been no specific cell surface markers recognized for eosinophils, and the tinctorial property visible by light microscopy gives a characteristic appearance and has remained the main identifying feature of eosinophils (Gleich *et al.*, 1993).

Eosinophil surface markers

Adhesion molecules

CD11a	CD44
CD11b	CD49d
CD11c	CD49f
CD11d	CD62L
CD18	CD156
CD29	β7 integrin

Immunoglobulin receptors and members of immunoglobulin superfamily

CD4	CD50
CD16	CD54
CD32	CD89
CD47	FcεRI
CD48	HLA-DR

Chemokine, complement and other chemotactic factor receptors

CCR1	CD35
CCR3	C3aR
CXCR1	LTB4R
CXCR2	PAFR
CRTH2	Histamine 4R

Cytokine receptors

CD25	CD124
CD116	CD125
CD117	CD129
CD119	CD131
CD120	CD132
CD123	TGFβR

Toll-like receptors

TLR1	TLR5
TLR2	TLR7
TLR4	TLR9

Eosinophil mediators

Eosinophil granule proteins

MBP
ECP
EDN
EPO

Cytokines	Chemokines
IL-1β	CCL2
IL-2	CCL5
IL-3	CCL11
IL-4	CXCL1
IL-5	CXCL5
IL-6	CXCL8
IL-9	CXCL9
IL-10	CXCL10
IL-12	CXCL11
IL-13	
IL-16	
IFN-γ	
TNF-α	
TGF-β	
GM-CSF	

Lipid mediators

PAF
LTC4
PGE2

Figure 1.3 Schematic diagram of human eosinophil morphology, surface markers and mediators (Modified from Hogan *et al.*, 2008).

1.2.3 Eosinophil apoptosis

Eosinophils are terminally differentiated cells with short life span and die quickly when they finished their functions. Under physiological conditions, eosinophils are constantly produced in the bone marrow. To keep cellular homeostasis, the same numbers of aged eosinophils undergo apoptosis, a programmed cell death, and then engulfed by phagocytes. Apoptosis is the major form of physiological cell death and a necessary process to maintain cell numbers in multicellular organisms, and is characterized by a series of typical morphological and biochemical features, such as shrinkage of cell, chromatin condensation, genomic DNA fragmentation into membrane-bound apoptotic bodies caused by caspase-activated DNase, changes in mitochondrial membrane permeability, and translocation of phosphatidylserine (PS) from the inner leaflet to outer leaflet of the plasma membrane (Krysko *et al.*, 2008; Saraste and Pulkki, 2000; Vignola *et al.*, 1999).

The disregulation of apoptosis can lead to pathophysiological changes. In many eosinophilic inflammatory diseases, reduced and delayed eosinophil apoptosis has been proposed as the central mechanism contributing to increased eosinophil numbers, a phenomenon called eosinophilia. Overexpression of activators, such as IL-5, GM-CSF and IL-3, has been shown to be crucial for delaying eosinophil apoptosis in many allergic disorders. However, eosinophils undergo apoptosis when removed from physiological environment or survival factor withdrawal, and the apoptosis can be further enhanced by certain mediators or chemicals (e.g. glucocorticoid). Besides, eosinophil apoptosis is also regulated by death factors, the tumor necrosis factor (TNF)/nerve growth factor receptor superfamily (e.g. Fas receptor, CD95) (Matsumoto *et al.*, 1995; Simon, 2006). Drugs, which could specifically induce eosinophil apoptosis, might be useful for triggering the resolution of eosinophilic inflammatory responses.

1.2.4 Eosinophil granule proteins

Human eosinophils are characterized by four different populations of secretory organelles: crystalloid (secondary) granules, primary granules, small granules, and secretory vesicles (Gleich and Adolphson, 1986; Hogan *et al.*, 2008). The crystalloid granules are the largest secretory organelles in eosinophils, which mainly contain four types of basic granule proteins, including MBP, EPO, eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN). MBP is localized to the crystallized core of the crystalloid granule, where it accounts for the largest percentage of all the basic protein (Gleich *et al.*, 1973; Hogan *et al.*, 2008; Lewis *et al.*, 1978), while EPO, ECP, and EDN are present in the granule matrix compartment (Egesten *et al.*, 1986; Peters *et al.*, 1986). The primary granules appear during the promyelocytic stage of eosinophil development and are unique unicompartmental organelles enriched in Charcot-Leyden crystal (CLC) protein by immunogold analysis (Dvorak *et al.*, 1988). Small secretory vesicles have also been identified that overlap in their contents with those of small granules, which are packed densely in the cytoplasm but do not contain MBP or CLC protein in eosinophils (Gleich and Adolphson, 1986; Walsh, 2001).

The basic proteins were first recognized for their cytotoxicity and now are known to play pleiotropic functions. The cationic nature of these molecules allows them to interact with the anionic barrier found on endothelial cells and basement membranes and to influence microvascular permeability. MBP and ECP have been shown to be toxic toward helminthic parasites and mammalian various epitheliums (Ackerman *et al.*, 1985; Furuta *et al.*, 2005). MBP could stimulate the release of a variety of preformed and newly synthesized mediators by inflammatory cells and potentiate ionomycin-induced LTC4 production (Kita *et al.*, 1995). ECP possesses bactericidal and antiviral activity, and promotes degranulation from mast cells (Lehrer *et al.*, 1989; Zheutlin *et al.*, 1984). EDN and ECP share high sequence homology of 70% at the amino acid level for the pre-formed proteins but EDN is less cationic than ECP (Rosenberg *et al.*, 1989). EDN also only shows very limited cytotoxicity (Ackerman *et al.*, 1985). In contrast, EDN displays antiviral activity, similar to that of ECP. EPO is cytotoxic for

bacteria and various mammalian cells and degradative toward connective tissue via the ability to form hypohalous acids (Brottman *et al.*, 1996; Slungaard and Mahoney, 1991; Wang and Slungaard, 2006).

1.2.5 Cytokines and chemokines from eosinophils

Experimental investigations have shown that eosinophils could produce and secret a variety of inflammatory and regulatory cytokines, chemokines, and growth factors upon a number of physiological or pathophysiological agonists stimulation (Figure 1.3). Cytokines are small proteins that are secreted by specific cells and carry signals within different cells. In contrast to other effector cell-derived cytokines, many of eosinophil-derived cytokines are characterized to be stored as preformed mediators within crystalloid granules and small secretory vesicles, which allow eosinophils to release these immunoregulatory factors immediately following their recruitment and activation. Acting in an autocrine, paracrine or juxtacrine manner, many of these cytokines are potent inducers of immune responses in allergic diseases, including asthma, atopic dermatitis, eczema, allergic rhinitis, and other inflammatory diseases. Chemokines are a family of chemotactic cytokines serving as potent chemoattractants that guide the migration and accumulation of leukocytes into the inflamed tissues in response to the chemokine gradient, contributing to the pathogenesis of allergic inflammation (Rothenberg *et al.*, 1999; Rothenberg and Hogan, 2006). Chemokines could be categorized into four different subtypes according to the number and spacing of conserved N-terminal cysteine residues, namely CXC, CC, CX3C and C (Rossi and Zlotnik, 2000). In addition, similar to cytokines, some chemokines are potent cellular activating factors that could control cells of the immune system during processes of immune surveillance, activate leukocytes, endothelial cells and epithelial cells, modulate leukocyte adhesion molecule interaction affinity, and induce activation of specific intracellular signal transduction cascades in allergic inflammation (Rothenberg *et al.*, 1999; Wong *et al.*, 2004).

1.2.5.1 IL-1β

IL-1, one of the first cytokines ever described, was initially discovered as a factor that could induce fever, control lymphocytes, increase the number of bone marrow cells and cause degeneration of bone joints (Dinarello, 1994). IL-1 was composed of two distinct proteins, IL-1α and IL-1β, which play central roles in acute and chronic inflammation locally and systemically. Both IL-1α and IL-1β are produced by various cell types, including macrophages, monocytes, fibroblasts, dendritic cells (DCs), keratinocytes, and eosinophils. IL-1β is an important mediators in the inflammatory responses, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. IL-1β was found to induce differentiation and development of Th17 cells, which is one of novel T helper subsets and have been linked to the pathogenesis of inflammatory and autoimmune diseases (Acosta-Rodriguez *et al.*, 2007). IL-1β could also promote IgG and IgE synthesis, Th2 cell activation and Th2 cytokine production, as well as development of airway hypersensitivity responses in ovalbumin (OVA)-induced airway hypersensitivity mouse model (Nakae *et al.*, 2003; Nambu *et al.*, 2006). In addition, it was reported that IL-1β concentration in nasal secretions of allergic rhinitis patients was significant elevated, which also implied that IL-1β could be involved in allergic inflammation (Bachert *et al.*, 1999).

1.2.5.2 IL-6

IL-6 could be synthesized by a variety of cells, including macrophages, T cells, B cells, monocytes, fibroblasts, keratinocytes, eosinophils and endothelial cells. IL-6 is a pleiotropic cytokine that involves in the regulation of immune responses, controls the resolution of acute innate immunity, and steers transition to an acquired immune response (Jones, 2005; Kishimoto, 2006). IL-6 is also one of the most important mediators of fever and of the acute phase response (Naka *et al.*, 2002; Nishimoto and Kishimoto, 2006). IL-6 protects against septic shock and directs resolution of acute inflammation and conversely, elicits detrimental

consequences in more progressive chronic diseases (Rose-John *et al.*, 2006). IL-6 could induce growth of T lymphocytes, differentiation of cytotoxic T cells and macrophages, polarization of Th2 cells, formation of multilineage blast cell colonies in hematopoiesis, and promote growth of epidermal keratinocytes (Chomarat *et al.*, 2000; Doganci *et al.*, 2005; Kishimoto, 2006; Mitani *et al.*, 2000). Similar to IL-1β, IL-6 was also reported to mediate differentiation of Th17 cells, which is the pathogenesis of several inflammatory and autoimmune diseases (Acosta-Rodriguez *et al.*, 2007). It has been shown the therapeutic potential of targeting IL-6 as a strategy for the treatment of chronic inflammatory diseases. Previous studies demonstrated that allergic asthmatic patients showed higher plasma IL-6 concentration and increased levels of sIL-6R in the airways than normal controls, implying its importance in allergic inflammation (Wong *et al.*, 2001; Doganci *et al.*, 2005).

1.2.5.3 CCL2

CCL2, also known as monocyte chemotactic protein-1 (MCP-1) and belonging to the CC chemokine family, is synthesized by multiple types of cells, including macrophages, DCs, lymphocytes, basophils, epithelial cells, endothelial cells, and fibroblasts, upon induction by diverse proinflammatory stimuli like IL-1β, IL-6, TNF-α and lipopolysaccharide (LPS). CCL2 is a low molecular weight monomeric polypeptide whose primary function was identified as promoting monocyte, macrophage, and basophil migration to sites of inflammation mediated by the high affinity CCL2 receptor, CCR2 (Craig and Loberg, 2006; Sarafi *et al.*, 1997). CCL2 can trigger chemotaxis of eosinophils via the receptor CCR2 (Dunzendorfer *et al.*, 2001). Besides, CCL2 also binds to the receptor CCR4, which mainly expressed on Th2 lymphocytes and binds to CCL5 and CCL20 (Graves *et al.*, 1999). In addition to the recruitment of regulatory and effector leukocytes, CCL2 could provoke aggregation and induce histamine and leukotriene release from both mast cells and basophils for IgE-mediated hypersensitivity, as well as production of transforming growth factor

(TGF)-β from fibroblasts (Conti *et al.*, 1995; Rose *et al.*, 2003). Clinical observation also found that CCL2 in bronchoalveolar lavage (BAL) fluid of asthma patients was significantly higher than that of control subjects. Taken together, CCL2 might act as a critical cytokine for the pathogenesis of allergic inflammation (Alam *et al.*, 1996).

1.2.5.4 CXCL1

CXCL1, belonging to the CXC chemokine family, was previously called growth related oncogene 1 (GRO1), GROα, KC, Neutrophil-activating protein 3 (NAP-3) and melanoma growth stimulating activity, alpha (MSGA-α). CXCL1 is synthesized by a variety of cells, including macrophages, DCs, neutrophils, epithelial cells, eosinophils, and human melanoma cells. CXCL1 could bind to the chemokine receptor CXCR2 to elicit its effects (Haghnegahdar *et al.*, 2000; Tsai *et al.*, 2002). CXCL1 could potently chemoattract human neutrophils and also play an important role in spinal cord development by inhibiting the migration of oligodendrocyte precursors (Rossi and Zlotnik, 2000; Tsai *et al.*, 2002). Besides, CXCL1 is always involved in the processes of angiogenesis, inflammation, wound healing, and tumorigenesis (Haghnegahdar *et al.*, 2000; Rossi and Zlotnik, 2000).

1.2.5.5 CXCL8

CXCL8, also named IL-8, is a proinflammatory CXC chemokine mainly produced by macrophages, monocytes, epithelial cells, keratinocytes, lymphocytes, eosinophils, and endothelial cells. CXCR1 and CXCR2 are the receptors that are capable to bind CXCL8, and the expression and affinity of CXCR1 is much higher than the receptor of CXCR2 for CXCL8 (Li *et al.*, 2002; Rothenberg *et al.*, 1999). The primary function of CXCL8 is the induction of chemotaxis, migration and recruitment of neutrophil to the site of inflammation. Recent studies found that CXCL8 could also potentiate neutrophil oxidative burst and thus result in tissue destruction at the site of inflammation, as well as modulate the expression of

adhesion molecules for promoting transmigration of leukocytes into the inflamed tissues (Rothenberg *et al.*, 1999; Kobayashi, 2008). It has been reported that IL-8 played an important role in viral infection-induced bronchiolitis, one of common respiratory tract disease, and the serum concentration of CXCL8 was significantly higher in severe asthmatics than that in controls, implying its potential role in mediating allergic responses (Hogg, 2001; Silvestri *et al.*, 2006).

1.2.6 The surface phenotype of eosinophils

Like other cells, eosinophils have specific characterization of the surface molecules, which involved in immune regulation and diseases. Eosinophils express a large number of cell-surface markers including adhesion molecules, apoptotic signaling molecules, chemokine, complement and chemotactic factor receptors, cytokine receptors, and immunoglobulin (Ig) receptors (Hogan *et al.*, 2008). In addition, eosinophils express several types of toll-like receptors (TLR), including TLR1, -2, -4, -5, -7, and -9, on their cell surface for initiating microbe-induced innate immune responses via activation of intracellular signaling mechanisms (Wong *et al.*, 2007). Eosinophils also express inhibitory receptors, which could suppress eosinophils activation (Hogan *et al.*, 2008). Common surface markers found on eosinophils are illustrated in Figure 1.3.

1.2.6.1 Adhesion Molecules

Eosinophil migration from vascular circulation into specific tissues involves a variety of adhesion molecules, mainly including selectin family and integrin family (Hogan *et al.*, 2008).

1.2.6.1.1 Selectin

Selectins, a family of cell adhesion molecules, are composed of three members,

E-selectin (CD62E), P-selectin (CD62P), and L-selectin (CD62L). These selectins are single-chain transmembrane glycoproteins and share a unique structural characteristic in their extracellular region, including an N-terminal calcium-dependent lectin domain which is directly responsible for ligand binding, an epidermal growth factor-like domain, and two to nine short consensus repeat units (Tedder *et al.*, 1995). Selectins mainly bind to sugar moieties and so are considered to be a type of lectin, one of cell adhesion proteins that could bind sugar polymers (Tedder *et al.*, 1995). E-selectin is expressed only on endothelial cells activated by IL-1β and TNF-α and recognizes and binds to sialylated carbohydrates present on the surface proteins of certain leukocytes, including monocytes, granulocytes, and T cells (Laferriere *et al.*, 2002). E-selectin plays a crucial role in recruiting leukocytes to the site of injury during inflammation. P-selectin is initially stored in granules Weibel-Palade bodies of endothelial cells and α-granules in unactivated platelets, and mobilized rapidly after cell activation, such as histamine stimulation of endothelial cell and thrombin stimulation of platelets (Tedder *et al.*, 1995). P-selectin involves in the initial recruitment of leukocytes to the site of injury during inflammation. Eosinophils could bind to P-selectin on immobilized surfaces and nasal polyp tissue sections, and the ligands for P-selectin on eosinophils and neutrophils are similarly sialylated, protease-sensitive, endo-beta-galactosidase-resistant structures (Symon *et al.*, 1994; Wein *et al.*, 1995). L-selectin is constitutively expressed on most circulating leukocytes and acts as a "homing receptor" for leukocytes to enter secondary lymphoid tissues via high endothelial venules. Ligands for L-selectin include CD34 and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Tedder *et al.*, 1995). The surface expression of L-selectin is regulated by metalloprotease (also named sheddase)-dependent shedding of the extracellular domain upon cell activation (Peschon *et al.*, 1998; Preece *et al.*, 1996). L-selectin is also constitutively expressed on eosinophils, and in sites of inflammation, circulating eosinophils roll on activated endothelial cells through binding of L-selectin to its ligands (Knol *et al.*, 1994; Sriramarao *et al.*, 1994). L-selectin is shed within minutes of

eosinphil activation in vitro or during therir recuitment *in vivo*.

1.2.6.1.2 Integrins

Integrins are composed of α and β subunits, and form non-covalently linked αβ heterodimers that are widely expressed on leukocytes and tissue cells. Each integrin subunit has a large extracellular domain, a single transmembrane domain, and a short cytoplasmic domain (Wardlaw, 2001). Human eosinophils express seven integrin heterodimers: α4β1 [CD49d/29; Very late activated antigen (VLA)-4], α6β1 (CD49f/29), αMβ2 [CD11b/18; macrophage-antigen (Mac)-1], αLβ2 [CD11a/18; eukocyte function-association antigen (LFA)-1], αXβ2 (CD11c/18), αDβ2 (CD11d/18), and α4β7 (CD49d/β7) (Barthel *et al.*, 2008). Each type of integrins could interact with the corresponding ligands, either in the extracellular matrix (ECM) or a counter-receptor on other cells for regulating leukocyte transmigration (Rosenberg *et al.*, 2007).

Among the β1 integrins, the α4β1 heterodimer binds to vascular cell adhesion molecule-1 (VACM-1; CD106) and plays an important role in selective eosinophil recuitment from the blood stream into tissue sites (Bochner, 1998). The other β1 integrin, α6β1, functions as a laminin receptor (Georas *et al.*, 1993). Among the β2 integrins, ligands for αLβ2 include intercellular adhesion molecule (ICAM)-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50), ICAM-5; while ligands for αMβ2 include albumin, fibrinogen, ICAM-1, VCAM-1, and vitronectin, which contribute to firm adhesion of leukocytes onto endothelium, promoting their accumulation at the site of inflammation (Wardlaw, 2001; Barthel *et al.*, 2008). αDβ2 on eosinophils binds to VCAM-1, an interaction seen most clearly following cellular activation (Rothenberg, 1998), while α4β7 is a ligand for MAdCAM-1 as wel as fibronectin and VCAM-1 (Berlin *et al.*, 1993; Walsh *et al.*, 1996). The expression levels of αMβ2, αLβ2, αXβ2 and αDβ2 are rapidly upregulated on the eosinophil surface upon activation, presumably the result of mobilization from the preformed intracellular stores (Grayson *et al.*,

1998; Kroegel *et al.*, 1994). In addition, interactions via β2 integrins and their ligands are important for degranulation, leukocyte-endothelial adhesion, and transendothelial migration (Kato *et al.*, 1998).

1.2.6.2 Immunoglobulin receptors and members of immunoglobulin superfamily

Eosinophils could express Fc receptors for IgA, IgD, IgG, and IgM (Giembycz and Lindsay, 1999). Among the three types of IgG receptors (CD16, CD32, CD64), CD32 (FcγRII) is constitutively expressed on resting human eosinophils and upregulated by interferon (IFN)-γ stimulation (Hartnell *et al.*, 1990; Hartnell *et al.*, 1992). CD32 does not bind monomeric IgG, but instead binds aggregated IgG, with a preference for IgG3 and IgG1 over IgG2 and IgG4 (Ravetch and Kinet, 1991). Not only function as IgG receptors, these receptors could also modulate eosinophil functions, including stimulation of eosinophil survival, degranulation, and genération of leukotrienes (Cromwell *et al.*, 1988; Kim *et al.*, 1999; Kita *et al.*, 1991). However, eosinophils do not constitutively express high-affinity IgG receptor CD64 (FcγRI) or low-affinity CD16 (FcγRIII). CD16 expression could be induced and upregulated by cytokine IFN-γ and chemoattractants complement (C5a), formyl peptide (FMLP) and PAF (Hartnell *et al.*, 1992; Zhu *et al.*, 1998). Eosinophils appear to express IgA receptors (CD89) and a receptor for secretary component (Lamkhioued *et al.*, 1995; Monteiro *et al.*, 1993). The expression of CD89 could be upregulated by ionomycin stimulation and eosinophil degranulation could be induced by IgA-coated particles (Abu-Ghazaleh *et al.*, 1989; Monteiro *et al.*, 1993). Until now, there remains to be controversial about the expression of either the low-affinity IgE receptor (CD23) or the high-affinity IgE receptor on eosinophils (Kita and Gleich, 1997). Though previous studies suggest that eosinophils could bind to IgE (Capron *et al.*, 1995), however, more recent investigations suggest that eosinophils express little, if any, α or β chains for the high-affinity receptor or the low-affinity IgE receptor (Seminario *et al.*, 1999; Ying *et al.*, 2000).

Eosinophils also express a number of immunoglobulin superfamily (IgSF) members, which possess a structural domain known as an immunoglobulin domain. One of most important IgSF for eosinophils is ICAM family, which consists of five members, namely ICAM-1, ICAM-2, ICAM-3, ICAM-4, and ICAM-5, belonging to type I transmembrane glycoproteins. Eosinophils express ICAM-1 and ICAM-3, but only little, if any, ICAM-2. ICAM-1 has been implicated in cell adhesion and complement binding (Hansel and Walker, 1992). Inflammatory cytokines, such as TNF-α, IL-5, GM-CSF, IL-25 and etc., could induce ICAM-1 expression on normal circulating eosinophils by a mechanism that involves de novo protein synthesis (Wong *et al.*, 2005). ICAM-1 also mediates adhesion of eosinophils to human bronchial epithelial cells which would facilitate their accumulation and retention in the airways in diseases such as asthma (Giembycz and Lindsay, 1999).

Like other leukocytes, eosinophils have been shown to express MHC class II protein, one of members of immunoglobulin superfamily. The expression of human leukocyte antigen (HLA)-DR on eosinophils has been shown to be regulated by IL-3, IL-4, GM-CSF, and IFN-γ (Lucey *et al.*, 1989; Weller *et al.*, 1993).

1.2.6.3 Chemokine, complement and other chemotactic factor receptors

Chemokine receptors are G protein-coupled receptors containing seven transmembrane domains that are found predominantly on the surface of leukocytes to mediate their migration in response to the chemokine gradient towards the site of inflammation (Rothenberg *et al.*, 1999). Following interaction with their specific chemokine ligands, chemokine receptors trigger a flux in intracellular calcium ions (calcium signaling). This causes cell responses, including the onset of a process known as chemotaxis that traffics the cell to a desired location within the organism. Chemokine receptors have been divided into four subfamilies, including CXC chemokine receptors, CC chemokine receptors, CX3C chemokine receptors and XC chemokine receptors that correspond to the distinct subfamilies of chemokines they bind.

Eosinophils constitutively express the chemokine receptor CCR3 and CCR1 (Phillips *et al.*, 2003; Ponath *et al.*, 1996) and could bind and migrate in response to CCR1 and CCR3 ligands, including macrophage inflammatory protein (MIP)-1α/CCL3, regulated upon activation normal T cell expressed and secreted (RANTES)/CCL5, MCP-2/CCL8, MCP-3/CCL7, MCP-4/CCL-13, eotaxin-1/CCL11, eotaxin-2/CCL24 and eotaxin-3/CCL26, and mucosa-associated epithelial chemokine (MEC)/CCL28. Experimental investigations have also shown that eosinophils could express a variety of other chemokine receptors including CXCR3, CXCR4, CCR5, CCR6, and CCR8 following activation by IL-5 (Nagase *et al.*, 2000; Oliveira *et al.*, 2002; Sullivan *et al.*, 1999). Through their receptors, chemokines not only regulate the migration of eosinophils, but also could modulate eosinophil function (Zimmermann *et al.*, 2003). It has been found that RANTES/CCL5 and eotaxin-1/CCL11 could promote cellular activation and release of reactive oxygen species (ROS) in eosinophils (Elsner *et al.*, 1997; Elsner *et al.*, 1999).

Besides, receptors for complement and some lipid mediators are also expressed on eosinophils. Eosinophils express receptors for complement C3a and C5a, as well as CR1 (CD35) for complement fragments C3b, C4b, iC3b, and C1q (Daffern *et al.*, 1995; DiScipio *et al.*, 1999; Fischer *et al.*, 1986). The activation of the complement system can induce eosinophil chemotaxis. Experimental investigation have also shown eosinophils express receptors for leukotrienes (LTB4, LTD4, LTE4) and 5-oxo-6,8,11,14-eicosatetraenoic acid, which are potent chomoattactants for eosinophil recruitment (Ohshima *et al.*, 2002; Powell *et al.*, 1995).

1.2.6.4 Cytokine receptors

With the advent of finding novel cytokines, their corresponding receptors are identified and their functions are characterized. For eosinophils, the three cytokines, IL-3, IL-5, and GM-CSF, are particularly important in regulating eosinophil development, survival and

activation. Eosinophil constitutively expresses the common β chain (CD131) shared by the three cytokines. In addition, eosinophil expresses the specific cytokine receptor subunit for IL-3 (IL-3Rα, CD123), IL-5 (IL-5Rα, CD125) and GM-CSF (GM-CSFRα, CD116) (Lopez *et al.*, 1986; Lopez *et al.*, 1988; Takatsu *et al.*, 1994). Eosinophils also express a number of receptors for other cytokines, including tumor necrosis factor (TNF)-α (via TNF-α receptor I and II, CD120a and CD120b), IFN-γ (CDw119), stem cell factor (via the c-kit receptor, CD117), IL-4 [via the IL-4 receptor, a heterodimer consisting of IL-4Rα chain (CD124) and the common γc chain (CD132)], IL-9 [via the IL-9 receptor, a heterodimer consisting of IL-9Rα chain (CD129) and the common γc chain (CD132)], IL-13 [via the IL-13 receptor, a heterodimer consisting of the IL-4Rα chain (CD124) and the IL-13Rα chain] (Dubois *et al.*, 1998; Hogan *et al.*, 2008; Yuan *et al.*, 1997). Recently, the receptors of novel finding Th2-related cytokines, including IL-25, IL-31 and IL-33, were also demonstrated to express on eosinophils (Cheung *et al.*, 2010; Chow *et al.*, 2010; Wong *et al.*, 2005). Activation of these receptors could modulate eosinophil functions.

1.2.6.5 Toll-like receptors

Toll-like receptors have been identified in mammals as functionally important receptors for the recognition of conserved motifs in pathogens termed pathogen-associated molecular patterns (PAMPs). TLRs share the Toll/IL-1R (TIR) homology domain, and are essential for the recognition of microbial products and result in the activation of innate immune response against microbial infections and the development of antigen-specific acquired immunity (Akira and Takeda, 2004). Eosinophils express mRNA for a number of TLRs, including TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR10 (Nagase *et al.*, 2003; Plötz *et al.*, 2001; Sabroe *et al.*, 2002), and protein for TLR5, TLR7 and TLR9. Functional analysis using TLR-specific ligands revealed that TLR5 ligand (flagellin), TLR7/8 ligand (Imiquimod R837 and R848) and TLR9 ligand (CpG) could prolong eosinophil survival,

modulate eosinophil surface expression of adhesion molecules and induce the release of cytokines and chemokines. R-848 could also induce the release of superoxides from eosinophils, while CpG induced release of EDN degranulation (Månsson and Cardell, 2009; Nagase *et al.*, 2003). The expression of TLR7/TLR8 on eosinophils has been shown to be regulated by cytokines including IFN-γ (Månsson and Cardell, 2009; Nagase *et al.*, 2003, Wong *et al.*, 2007). However, there are still controversial and contradictory results about TLR2 and TLR4 expression on eosinophils (Bonini *et al.*, 2005; Komiya *et al.*, 2006; Plötz *et al.*, 2001; Sabroe *et al.*, 2002; Wong *et al.*, 2007).

1.2.7 Eosinophil trafficking

Eosinophils develop in the bone marrow, circulate in the peripheral blood, and migrate into specific tissues, including thymus, mammary gland, ovary, uterus, gastrointestinal tract, spleen, and lymph nodes. In response to diverse stimuli, eosinophil accumulation could be recruited from the circulation into inflammatory loci where they modulate immune responses through an array of mechanisms. Transmigration of eosinophils through the vascular endothelium is the result of a multi-step process involving rolling, tethering, firm adhesion, and transendothelial migration, which involves sequential interactions of numerous adhesion molecules expressed on both eosinophils and endothelium (Kitayama *et al.*, 1997; Rosenberg *et al.*, 2007; Wardlaw, 2001) (Figure 1.4). The initial step of circulating eosinophil rolling and tethering is regulated by selectins and their counter glycosylated mucin-like ligands expressed on the endothelium (Rosenberg *et al.*, 2007; Tedder *et al.*, 1995; Wardlaw, 1999), though the initial interaction is weak. Subsequent firm adhesion of eosinophils onto and transmigration across endothelium is mediated by integrins and coordinated integrin receptors such as VCAM-1, MAdCAM-1 and ICAM-1 expressed on vascular endothelial cells (Rosenberg *et al.*, 2007; Tedder *et al.*, 1995; Wardlaw, 1999). The interactions facilitate diapedesis of eosinophils and then transendothelial migration and accumulation at the inflamed tissues

(Rosenberg *et al.*, 2007; Wardlaw, 2001).

In addition to the regulation by adhesion molecules, chemokines could also attract eosinophils for their accumulation through chemokine receptors expressed on the eosinpohils. For example, chemoattractants like CCL5 and CCL11, which are specific binding the receptors of CCR1 and CCR3 expressed on eosinophils, are also critical for mediating eosinophil transmigration into local vascular endothelium (Phillips *et al.*, 2003; Rosenberg *et al.*, 2007). Besides, Th2 cytokines also play a role in trafficking of eosinophils into inflammatory sites (Hogan *et al.*, 2008). IL-5 not only regulates growth, differentiation, activation, and survival of eosinophils but has also been shown to provide an essential signal for the expansion and mobilization of eosinophils from the bone marrow into the lung following allergen stimulation (Collins *et al.*, 1995). IL-5 also cooperates with CCL11 in the induction of tissue eosinophilia. IL-5 could prime eosinophils to respond to CCR3 lignads and increase the pool of CCL11-responsive cells (Collins *et al.*, 1995; Zimmermann *et al.*, 2003).

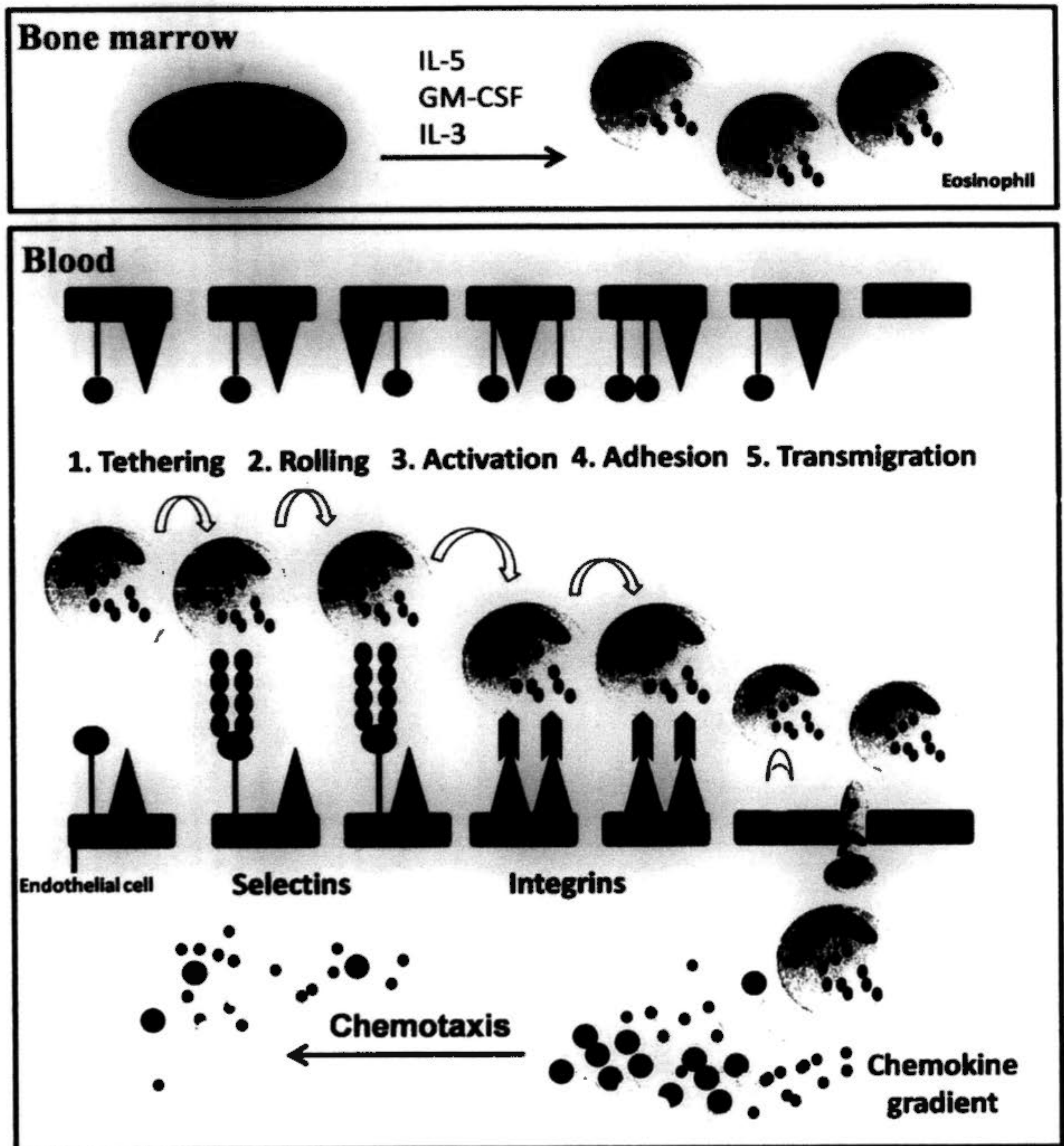


Figure 1.4 Schematic diagram of human eosinophil trafficking (Modified from Hogan *et al*., 2008).

1.3 Immunopathological roles of eosinophil in allergic inflammation

Eosinophils have long been regarded as one of the immune system components responsible for host defense against multicellular parasites and certain infections in vertebrates (Rothenberg and Hogan, 2006; Rosenberg *et al.*, 2007). Previous studies also revealed the significance of eosinophils in later immune responses of allergic inflammation (Hogan *et al.*, 2008). Although eosinophils normally account for only 1 - 3% of circulating leukocytes, their numbers markedly increase in the peripheral blood and tissues in parasite infections and allergic diseases.

1.3.1 Eosinophil in allergic asthma

Asthma is a chronic, inflammatory condition of the lower airways characterized by largely reversible airflow obstruction, increased lung and airway hyperresponsiveness (AHR), and episodic respiratory symptoms, including wheezing, productive cough, and the sensations of breathlessness and chest tightness (Buc *et al.*, 2009). The inflammatory milieu promotes the survival of eosinophils by delaying apoptosis. Increases of eosinophils in the airway, blood, and bone marrow are a hallmark and pathogenesis of atopic asthma and, in general, elevated numbers correlate with disease severity. This has led to the hypothesis that eosinophils are the central effector cells responsible for ongoing airway inflammation. Eosinophils traffic and transmigration from the bone marrow into the lung and respiratory tract in response to allergen challenge. Eosinophils exert a primary effector function in allergic inflammation through the release of a variety of mediators, including cytokines, such as IL-6, IL-1β, TNF-α, IL-5, and GM-CSF; chemokines like CCL2, CCL11, CCL5, CXCL8, and CXCL1; as well as ROS. These mediators have pro-inflammatory effects including upregulation of adhesion systems, modulation of cellular trafficking, activation and regulation of vascular permeability, stimulation of mucus secretion and smooth muscle constriction and fibrosis, mediating bronchospasm and airway hyperresponsiveness, leading to the progression of allergic asthma

(Trivedi and Lloyd, 2007). Furthermore, eosinophils can serve as major effector cells inducing airway epithelial cells damage and dysfunction by releasing toxic granule proteins, such as ECP and MBP, and lipid mediators (PAF and LTC4). Elevated numbers of eosinophils in the sputum and airway epithelium was correlated with the severity of asthma symptoms (Trivedi and Lloyd, 2007). An increase in blood or sputum eosinophils often predicts deterioration in symptoms and lung function of asthma (Hogan *et al.*, 2008). Not only the number differences, there exits other alterations of eosinophils between asthma and healthy controls, such as phenotype, adhesive properties, and mediator release. Eosinophils from patients with asthma showed higher expression of collagen receptors α1β1 and α2β1 and contained significantly more intracellular eosinophil-derived neurotoxin when compared with eosinophils from healthy control subjects (Bazan-Socha *et al.*, 2006; Sedgwick *et al.*, 2004). Besides, airway eosinophils from allergic asthmatic patients recovered after antigen challenge have enhanced adhesion to VCAM-1 and other ligands including albumin, ICAM-1, fibrinogen, and vitronectin through upregulated and activated αMβ2 (Barthel *et al.*, 2006). In addition, eosinophils also have the ability to release a variety of fibrogenic and growth factors, including TGF-α, TGF-β, fibroblast growth factor (FGF)-2, vascular endothelial growth factor (VEGF), and matrix metalloproteinase (MMP)-9, all of which are associated with and involved in airway remodeling processes of asthma (Buc *et al.*, 2009). The above findings suggest that eosinophil is a central effector cell responsible for asthmatic airway inflammation.

1.3.2 Eosinophil in atopic dermatitis

AD is an inflammatory, chronically relapsing, non-contagious and pruritic skin disease and the incidence rates are rising during the last decades (De Benedetto *et al.*, 2009). Histological features of AD leisonal skin are characterized by perivascular lymphocytes, spongiosis of keratinocytes in acute lesions, infiltrated DCs and eosinophils. DCs and

keratinocytes are critical elements in the regulation of skin pathology in AD. Keratinocytes produce antimicrobial peptides, and inflammatory mediators in response to invading microbes and allergen (De Benedetto *et al.*, 2009; McGirt and Beck, 2006). Eosinophils also play important roles in AD and aggravate the inflammatory reaction (Kapp, 1993; Simon *et al.*, 2004). Eosinophil numbers as well as eosinophil granule protein levels, especially ECP, in peripheral blood are elevated in most AD patients and appear to correlate with clinical severity of the disease (Czech *et al.*, 1992; Simon *et al.*, 2004). Inhibition of eosinophil apoptosis in AD, probably mediated by Th2 cytokines IL-5 and GM-CSF, appears to be one of mechanisms for the eosinophil accumulation observed in AD. Eosinophils are recruited to tissue sites mainly by chemokines such as CCL5 and CCL11 through the receptor CCR3, the expression level of which is elevated in eosinophils from AD patients (Yawalkar *et al.*, 1999). Besides, the interactions of eosinophil surface adhesion molecules such as selectins (L-selectin) and integrins (α4β1 and α4β7) with VCAM-1 and ICAM-1 on endothelial cell are particularly important for eosinophil extravasation and activation (Simon *et al.*, 2004). In summary, eosinophils infiltrate the skin of AD patients and aggravate the inflammatory responses.

1.3.3 Eosinophil in other allergic diseases

In addition to allergic asthma and atopic dermatitis, eosinophils also prominently involves in other allergic diseases, including allergic rhinitis, allergic conjunctivitis and etc. Allergic rhinitis, also often associated with conjunctival symptoms, is a disorder of the upper airways resulting from IgE-mediated inflammation of the nose upon contact of the nasal mucosa with allergens (Jeffery and Haahtela, 2006). The symptoms include rhinorrhea, nasal itching, sneezing, and nasal obstruction. Nasal biopsies demonstrate that allergic rhinitis is characterized by the accumulations of mast cells, eosinophils, and basophils in the epithelium and accumulations of eosinophils in the deeper subepithelium (Braunstahl *et al.*, 2003).

Besides, eosinophil granule proteins, such as MBP, ECP and EPO, could also be found in nasal biopsies and lavage fluid of allergic rhinitis patients and cause direct damage to epithelium from the luminal side (Erjefält *et al.*, 1999; Ponikau *et al.*, 2005). Allergic conjunctivitis is a bilateral, self-limiting conjunctival inflammatory process and involves early phase and late phase reactions (Bielory, 2000). The early phase reaction is mediated by IgE antibody that are bound to FcεRI receptors on conjunctival mast cells, while the late phase reaction is IgE-independent and mediated by inflammatory cells, especially eosinophils (Niederkorn, 2008). Eosinophils infiltrated in the conjunctiva are mediated by adhesion molecules integrin α4β1, and the coordinate receptor VCAM-1 expressed on the activated vascular endothelial cells (Fukushima *et al.*, 2006). In addition, significant amounts of LTC4, EPO, ECP and histamine could be detected in the later phase reaction of allergic conjunctivitis (Bonini *et al.*, 1989).

1.4 Intracellular signaling transduction pathways of eosinophils

Eukaryotic cells respond to extracellular stimuli and transmit biochemical information from one part of the cell to another through transmitting intracellular signals to coordinate appropriate responses. The reactions of eosinophils upon a variety of stimulations and in allergic responses are also induced, in part, by a diverse array of intracellular signaling pathways (Figure 1.5). Intensive studies demonstrated that various intracellular signal transduction pathways, including Ras-Raf-mitogen-activated protein kinase (MAPK), Janus kinase (JAK)-signal transducer and activator of transcription (STAT), phosphatidylinositol 3-kinase (PI3K), and nuclear factor-kappa B (NF-κB) pathways, are activated and involved in inflammatory response, apoptosis, degranulation, chemotaxis, expression of adhesion molecules, and the pathogenesis of allergic inflammation (Wong *et al.*, 2002).

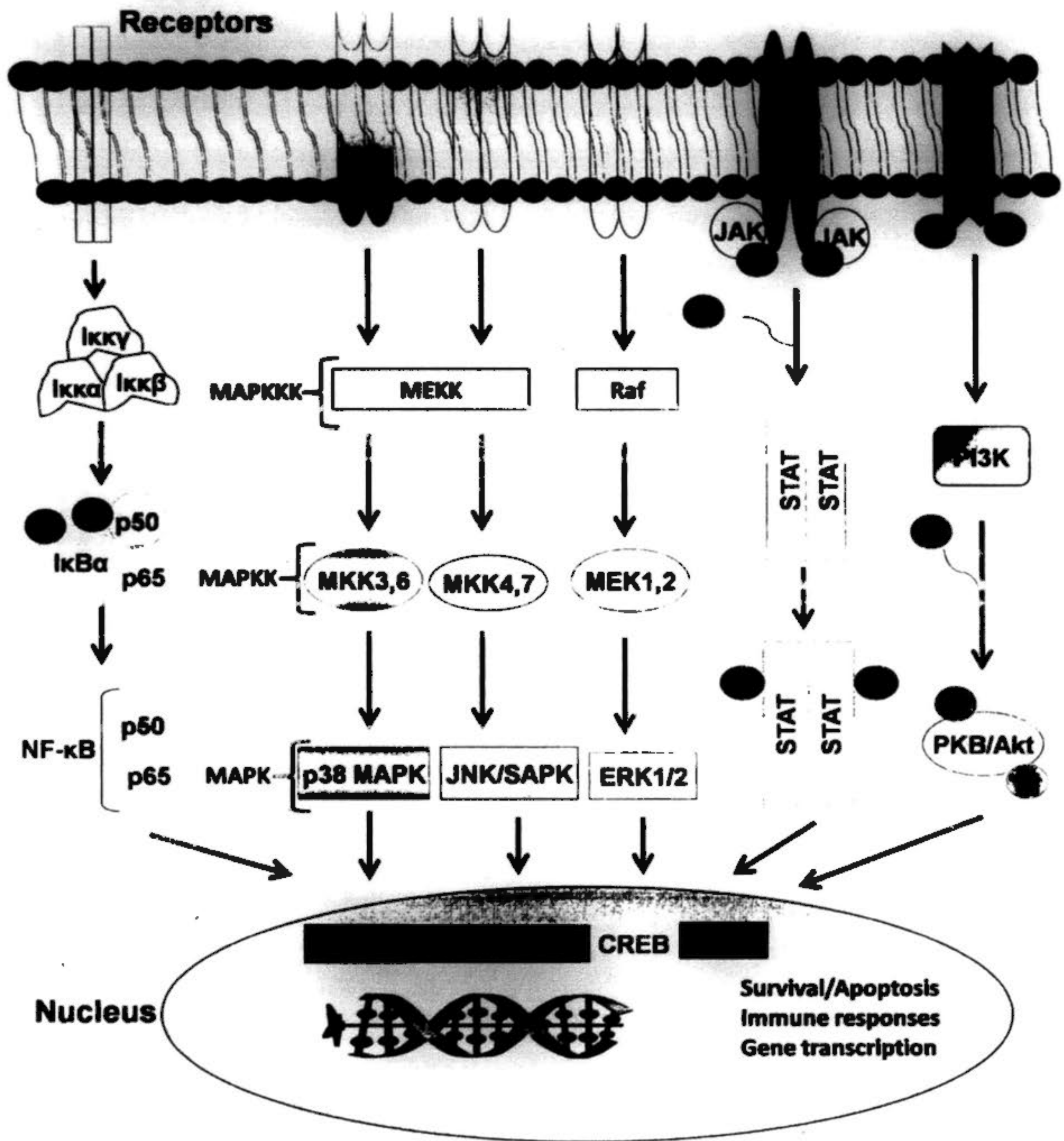


Figure 1.5 Schematic diagram of intracellular signal transduction pathways in human eosinophils.

1.4.1 Ras-Raf-MAPK pathway

The MAPKs are evolutionarily conserved serine and threonine kinases that could be activated by a variety of stimuli, including pattern-recognition receptors, proinflammatory cytokines, ROS, UV radiation and etc. MAPK pathways are composed of a three-tier kinase module in which MAPKs are activated upon phosphorylation by MAPK kinases (MAPKK), which in turn are activated when phosphorylated by upstream MAPK kinase kinases (MAPKKK) (Kumar *et al.*, 2003; Wong *et al.*, 2002). There are three major MAPK members in mammalian cells, including, extracellular signal regulated kinase (ERK) (p42/p44), c-Jun amino terminal kinase [JNK, also referred to as stress-activated protein kinases (SAPKs)], and p38 MAPK. Activated MAPKs phosphorylate numerous substrates in all cellular compartments, including various membrane proteins (CD120a, Syk, and calnexin), nuclear substrates (ATF1 and -2, Elk-1, NF-κB, Ets-1, Pax6, NF-AT, MEF2, c-Fos, c-Myc, and STAT3), cytoskeletal proteins (neurofilaments, paxillin and Tau), and several MKs (MSK1 and -2, MNK1 and -2, and MK2 and -3), and result in gene regulation (Roux and Blenis, 2004). The MAPKs are inactivated by phosphatases, especially the dual specificity phosphatases, also called MAPK phosphatases (Saklatvala, 2004). All the three members link extracellular signals to the machinery that control fundamental cellular processes such as growth, proliferation, differentiation, migration, transformation, apoptosis, and regulate pro-inflammatory cytokine expression. It has been reported that MAPKs play an important role in eosinophil apoptosis, chemotaxis, degranulation and cytokine production. Eotaxin could activate p38 MAPK and ERK2 to mediate the ECP release and chemotaxis of human eosinophils (Kampen *et al.*, 2000). Leptin-induced eosinophil apoptosis, cytokine and chemokine release, and chemokinetic migration are differentially regulated by the three MAPKs (Wong *et al.*, 2007).

1.4.2 JAK-STAT pathway

JAKs are a family of cytoplasmic tyrosine kinases that are associated with membrane-proximal regions (termed box 1 and box 2) of cytokine receptors and play a major role in the initial steps of cytokine signaling (Kisseleva *et al.*, 2002). There are four members of mammalian JAKs, including JAK1, JAK2, JAK3, and tyrosine kinase (Tyk) 2, and all JAKs have similar structures and molecular weight approximately at 120 - 135 kD. These kinases are widely expressed in a variety of different cell types, with the exception of JAK3, which is selectively expressed in cells of hematopoietic origin. The binding of cytokine ligands results in dimerization of receptor subunits and tyrosine auto-phosphorylation of JAKs. Activated JAKs then initiate the signal transduction cascade by phosphorylation of tyrosine motifs present in receptor cytoplasmic domains and in receptor-associated proteins. The phosphorylated tyrosine residues in the receptors then serve as the docking sites for recruiting Src homology 2 (SH2) domain-containing proteins like cytoplasmic transcription factor STATs. There are seven mammalian STATs: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. Once recruited to the receptor, STATs themselves are phosphorylated on a unique conserved carboxy-terminal tyrosine, dissociate from the receptor and form STAT:STAT dimers, which is mediated by reciprocal SH2-phosphotyrosine interactions between the dimer partners. Activated STAT:STAT dimers then rapidly translocate into the nucleus, where they bind to specific sequences in the promoter regions of their target genes and stimulate their transcription (Darnell, 1997; Leonard and O'Shea, 1998). Besides, most vertebrate STATs contain a serine phosphorylation site within a P(M)SP motif in their C-termini. The mutation of the phosphorylated residue serine to alanine could alter the transcription factor activity (Decker and Kovarik, 2000). IL-5, IL-3, and GM-CSF are hematopoietic cytokines which signal through a common β subunit (βc) of a heterodimeric receptor. JAK2-STAT5 activation mediated by IL-5, IL-3, and GM-CSF is critical for anti-apoptotic signal for human eosinophils (de Groot *et al.*, 1998). IL-5 and GM-CSF promote survival-associated kinase Pim-1 and cell cycle regulator Cyclin D3 expression in

human eosinophils through the activation of STAT3 and STAT5 (Stout *et al.*, 2004).

STATs are activated by tyrosine phosphorylation, while STAT deactivation is mediated by phosphotyrosine phosphatases. Three families of tyrosine phosphatases could negatively regulate JAK/STAT signaling pathways, including the SH2-containing tyrosine phosphatases SHP1 (PTP1C, Phospho-Tyrosine Phosphatase 1C) and SHP2 (PTP1D), transmembrane tyrosine phosphatase CD45, and PTP1B and TC-PTP (T Cell Protein Tyrosine Phosphatase) tyrosine phosphatases. In addition, mammalian protein inhibitors of activated STAT (PIAS) and the inducible SH2 containing proteins belonging to the SOCS (suppressor of cytokine signaling) family were identified as negative regulators of STAT signaling (Valentino and Pierre, 2006).

1.4.3 PI3K pathway

PI3K, the family of enzymes responsible for phosphorylation at the 3-position of the inositol ring within specific phosphoinositides (PIs) and phosphatidylinositol (PtdIns), are crucial components of many signaling pathways playing a pivotal role in many different physiological events (Lindmo and Stenmark, 2006). Mammalian PI3Ks are divided into three different classes, in which class I PI3Ks have been extensively studied. Class I PI3Ks seem to use phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2, PIP2] as preferential substrate in vivo and therefore their main product is phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3, PIP3], a ubiquitous second messenger. Their activity is acutely regulated by agonist activation through tyrosine kinase receptors (RTKs), such as growth factors and insulin receptor, or G-protein-coupled receptors, followed by phosphorylation of upstream Src-family tyrosine kinase, such as Lyn. PIP3 could recruit downstream pleckstrin homology (PH) domain-containing signaling molecules to the cell membrane and interact with these proteins, such as the protein kinase B (PKB)/Akt of protein serine/threonine kinases, driving their conformational change and resulting in their phosphorylation by the constitutively active

phosphoinositide-dependent kinase 1 (PDK1) at threonine 308 and by PDK2 [mammalian target of rapamycin complex 2 (mTORC2)] at serine 473. Activated AKT translocates to the cytoplasm and nucleus, activates and phosphorylates several downstream cellular target proteins, including glycogen synthase kinase 3α (GSK3α), GSK3β, forkhead box O transcription factors (FoxO), MDM2, BCL2-interacting mediator of cell death (BIM) and BCL2-associated agonist of cell death (BAD) to facilitate cell survival and cell cycle entry. AKT is negatively regulated by the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10), which dephosphorylates PIP3 (Engelman *et al.*, 2006; Yap *et al.*, 2008). PI3K regulate IL-5-induced chemotaxis and survival of eosinophils as well as the ability of eosinophils adhesion to ICAM-1-dependent substrata (Hall *et al.*, 2001). PI3K is also required for group IV cytosolic phospholipase A(2) (gIV-PLA 2) activation and hydrolytic production of arachidonic acid in activated eosinophils (Myou *et al.*, 2003). Furthermore, IL-3, IL-5, and GM-CSF, as well as chemoattractants PAF, RANTES, and C5a could all induce PI3K activity (Coffer *et al.*, 1998).

1.4.4 NF-κB pathway

NF-κB was first identified as a transcription factor that binds to the intronic enhancer of the kappa light chain gene (the κB site) in B cells. The mammalian Rel/NF-κB family of transcription factors consists of RelA (p65), c-Rel, RelB, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100). These proteins share a conserved amino-terminal 300-amino acid Rel homology domain and form homo- and heterodimers through the Rel-homology domain. NF-κB activity is tightly controlled by a collection of IκB inhibitory proteins characterized by the presence of multiple ankyrin repeats, including IκBα, IκBβ, IκBε, p100, p105, IκBζ, IκBNS (NF-κBδ), and Bcl-3. In normal cells, the NF-κB family is normally kept inactive in the cytoplasm by interaction with inhibitors IκBs or the unprocessed forms of NF-κB1 and NF-κB2 (Ghosh and Hayden 2008; Vallabhapurapu and

Karin, 2009). NF-κB activation can be induced by a plethora of extracellular signals. Upon cell activation, IκB molecules undergo rapid ubiquitin-mediated proteasomal degradation that results in the release of the bound, cytoplasmic NF-κB dimers. The major and most well-studied activation pathway by extracellular stimuli is the canonical NF-κB signaling pathway, which are mainly RelA:p50 and c-Rel:p50 heterodimers. p50 and RelA heterodimer ranslocates to the nucleus and bind to κB sites in promoters or enhancers of target genes. Unlike p50, RelA contains a transactivation domain in the C-terminal end of the protein, which initiates gene transcription through direct interaction with the basal transcription apparatus and the recruitment of transcriptional co-activators and transcriptional co-repressors (Ghosh and Hayden 2008; Wong *et al.*, 2002; Wong *et al.*, 2004). NF-κB plays an important role in innate and adaptive immunity and inflammatory responses, the development and survival of lymphocytes and lymphoid organs, as well as malignant transformation. Activation of NF-κB can enhance the transcription of a variety of genes, including cytokines and growth factors, adhesion molecules, immunoreceptors, and acute-phase proteins, all of which serve to coordinate the body's response to injury and infection through the recruitment and activation of immune cells and the stimulation of repair processes.

NF-κB also appears to be important in the pathogenesis of allergic response. In OVA sensitisation and challenge model, NF-κB from total lung extracts showed enhanced activation in Brown-Norway rats (Lin *et al.*, 2000). Mice that lack NF-κB p50 have defective in the production of IL-5 and CCL11 and reduced eosinophilic responses to aerolised allergen (Yang *et al.*, 1998). NF-κB has also been demonstrated to involve in eosinophil activation mediated by a variety of inflammatory stimuli, such as IL-25, IL-31 and IL-33 (Cheung *et al.*, 2006; Cheung *et al.*, 2010; Chow *et al.*, 2010).

1.5 Aim of the study

The incidences of allergic diseases are increasing rapidly all over the world, including

Hong Kong as well as mainland China, and account for a significant portion of annual healthcare expenditures worldwide. The allergic inflammation involves the accumulation and infiltration a variety of allergic-related responding cells in the airway mucosa or the skin, including eosinophils, CD4+ T cells, DCs, langerhans cells, mast cells, and basophils. Eosinophils are the principal effector cells involved in late-phase reaction of allergic inflammation and sensitively regulated by multiple mediators at the reaction site. Therefore, the aims of the research are to address the activation of eosinophils by novel found diverse stimuli and elucidate the underling intracellular immunological mechanisms. The activation of eosinophils was examined in the aspects of survival enhancement, modulation of surface adhesion molecule expression, as well as release of allergic-related mediators such as inflammatory cytokines, chemokines, and granule proteins. The underling immunological mechanisms were revealed by studying the intracellular signaling transduction pathways.

Epithelial cells at mucosal surface, such as in the skin, airways, gut and intestine, are the first line of defense of the organism against microbes and foreign antigens. Recent work strongly suggests that cytokines produced by epithelial cells play a critical role in shaping the immune response and in the pathogenesis of immune-mediated diseases (Saenz *et al.*, 2008). Thymic stromal lymphopoietin (TSLP) is an epithelial cell-derived cytokine expressed in skin, gut, lungs, and thymus, and is highly expressed by bronchial epithelial cells and skin keratinocytes in allergic diseases but not detectable in healthy control subjects (Comeau and Ziegler, 2010). TSLP acts as a master switch for allergic inflammation through the activation of DCs and mast cells for initiating inflammatory Th2 responses. To elucidate the immunological cascades of epithelium/keratinocyte-eosinophil mediated allergic inflammation, we have examined the modulating effects of TSLP on human eosinophils in Chapter 3.

IL-27 is a novel IL-12 family cytokine, which also includes previous found members IL-12 and IL-23 (Kastelein *et al.*, 2007). IL-27 has pleiotropic effects in that although it

induces Th1 differentiation and inflammatory cytokines from monocytes and mast cells, IL-27 also has an immunosuppressive function such as inhibiting Th1, Th2, and Th17 responses, and suppresses production of inflammatory cytokines (Yoshida *et al.*, 2009). In addition, though belonging to IL-12 family cytokines, IL-12 and IL-23 was reported to exert opposite effects on eosinophils. IL-23 could potentially enhance eosinophil survival whereas IL-12 increased in vitro human eosinophil apoptosis (Cheung *et al.*, 2008; Nutku *et al.*, 2001). In an attempt to further elaborate the role of IL-12 family members in allergic responses, we have investigated the biological effects of IL-27 on human peripheral blood eosinophils and the underlying mechanisms in Chapter 4.

Although eosinophils are key effector cells in allergic diseases, however, the complicated features of allergic responses cannot be attributed to a single cell type only. The complex interaction among different cell types therefore gives a more comprehensive view on the potential mechanisms for the pathogenesis of allergic diseases. In addition, experimental and epidemiological studies have suggested that bacterial and viral infections could modulate allergic inflammation. Therefore, we investigated the *in vitro* co-culture system to study the interaction between eosinophils and bronchial epithelial cells, the first line barrier defence against microbial organisms in airways, upon novel found pattern recognition receptor nucleotide-binding oligomerization domain (NOD) like receptor (NLR) protein NOD1 and NOD2 ligand stimulation in Chapter 5. The investigation could give clear information and unraveling more immunological roles of NOD1 and NOD2 in allergic asthma.

In summary, we herein investigated the immunological mechanisms of eosinophil activation by diverse novel stimuli to improve our understanding of their expanding roles in the pathogenesis of allergic diseases and thereby identifying better approaches of targeting eosinophils for potential therapies. Only when these complicated interactions and immunological mechanisms are clarified can the comprehensive roles of eosinophils in allergic diseases be clearly defined.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Human eosinophils

2.1.1.1 Human Buffy Coat

Fresh human buffy coat was obtained from healthy adult volunteers of Hong Kong Red Cross Blood Transfusion Service. Eosinophils were purified from buffy coat within 48 h after donation.

2.1.1.2 Phosphate-Buffered Saline (PBS) Solution (1 ×)

PBS (1 ×, pH 7.4) was prepared by dissolving PBS powder (Sigma-Aldrich, St. Louis, MO, USA) containing 0.2 g monobasic potassium phosphate, 0.2 g potassium chloride, 8 g sodium chloride and 1.15 g dibasic sodium phosphate in 1 Liter (L) double distilled water (ddH_2O), and was then sterilized by autoclaving at 121°C for 15 min and kept at 4°C.

2.1.1.3 Wash Buffer

Sterilized 1 × PBS solution was supplemented with 2% heat-inactivated fetal bovine serum (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) and used as wash buffer in MACS.

2.1.1.4 Sodium Chloride (NaCl) Solution (1.5 M)

NaCl solution (1.5 M) was prepared by dissolving 87.6 g NaCl (Sigma-Aldrich) in 1 L ddH_2O, sterilized and then kept at 4°C. The concentrated stock solution was further diluted to

1.8% (w/v) and 0.9% sterile normal saline.

2.1.1.5 Percoll gradient Solution (1.082 g/ml)

Percoll gradient solution (150 ml, density 1.082 g/ml) was freshly prepared by mixing 87.9 ml Percoll stock solution (density 1.130 g/ml, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA), 15 ml sterilized 1.5 M NaCl and 47.1 ml sterilized ddH_2O and then stored at 4°C.

2.1.1.6 Magnetic-activated cell sorting (MACS) and Anti-CD16 Magnetic MicroBeads

The MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) composed of three components, including MidiMACS Separation Unit, MACS MultiStand, and LS Separation Column which could separate up to 2×10^9 total cells and 10^8 magnetically-labeled cells. The anti-CD16 magnetic MicroBeads (Miltenyi Biotec) were conjugated with monoclonal mouse anti-human CD16 antibodies.

2.1.2 Cell culture

2.1.2.1 Human Epidermal Keratinocytes (HEK)

Primary human epidermal keratinocytes (ScienCell Research Laboratories, Carlsbad, CA, USA) were isolated from human epidermal tissue. HEK were tested negative for HIV-1, HBV, HCV, mycoplasma, bacteria, yeast and fungi.

2.1.2.2 Primary Human Bronchial Epithelial Cells (HBEpiC)

Primary human bronchial epithelial cells (ScienCell Research Laboratories) were isolated from human lung tissue. HBEpiC were tested negative for HIV-1, HBV, HCV, mycoplasma, bacteria, yeast and fungi.

2.1.2.3 Human Bronchial Epithelial Cell Line BEAS-2B

BEAS-2B cells (ATCC, Manassas, VA, US) were derived from normal human bronchial epithelium transformed by an Adenovirus12-SV40 hybrid virus (Reddel *et al.*, 1988). BEAS-2B cells retain electron microscopic features of epithelial cells and show positive staining with antibodies to cytokeratin but do not form tight junctions.

2.1.2.4 Serum Supplements

Fetal bovine serum (FBS) was purchased from Invitrogen Corporation. Heat-inactivated fetal bovine serum (HI-FBS) was prepared by heating at 56^{0}C for 30 min and stored at -20^{0}C until use.

2.1.2.5 Culture Medium

Rosewell Park Memorial Institute (RPMI) 1640 medium containing L-glutamine and 25 mM hydroxyl-ethyl-piperazine-N'-2-ethene-sulfonic acid (HEPES), and Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) medium, with L-glutamine, 15 mM HEPES and pyridoxine HCl were purchased from Invitrogen Corporation. The LHC-9 Medium (1 ×) specific for human bronchial epithelial cells was purchased from Invitrogen Corporation. EpiLife Medium was also purchased from Invitrogen and contained 60 μM of calcium chloride for the growth of normal human epidermal keratinocytes. Human Keratinocyte Growth Supplement, an ionically balanced supplement containing bovine pituitary extract, bovine insulin, hydrocortisone, bovine transferrin, and human epidermal growth factor, was also used for maintaining complete culture environment for the growth of normal human epidermal keratinocytes.

No antibiotic was added in all culture solutions and they were free of detectable endotoxin (< 0.1 EU/ml) as determined by the Limulus amebocyte lysate assay (sensitivity limit 12 pg/ml;

Biowhittaker Inc., Walkersville, MD).

2.1.2.6 Trypan Blue Solution

Trypan blue solution (Sigma-Aldrich) was prepared containing 0.4% (w/v) trypan blue dissolved in 0.81% sodium chloride and 0.06% dibasic potassium phosphate.

2.1.2.7 Harleco Hemacolor Staining Solutions

Harleco hemacolor staining solutions (E Merck Diagnostica, Darmstadt, Germany) were used to stain peripheral blood eosinophils after cytospin. Hemacolor solution 1 was methanol for cell fixation; solution 2 was a buffered color reagent red containing eosin; solution 3 was phosphate buffered thiazine solution for nuclei detection. All the staining solutions were and stored at room temperature and protected from light.

2.1.2.8 Recombinant Human Cytokines

Recombinant human TSLP, IL-25, IL-27, IL-31 and IL-33 were purchased from R & D Systems Inc., Minneapolis, MN, USA. Recombinant human GM-CSF, IL-4 and IL-5 were purchased from PeproTec Inc., Rocky Hill, NJ, USA.

2.1.2.9 Toll-like receptor ligands and NOD-like receptor ligands

TLR2 ligand Peptidoglycan (PGN) from *Staphylococcus aureus*, TLR3 ligand Polyinosine-polycytidylic acid [poly(I:C)], a synthetic analog of dsRNA, TLR4 ligand Lipopolysaccharides (LPS) from *Escherichia coli* serotype 0111:B4, NOD1 ligand γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and iE-DAP negative control γ-D-glutamyl-lysine (iE-Lys), and NOD2 ligand N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide/MDP) and MDP negative control (D-D isomer) were purchased from

InvivoGen (San Diego, CA, USA).

2.1.2.10 Protein Synthesis Inhibitors

RNA transcription inhibitor actinomycin D and mRNA translation inhibitor cycloheximide were purchased from Sigma-Aldrich.

2.1.2.11 Signal Transduction Pathways Inhibitors

ERK inhibitor U0126, p38 MAPK inhibitor SB203580, JNK inhibitor SP600125, IkBα phosphorylation inhibitor BAY11-7082, JAK inhibitor AG490, and PI3K inhibitor LY294002 were purchased from Calbiochem, Darmstadt, Germany. Double distilled water was used to dissolve SB203580, while dimethyl sulfoxide (DMSO) was used to dissolve U0126, SP600125, BAY11-7082, AG490, and LY294002. The concentration of DMSO was kept at 0.1% (v/v) in all studies.

2.1.3 RNA extraction, Reverse transcription–polymerase chain reaction (RT-PCR), and real-time quantitative PCR (qPCR)

2.1.3.1 TRI-Reagent

Total RNA was extracted using TRI-Reagent® (Molecular Research Center Inc., Cincinnati, OH, USA). RNA was phase-separated using bromochloropropane (BCP) (Molecular Research Center Inc.) and precipitated by isopropanol.

2.1.3.2 Reverse Transcription (RT)

The first-stand cDNA synthesis kit was purchased from Applied Biosystems Inc., Foster City, CA, USA. The kit consisted of the following components: MultiScribe Reverse Transcriptase (50 U/μL), RNase Inhibitor (20 U/μL), dNTP Mixture (2.5 mM each for dATP, dCTP, dGTP,

dTTP), Oligo $d(T)_{16}$ (50 μM), Random Hexamers (50 μM), 10 × RT buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), $MgCl_2$ solution (25 mM).

2.1.3.3 PCR

2.1.3.3.1 PCR primers

The lyophilized PCR primers (Invitrogen) for different human genes were reconstituted in TE buffer to obtain a stock at concentration of 100 mM and kept at -20°C. The primer sequences and their PCR product sizes were as follows:

Primer	Sequence of primers	Product Size
hTSLPR	Forward 5'-TGAGATTTTCGTGGCATCAG-3'	238 bp
	Reverse 5'-GACCAGTCGCTTGGGTATGT-3'	
hIL-7R	Forward 5'-GAAGGTTGGAGAAAAGAGTC-3'	418 bp
	Reverse 5'-CAAAATGCTGATGGTTAGTAAG-3'	
hWSX-1	Forward 5'-TGGACTTTTCCGAGGATGAC-3'	451 bp
	Reverse 5'-GGAGCAGCAGCAGGTAATTC-3'	
hgp130	Forward 5'-TGCTGATTGCAAAGCAAAAC-3'	452 bp
	Reverse 5'-CCCACTTGCTTCTTCACTCC-3'	
β-actin	Forward 5'-CTTCCTTCCTGGGCATGGAG-3'	300 bp
	Reverse 5'-TGGAGGGGCCGGACTCGTCA-3'	

2.1.3.3.2 [Tris-ethylenediamine tetra-acetic acid (EDTA)] (TE) Buffer

TE buffer consisted of 10 mM Tris-HCL (pH 7.5) and 0.1 mM EDTA in ddH_2O. The buffer was used for primer reconstitution and kept at room temperature.

2.1.3.3.3 Polymerase chain reaction

AmpliTaq Gold PCR Master Mix was purchased from Applied Biosystems. The kit consisted of the following components: AmpliTaq Gold DNA polymerase 250 U (0.05 U/μl), GeneAmp PCR Gold Buffer, $MgCl_2$ (5 mM), and dNTP (400 μM each).

2.1.3.3.4 Ethidium Bromide Solution and Tris-Acetate-EDTA (TAE) Electrophoresis Buffer (10 ×)

Ethidium Bromide (10 mg/ml) (Invitrogen) was used for agarose gel electrophoresis of nucleic acids at 0.5 μg/ml. TAE Buffer (10 × Ultra Pure) was also obtained from Invitrogen contained 400 mM Tris-acetate and 10 mM EDTA at pH 8.3 was diluted to 1 × TAE Buffer for agarose DNA electrophoresis.

2.1.3.3.5 100 bp DNA Ladder

The 100 bp DNA ladder (Invitrogen) was dissolved in TE Buffer at concentration of 1 μg/μl and consists of 15 blunt-ended fragments between 100 and 1500 bp in multiples of 100 bp and an additional fragment at 2072 bp. This reagent was 10-fold diluted with loading buffer, and 10 μl of the diluted DNA ladder (0.1 μg) was used per lane.

2.1.3.3.6 Agarose Gel

Agarose gels (2%) (Invitrogen) were prepared by dissolving 2 g agarose in 100 ml 1 × TAE buffer (Invitrogen) and were used to analyze RNA and PCR products.

2.1.3.4 Real-time quantitative PCR

2.1.3.4.1 Primers for q PCR

The following lyophilized qPCR primers (Invitrogen) for different human genes were used:

Primer	Sequence of primers
NOD1	Forward 5'-TTCCCTGCTCACTCAGAGCAAAG-3'
	Reverse 5'-TAGCACAGCACGAACTTGGAGTCA -3'
NOD2	Forward 5'-CGAGGCATCTGCAAGCTCATTGAA -3'
	Reverse 5'-GTGCACAGCCGTCAGTCAATTTGT-3'
GAPDH	Forward 5'-ATGGGGAAGGTGAAGGTCG-3'
	Reverse 5'- GGGGTCATTGATGGCAACAATA-3'

2.1.3.4.2 Real-time qPCR

The FastStart Universal SYBR Green Master (ROX) kit for qPCR was purchased from Roche Applied Science, Mannheim, Germany. The kit was a ready-to-use, 2 × concentrated master mix that contains all the reagents (except primers and template) and a special ROX reference dye in the SYBR Green I detection format.

2.1.4 Cell surface and intracellular immunofluorescence staining

2.1.4.1 Fluorescence-activated cell sorting (FACS) Flow Sheath Fluid

FACS Sheath Fluid (BD Biosciences, San Jose, CA, USA), a balanced electrolyte solution, contains sodium chloride, disodium EDTA, potassium chloride, monobasic potassium phosphate, dibasic sodium phosphate, and anti-microbial agent as preservatives.

2.1.4.2 FACS Staining Medium

The FACS staining medium was composed of 0.5% BSA (Sigma-Aldrich) and 0.01% sodium azide (NaN_3) in 1 × PBS. The buffer was kept at 4°C and used for washing in immunofluorescence staining.

2.1.4.3 Fixation and Permeabilization Buffers for intracellular staining

Formaldehyde (Sigma-Aldrich) (36%) was used as fixation buffer, and methanol (Fisher Scientific, Leicestershine, UK) was used for permeabilization for intracellular fluorescent eosinophil staining.

2.1.4.4 Antibodies for flow cytometry

2.1.4.4.1 Fluorescein isothiocyanate (FITC)-conjugated mouse IgG_1 anti-human ICAM-1 (CD54) monoclonal antibody

The antibody (Clone BBIG-I1, R & D Systems Inc.) at concentration of 50 μg/ml was used with 5 μl per 5 × 10^5 cells in direct immunofluorescence staining.

2.1.4.4.2 Phycoerythrin (PE)-conjugated mouse IgG_1 anti-human ICAM-1 (CD54) monoclonal antibody

The antibody (Clone HA58, BD Biosciences) was used with 5 μl per 5 × 10^5 cells in direct immunofluorescence staining.

2.1.4.4.3 FITC-conjugated mouse IgG_1 anti-human ICAM-3 (CD50) monoclonal antibody

The antibody (Clone Cal 3.10, R & D Systems Inc.) at concentration of 25 μg/ml was used with 5 μl per 5 × 10^5 cells in direct immunofluorescence staining.

2.1.4.4.4 FITC-conjugated mouse IgG_1 anti-human L-selectin (CD62L) monoclonal antibody

The antibody (Clone 4G8, R & D Systems Inc.) at concentration of 25 μg/ml was used with 5 μl per 5×10^5 cells in direct immunofluorescence staining.

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

The antibody (Clone 6.7, BD Biosciences) was used with 5 μl per 5×10^5 cells in direct immunofluorescence staining.

2.1.4.4.6 PE-conjugated mouse IgG_1 anti-human IL-7Rα (CD127) monoclonal antibody

The antibody (Clone HIL-7R-M21, BD Biosciences) at concentration of 0.2 mg/ml was used with 2 μl per 5×10^5 cells in direct immunofluorescence staining.

2.1.4.4.7 PE-conjugated mouse IgG_{2a} anti-human TSLPR monoclonal antibody

The antibody (Clone 1D3, Biolegend, CA, USA) was used with 5 μl per 5×10^5 cells in direct immunofluorescence staining.

2.1.4.4.8 FITC-conjugated mouse IgG_1 monoclonal Ig isotype control

The antibody (Clone MOPC-21, BD Biosciences) was used with 5 μl per 5×10^5 cells in direct immunofluorescence staining.

2.1.4.4.9 PE-conjugated mouse IgG_1 monoclonal Ig isotype control

The antibody (Clone MOPC-21, BD Biosciences) was used with 5 μl per 5×10^5 cells in direct immunofluorescence staining.

2.1.4.4.10 PE-conjugated mouse IgG_{2a} monoclonal Ig isotype control

The antibody (Clone MOPC-173, Biolegend) was used with 5 µl per 5 × 10^5 cells in direct immunofluorescence staining.

2.1.4.4.11 Mouse IgG_1 anti-human phospho-IκB monoclonal antibody

The antibody (Clone 39A1413, BD Biosciences) at concentration of 50 µg/ml was used with 8 µl per 5 × 10^5 cells.

2.1.4.4.12 Mouse IgG_1 anti-human phospho-ERK1/2 monoclonal antibody

The antibody (Clone 20A, BD Biosciences) at concentration of 50 µg/ml was used with 8 µl per 5 × 10^5 cells.

2.1.4.4.13 Mouse IgG_1 anti-human phospho-p38 monoclonal antibody

The antibody (Clone 30, BD Biosciences) at concentration of 50 µg/ml was used with 8 µl per 5 × 10^5 cells.

2.1.4.4.14 Mouse IgG_1 anti-human phospho-JNK monoclonal antibody

The antibody (41/JNK/SAPK, BD Biosciences) at concentration of 50 µg/ml was used with 8 µl per 5 × 10^5 cells.

2.1.4.4.15 Mouse IgG_1 anti-human AKT monoclonal antibody

The antibody (Clone J1-223.371, BD Biosciences) at concentration of 50 µg/ml was used with 8 µl per 5 × 10^5 cells.

2.1.4.4.16 Mouse IgG_1 anti-human gp130 monoclonal antibody

The antibody (Clone 28126, R & D Systems Inc.) at concentration of 50 μg/ml was used with 8 μl per 5 × 10^5 cells.

2.1.4.4.17 Mouse IgG_{2a} anti-human NOD2 monoclonal antibody

The antibody (Clone NOD-15, Biolegend) at concentration of 50 μg/ml was used with 8 μl per 5 × 10^5 cells.

2.1.4.4.18 Mouse IgG_1 monoclonal Ig isotype control

The antibody (Clone MOPC-31C, BD Biosciences) at concentration of 50 μg/ml was used with 8 μl per 5 × 10^5 cells.

2.1.4.4.19 FITC-conjugated goat anti-mouse IgG antibody

The antibody of goat anti-mouse IgG (H+L) (Invitrogen) at a dilution of 1:250 in FACS staining medium was used as secondary antibody with 80 μl per 5 × 10^5 cells in indirect immunofluorescence staining.

2.1.4.5 Human Serum

Human serum obtained from venous blood of Chinese healthy volunteers was used for blocking step in immunofluorescence staining.

2.1.5 Detection of inflammatory mediator release

2.1.5.1 Protein Array

The expression profile of 79 different cytokines in culture supernatant of eosinophils with or without treatment was semi-quantitatively assessed using antibody based RayBio® human cytokine antibody array V (RayBiotech Inc., Norcross, GA, USA). The kit contains 8 Array

Membranes, Biotin-conjugated anti-cytokines, 1000 × HRP-conjugated streptavidin, 1 × Blocking buffer, 20 × Wash buffer I, 20 × Wash buffer II, Detection buffer C, Detection buffer D and Eight-well tray (1 each). The cytokine array map was listed in Appendix.

2.1.5.2 Enzyme-linked Immunosorbent Assay (ELISA)

Concentrations of inflammatory cytokine IL-6 and chemokine CXCL8/IL-8 and CCL2/MCP-1 in culture supernatant were quantitated using ELISA kit from BD Biosciences, with detection limits of 2.2, 0.8, 1.0 pg/ml for IL-6, CXCL8/IL-8, and CCL2/MCP-1, respectively. CXCL1/GRO-α was measured using ELISA kit from R & D Systems and the detection limit is 10 pg/ml. TSLP concentration was also measured using ELISA kit from R & D Systems. Absorbance at 450 nm was read using VICTOR^3 Multilabel Counter model 1420-032 (PerkinElmer, Waltham, MA, USA), and the values were adjusted with the corresponding reading at 570 nm.

2.1.5.3 Cytometric beads array (CBA)

The BD CBA system uses the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analytes in a particle-based immunoassay. Each bead in a CBA provides a capture surface for a specific protein and is analogous to an individually coated well in an ELISA plate. The BD™ CBA Human Inflammation Kit can be used to quantitatively measure IL-8, IL-1β, IL-6, IL-10, TNF-α, and IL-12p70 protein levels in a single sample with a wide range of measured concentration from 20 pg/ml to 5000 pg/ml. The BD™ CBA Human Chemokine Kit can be used to quantitatively measure CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, and CXCL10/IP-10 levels in a single sample with a wide range from 10 pg/ml to 2500 pg/ml.

2.1.5.4 Assay for ECP degranulation

The release of ECP was measured by fluorescence enzyme immunoassay (FEIA) using AutoCAP analyzer (Pharmacia Diagnostics AB, Uppsala, Sweden).

2.1.6 Detection of cell viability and apoptosis

2.1.6.1 Annexin V and propidium iodide (PI) staining

Apoptosis of eosinophils was assessed by the TACS^{TM} Annexin V-FITC Apoptosis Detection Kit (Trevigen Inc., Gaithersburg, MD, USA), which contained 10 × binding buffer, propidium iodide (PI), and Annexin V-FITC.

2.1.6.2 DNA ladder assay

Genomic DNA from eosinophils was harvested using quick apoptotic DNA ladder detection kit (BioVision Inc., Mountain View, CA, USA), containing TE lysis buffer, Enzyme A solution, Enzyme B (lyophilized), Ammonium Acetate solution, and DNA Suspension buffer.

2.1.7 Protein extraction

2.1.7.1 Cell Lysis Buffer

RIPA buffer (Cell Signaling Technology, Beverly, MA, USA) contains 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 μg/ml leupeptin. The cell lysis buffer used for total protein extraction was prepared by 1 × RIPA buffer with fresh protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany) and PMSF just prior to use.

2.1.7.2 Nuclear protein extract

Nuclear protein extracts were prepared using Nuclear Extraction Kit (Panomics Inc., Fremont, CA, USA), which contained Buffer A, Buffer B, 100 mM DTT, Protease inhibitor, Phosphates inhibitor I, and Phosphates inhibitor II.

2.1.7.3 BCA Protein Assay

The Pierce BCA Assay (Thermo, Rockford, IL, US), which consists of BCA Reagent A, Reagent B, and Albumin Standard Ampules (2 mg/ml), can be used to determine the concentration of proteins from the whole cell lysates and nuclear protein extract fractions.

2.1.8 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.1.8.1 Acrylamide Solution [30% (w/v)]

Acrylamide/Bis solution (30%) (29:1) was purchased from Bio-Rad Laboratories, Hercules, CA, USA and stored at 4°C.

2.1.8.2 Ammonium Persulfate (APS) Solution [10% (w/v)]

APS (Sigma-Aldrich) (10%) was dissolved in double distilled water and stored at -20°C until use.

2.1.8.3 Tris-HCl Buffer (pH 7.5)

Tris base (Sigma-Aldrich) was prepared as 1 mol/L stock solution in DEPC-treated double distilled water, and then adjusted to pH 7.5 with HCl and stored at 4°C.

2.1.8.4 Upper Buffer for Stacking Gel

The upper buffer was prepared as 0.5 mol/L Tris-HCl (pH 6.8) and kept at 4°C.

2.1.8.5 Lower Buffer for Separating Gel

The lower buffer was prepared as 1.5 mol/L Tris-HCl (pH 8.8) and kept at 4°C.

2.1.8.6 SDS Solution [10% (w/v)]

SDS (Sigma-Aldrich) 5 g was dissolved in 50 ml double distilled water and kept at room temperature.

2.1.8.7 N, N, N', N'-Tetra-methylethylenediamine (TEMED)

TEMED (Bio-Rad Laboratories) used for initiating polymerization of SDS-PAGE was stored at 4°C in dark.

2.1.8.8 Tris-Glycine-SDS Electrophoresis Buffer (10 ×)

Tris-glycine-SDS electrophoresis buffer (10 ×) was composed of 250 mM Tris-HCl (pH 7.5), 1.92 M glycine, and 1% SDS in double distilled water. The concentrate was freshly diluted to 1 × working buffer solution using double distilled water for SDS-PAGE.

2.1.8.9 Laemmli Sample Buffer

Laemmli sample buffer (Bio-Rad Laboratories) contained 62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, and 0.01% bromophenol blue. Before use, 50 μl of β-mercaptoethanol was added per 950 μl of sample buffer for a final concentration of 5% β-mercaptoethanol, 710 mM. The prepared buffer was 2 × concentrate and 1 part sample was diluted with 1 part Laemmli sample buffer for loading.

2.1.8.10 BenchMark™ Pre-stained Protein Ladder

The BenchMark™ Pre-stained protein ladder (Invitrogen) allows easy visualization of protein

molecular weight ranges during electrophoresis and evaluation of western transfer efficiency. The protein ladder was stored at -20°C and composed of 10 pre-stained protein bands in the range of 6 - 180 kDa, including 180 kDa, 115 kDa, 82 kDa, 64 kDa, 49 kDa, 37 kDa, 26 kDa, 19 kDa, 15 kDa, 6 kDa. The proteins are covalently coupled to a blue or pink dye, and the fourth protein band from the top (64 kDa) is coupled to a pink dye for easy orientation and to ensure proper identification of each protein

2.1.9 Western blot analysis

2.1.9.1 Antibodies

Goat anti-human TSLPR antibody, normal goat IgG, and mouse anti-human TCCR antibody were purchased from R & D Systems. Rabbit anti-human STAT3 and STAT5, anti-phospho-STAT3 (Ser 727), anti-phospho-STAT3 (Tyr 705), anti-phospho-STAT5 (Tyr 694), and anti-NOD1 antibodies were purchased from Cell Signaling Technology. Anti-pan ERK, anti-phospho-ERK1/2, anti-p38MAPK, anti-phospho-p38MAPK, anti-IκBα and anti-phospho-IκBα were purchased from BD Pharmingen (San Diego, CA, USA). Mouse anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody and rabbit anti-human gp130 antibody were purchased from Millipore (St. Charles, MO, USA). Donkey anti-mouse Ig and donkey anti-rabbit Ig secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from GE Healthcare. Donkey anti-goat Ig was purchased from R & D systems.

2.1.9.2 Polyvinylidene Difluoride (PVDF) Western Blotting Membranes

The PVDF membranes (pore size of 0.45 μm) were purchased from GE Healthcare Corp.

2.1.9.3 Tris-Glycine Buffer (10 ×)

Tris-glycine buffer (10 ×) consisted of 0.25 M Tris-HCl and 1.92 M glycine, pH 8.3 in double distilled water and stored at 4°C.

2.1.9.4 Tris-Glycine-Methanol Transfer Buffer (1 ×)

The transfer buffer was prepared by freshly mixing 20% methanol in 1 × Tris-glycine buffer and kept at 4°C.

2.1.9.5 Tris-Buffered Saline Tween 20 (TBST) washing buffer

The washing buffer for Western blot consisted of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.05% (v/v) Tween 20 (Sigma-Aldrich).

2.1.9.6 Non-fat Milk Solution (5%)

Non-fat milk powder was purchased from San Hua Co., Hong Kong, China. Non-fat milk solution (5%) was freshly prepared by dissolving 5 g milk in 100 ml TBST washing buffer. The solution was used as blocking solution.

2.1.9.7 Enhanced Chemiluminescent (ECL) Plus™ Western Blotting Detection

The ECL Plus™ Western blotting detection kit (GE Healthcare) consisted of two components: Lumigen™ PS-3 detection reagent A, an ECL Plus substrate solution containing Tris buffer, and Lumigen™ PS-3 detection reagent B, a stock acridan solution in dioxane and ethanol. The kit was stored at 4°C. Reagent A and Reagent B were mixed in a ratio of 40:1 for detection use.

2.1.9.8 ECL Films (Hyperfilm™ ECL™)

The Hyperfilm™ ECL™ (18 × 24 cm) purchase from GE Healthcare was a high

performance chemiluminescence film used for Western blot analysis.

2.1.10 Detection of activities of nuclear transcription factors

2.1.10.1 Tris-Borate-EDTA (TBE) Electrophoresis Buffer (5 ×)

The 5 × TBE electrophoresis buffer (pH 8.0) stock was prepared by dissolving 54 g Tris base, 27.5 g boric acid and 20 µl 0.5 M EDTA in 1 L double distilled water. The working buffer 0.5 × TBE was prepared by diluting one part of the 5 × TBE stock buffer with nine parts of double distilled water. Both working and stock solutions were stored at 4°C.

2.1.10.2 Hybond-N+ Nylon Membrane

The Nylon membranes (pore size of 0.45 µm) with positive charge were purchased from GE Healthcare Corp.

2.1.10.3 Electrophoretic Mobility Shift Assay (EMSA, Gel Shift) kit

The NF-κB (1) EMSA Kit (Panomics) was used to detect the activation and nuclear translocation of NF-κB. The kit was consisted of three set of components. The first set contained 5 × binding buffer, poly d(I-C), loading dye, 2 × blocking buffer, control nuclear extract, control probe, and cold (unlabeled) control probe, and were stored at -20°C. The second set contained distilled H_2O, solution I, Solution II, Solution III, streptavidin-HRP conjugate, 10 × Detection buffer, and 10 × Wash buffer, and were kept at 4°C. The third set stored at -20°C were transcription factor (TF) probe and cold TF probe with the consensus NF-κB oligonucleotides 5'-AGTTGAGGGGACTTTCCCAGGC-3'.

2.2 Methods

2.2.1 Purification of human eosinophils, neutrophils and peripheral blood

mononuclear cells

Fresh human buffy coat obtained from healthy adult volunteers was diluted 1:2 with PBS at 4°C and centrifuged using an isotonic Percoll solution (density 1.082 g/ml) for 30 min at 1,000 *g*. The peripheral blood mononuclear cells (PBMC) at the interface were collected firstly. The eosinophil-rich granulocyte fraction was collected and incubated with anti-CD16 magnetic beads at 4°C for 45 min. CD16-positive cells were depleted by passing through a LS+ column within a magnetic field. CD16+ neutrophils were immediately collected by pipetting the wash buffer and applying the plunger onto the column. The isolated eosinophils were cultured in RPMI 1640 medium supplemented with 10% HI-FBS.

With this preparation, the purity of the drop-through eosinophils was examined. Eosinophils (5×10^4) were centrifuged at 300 rpm for 3 min on microscopic slide by the Shandon Cytospin Centrifuge (Cometa Scientific, Nottingham, UK). The cells were air-dried, stained with Harleco hemacolor staining solutions, and examined using Nikon Eclipse E800 microscope (Nikon Corp., Tokyo, Japan). Only eosinophils with the purity more than 95% were used for functional study.

2.2.2 Cell culture

2.2.2.1 Primary Human Epidermal Keratinocyte Cell Culture

HEK were maintained in EpiLife Medium with Human Keratinocyte Growth Supplement. The cells were incubated at 37°C in a humidified incubator with the supply of 5% carbon dioxide (CO_2) and 95% air. HEK were subcultured when they were up to 90% confluent.

2.2.2.2 Primary Human Bronchial Epithelial Cell Culture

HBEpiC were maintained in LHC-9 medium, incubated at 37°C in a humidified incubator with the supply of 5% CO_2 and 95% air, and were subcultured when up to 90% confluent.

2.2.2.3 Human Bronchial Epithelial Cell line BEAS-2B Culture

BEAS-2B were maintained in DMEM/F12 medium supplemented with 10% HI-FBS, incubated at 37°C in a humidified incubator with the supply of 5% CO_2 and 95 % air, and were subcultured when they grown to 90% confluent.

2.2.2.4 Co-culture of Human Eosinophils with BEAS-2B cells

BEAS-2B (8×10^4 cells) cells were cultured in 24-well plate one day before the co-culture. The medium was replaced with RPMI 1640 medium containing 10% HI-FBS with or without eosinophils (5×10^5 cells).

2.2.2.5 Co-culture of Human Eosinophils with BEAS-2B cells in the presence of transwell inserts

To prevent direct interaction between eosinophils and BEAS-2B cells in the co-culture, transwell inserts (pore size: 0.4 μM) (BD Biosciences) were used to separate the cells into two compartments. BEAS-2B (8×10^4 cells) and eosinophils (5×10^5 cells) were cultured together in the presence of transwell inserts, in which eosinophils were placed in the upper compartment and BEAS-2B cells were in the lower one.

2.2.2.6 Co-culture of fixed Human Eosinophils with BEAS-2B cells

BEAS-2B cells (8×10^4 cells) or eosinophils (5×10^5 cells) were pre-treated with 1% paraformaldehyde in PBS on ice for 45 min 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 BEAS-2B cells or eosinophils were co-cultured in RPMI 1640 medium supplemented with 10% FBS with different treatment.

2.2.3 Total RNA extraction

Briefly, eosinophils (1 × 10^7 cells) were washed with 1 × PBS and then lysed and homogenized in 1 ml TRI-Reagent containing highly denaturing guanidinium isothiocyanate and phenol. BCP (200 μl) was used to extract phenol and protein in organic phase while RNA remained dissolved in aqueous phase. After acquiring the aqueous phase, isopropanol was used to precipitate the RNA and then washed with 75% ethanol. Extracted RNA was then treated with DNase I (Ambion Inc., Austin, TX, USA) to exclude genomic DNA contamination and dissolved in RNase-free water (QIAGEN, Valencia, CA, USA). The extracted total RNAs were finally dissolved in RNase-free water and stored at -80°C.

2.2.4 RT-PCR

Extracted RNA was reverse transcribed into first-strand complementary DNA using first-strand cDNA synthesis kit (Applied Biosystems). The RT reaction mixture final concentration included 1 × RT buffer, 5.5 mM $MgCl_2$, 500 μM dNTP, 2.5 μM Oligo $d(T)_{16}$, 0.4 U/μL RNase inhibitor, 1.25 U/μL MultiScribe Reverse Transcriptase, and 0.5 μg RNA. The reaction was performed at 25°C for 10 min, 48°C for 30 min and 95°C for 5 min.

PCR reaction was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems), containing 0.5 μM of 5' and 3' primers in PCR reaction buffer (1 min each at 95°C, 55°C and 72°C) for 30 cycles after an initial 5 min of denaturation at 95°C. After the amplification using PTC 100 thermal cycler (MJ Research Inc., Waltham, MA, USA), PCR products were electrophoresed on 2% agarose gel in TAE buffer and stained with ethidium bromide. The electrophoretic bands were documented with Gene Genius Gel Documentation System (Syngene Inc., Cambridge, UK).

2.2.5 Quantitation of mRNA expression

The quantitative expression of human NOD1 and NOD2 was performed using ABI Step One (Applied Biosystems), containing 30 μM of 5' and 3' primers, 1 × FastStart Universal SYBR Green Master (ROX), and appropriate cDNA template. Values were expressed as arbitrary units relative to GAPDH plus 10^4.

2.2.6 Cell surface and intracellular immunofluorescence staining

For assessing cell surface molecules, human eosinophils, HEK, HBEpiC, and BEAS-2B cells were washed and resuspended with cold 1 × PBS supplemented with 0.5% BSA. The cells were firstly blocked with 2% human pooled serum for 20 min. For direct immunofluorescence staining, the cells were incubated with FITC- or PE- conjugated mouse anti-human monoclonal antibody and FITC- or PE- conjugated mouse IgG isotype at 4°C in the dark for 45 min. For indirect immunofluorescence staining, the cells were first incubated with either mouse anti-human monoclonal antibody or mouse IgG isotype at 4°C for 45 min, following by incubation FITC-conjugated anti-mouse secondary antibody (1:250) at 4°C in the dark for further 30 min. The cells were then washed and re-suspended in 1% paraformaldehyde in 1 × PBS as fixative and subjected to analysis.

For studying the intracellular protein expression, eosinophils were fixed with 4% formaldehyde for 10 min at 37°C. After centrifugation, cells were permeabilized in ice-cold methanol for 30 min and followed by either direct or indirect immunofluorescence staining. Expression of cell surface markers, adhesion molecules, and intracellular proteins was analyzed by FACSCalibur flow cytometer using CellQuestPro software (BD Biosciences).

2.2.7 Detection of cell viability and apoptosis

2.2.7.1 Annexin V and PI staining

Eosinophil survival was assessed by TACS™ Annexin V-FITC assay (Trevigen Inc.) using

flow cytometry. After treatment eosinophils were washed with 1 × PBS, resuspended in 1 × binding buffer, and then incubated with FITC-Annexin V and PI in dark for 15 min at room temperature. Samples were analyzed by gating eosinophil population based on its forward and side light scatter with exclusion of any cell debris. The population of viable cells was characterized by low mean fluorescence intensity (MFI) of both FITC-Annexin V and PI.

2.2.7.2 DNA ladder assay for apoptosis

Genomic DNA was harvested using quick apoptotic DNA ladder detection kit (BioVision Inc.) according to the instruction and analyzed by electrophoresis with 2% agarose gel.

2.2.8 Cell adhesion assay

Eosinophils (1×10^6 cells/ml) with different treatments were cultured in 24-well plates coated with fibronectin (BD Bioscience) at 37°C, in a 5% CO_2, humidified atmosphere for 16 hours. After incubation, non-adherent cells were removed and the plates were washed three times with 37°C PBS. Eosinophil adherence in each well was assessed by counting the number of eosinophils adhered to the fibronectin-coated well in four high-power fields (magnification: 100 ×) under inverted microscope.

2.2.9 Protein extraction

Eosinophils, neutrophils and PBMC were washed twice with cold 1 × PBS and lysed in freshly prepared complete 1 × RIPA lysis buffer with brief sonication. Cell debris was removed by centrifugation at 14,000 g for 15 min and supernatant of total proteins were stored as aliquots at -80°C until use.

2.2.10 Western blot analysis

Equal amount of proteins determined by BCA protein assay was heat-denatured and subjected to SDS-PAGE. After electrophoresis, the proteins were electrotransferred onto PVDF membrane. The membrane was blocked with 5% non-fat milk and probed with primary antibody at 4°C overnight. After washing, the membrane was incubated with secondary antibody coupled to HRP for 1 h at room temperature. Antibody-antigen complexes were then detected using ECL Plus™ detection kit.

2.2.11 Detection of activities of transcription factor NF-κB

Eosinophil nuclear proteins were extracted using Nuclear Extraction Kit (Panomics). Equal amounts of nuclear proteins were subjected to EMSA to analyse the activity of transcription factor NF-κB using NF-κB (1) EMSA Kit. Briefly, nuclear extracts with the same protein amount were incubated with biotin-labeled NF-κB oligonucleotide for 20 min at room temperature to allow DNA/protein binding. The DNA/protein complexes were then resolved by 6% non denaturing polyacrylamide gel electrophoresis and transferred to Hybond-N+ membrane. The DNA/protein on the membrane was then fixed using a UV crosslinker. After blocking, the biotin-labeled DNA was detected using streptavidin-HRP and a chemiluminescent substrate according to the manufacturer's instructions.

2.2.12 Statistical analysis

All data were expressed as means +/± SD from three independent experiments. The statistical significance of differences was determined by one-way analysis of variance (ANOVA) or Student's t-test. When ANOVA indicated a significant difference, Bonferroni post hoc test was then used to assess the difference between groups. Any difference with p values less than 0.05 was considered significant. All analyses were performed using Graphpad PRISM software (version 5.01, GraphPad Software Inc., San Diego, CA, USA).

Chapter 3
Role of TSLP in Allergic Inflammation: TSLP-mediated Eosinophil Activation

3.1 Introduction

Thymic stromal lymphopoietin (TSLP), a novel IL-7-like cytokine, was firstly identified as a growth factor in the supernatant of thymic stromal cell line Z210R.1 which could support the proliferation and survival of mouse pre-B cell line NAG8/7 (Friend *et al.*, 1994). TSLP is mainly produced by keratinocytes, bronchial epithelial cells, stromal cells, lung fibroblasts, smooth muscle cells, allergen-activated basophils and IgE-primed mast cells (Kato *et al.*, 2007; Kinoshita *et al.*, 2009; Liu *et al.*, 2007; Medoff *et al.*, 2009; Sokol *et al.*, 2008; Zhang *et al.*, 2007). TSLP receptor is a heterodimer comprising TSLP-specific binding chain (TSLPR, also known as CRLF2) and the IL-7Rα chain, primarily expressed on CD11c+ DCs, mast cells, pre-activated CD4+ and CD8+ T cells (Pandey *et al.*, 2000; Reche *et al.*, 2001; Rochman *et al.*, 2007; Rochman and Leonard, 2008). Upon interaction with TSLPR/IL-7Rα receptor heterodimer, TSLP induces the activation of STAT3 and STAT5 (Liu *et al.*, 2007; Quentmeier *et al.*, 2001). TSLP could promote different stages of B cell development, expand autologous CD4+ T cells and induce the proliferation and differentiation of CD4+CD25+FOXP3+ regulatory T cells (Astrakhan *et al.*, 2007; Mazzucchelli *et al.*, 2005; Rochman and Leonard, 2008; Watanabe *et al.*, 2004; Watanabe *et al.*, 2005).

Though TSLP was involved in lymphocyte development and homeostasis, increasing evidences have emphasized its crucial roles in allergic disorders. Human TSLP is found on chromosome 5q22.1, and close to the atopy and asthma-associated cytokine cluster on

5q31-33 (Huston and Liu, 2006; Ober and Hoffjan, 2006), which encodes Th2-related cytokines and cytokine receptors, such as IL-3, IL-4, IL-5, IL-9, IL-13, and the IL-4 receptor (Walley *et al.*, 2001). Like other Th2 cytokines, TSLP expression is associated with allergic inflammation in both mice and humans. TSLP was expressed in acute and chronic lesions of AD patients but not detected in normal skin and non-lesional skin (Soumelis *et al.*, 2002). TSLP mRNA was highly expressed in epithelial and submucosa of asthmatic patients and the BAL concentration of TSLP was much higher in patients with asthma and chronic obstructive pulmonary disease compared to healthy control subjects (Ying *et al.*, 2005; Ying *et al.*, 2008). TSLP was also significantly up-regulated in nasal lavages of patients with allergic rhinitis compared to the normal controls (Xu *et al.*, 2010). Moreover, overexpression of TSLP in the airway epithelium of patients with allergic rhinitis and asthma has been shown to be correlated with the severity of these diseases (Al-Shami *et al.*, 2005; Mou *et al.*, 2009). In OVA-induced murine asthmatic models, TSLPR-/- mice have reduced BAL cells and eosinophils, and exhibit lower levels of Th2 cytokines, thereby reducing airway inflammation. Mice overexpressing TSLP under the control of the lung-specific surfactant protein-C promoter (SPC-TSLP) develop CD4+ Th2 cell airway infiltrates, eosinophilia, goblet cell hyperplasia, increased serum IgE, airway hyper-responsiveness, and remodeling (Zhou *et al.*, 2005). Mice engineered to over-express TSLP in the skin resulted in AD-like syndrome (Yoo *et al.*, 2005). Induced expression of TSLP in mouse keratinocytes can aggravate OVA-mediated allergic asthma (Zhang *et al.*, 2009). In addition, anti-TSLP and anti-TSLPR antibodies or TSLPR-Fc fusion protein could prevent Th2-mediated allergic skin inflammation and airway inflammation (Al-Shami *et al.*, 2005; He *et al.*, 2008; Shi *et al.*, 2008). All the above findings indicate that TSLP play important roles in mediating allergic inflammation.

TSLP has been demonstrated to involve in immune cell activation. TSLP potently activates immature human CD11c+ myeloid DCs to enhance the survival and up-regulate

co-stimulatory molecules OX40L, CD80 and CD86 and Th2 chemokines CCL17 and CCL22 for the induction of inflammatory Th2 responses characterized by producing high levels of IL-4, IL-5, IL-13 and TNF-α but low level of IL-10 (Soumelis *et al.*, 2002). It subsequently provokes allergic inflammation through the induction of IgE, activation of mast cells and recruitment of eosinophils. Uniquely, the interaction between TSLP-induced OX40L on DCs and OX40 on naïve T-cells was identified as the critical molecular signal for TSLP-primed inflammatory Th2 differentiation in the absence of IL-12 (Ito *et al.*, 2005). In the early phase allergic reaction, TSLP can synergistically react with inflammatory cytokines IL-1β and TNF-α to stimulate mast cells to release high levels of inflammatory Th2 cytokines and chemokines (Allakhverdi *et al.*, 2007). The synergistic effects of TSLP with IL-1β and TNF-α or novel Th2 cytokine IL-33 are also responsible for the activation of CD34+ progenitor hematopoietic cells, which can differentiate to other immune effector cells, including DCs, T cells, B cells, mast cells, basophils and eosinophils (Allakhverdi *et al.*, 2009). Eosinophils produce various inflammatory mediators and immunoregulatory cytokines for the late phase asthmatic response (Hogan *et al.*, 2008). However, the effects of TSLP on eosinophils have not been reported.

Ablation of lymphocytes does not completely abrogate allergic diseases, implying that other immune effector cells may be involved in allergic responses (Allakhverdi *et al.*, 2007; Li *et al.*, 2006; Yoo *et al.*, 2005; Zhou *et al.*, 2005). Eosinophils have been shown to play an essential role in inflammation in mice induced by intradermal administration of TSLP (Jessup *et al.*, 2008). In addition, large numbers of eosinophils have been found to infiltrate beneath skin epidermis in AD and bronchial epithelial cells in allergic asthma, while keratinocytes and bronchial epithelial cells can produce high expression level of TSLP in allergic inflammation (Simon *et al.*, 2004; Soumelis *et al.*, 2002; Ying *et al.*, 2005; Ying *et al.*, 2008). The above evidence therefore implicate that TSLP might exhibit direct effects on eosinophils. In order to further elucidate the pathophysiological link between the activated epithelial

cells/keratinocytes and eosinophils mediated inflammation in allergic diseases, we examined the *in vitro* effects of TSLP on eosinophil activation, in terms of apoptosis, release of cytokines and chemokines and degranulation, and explored the underlying intracellular regulatory mechanisms.

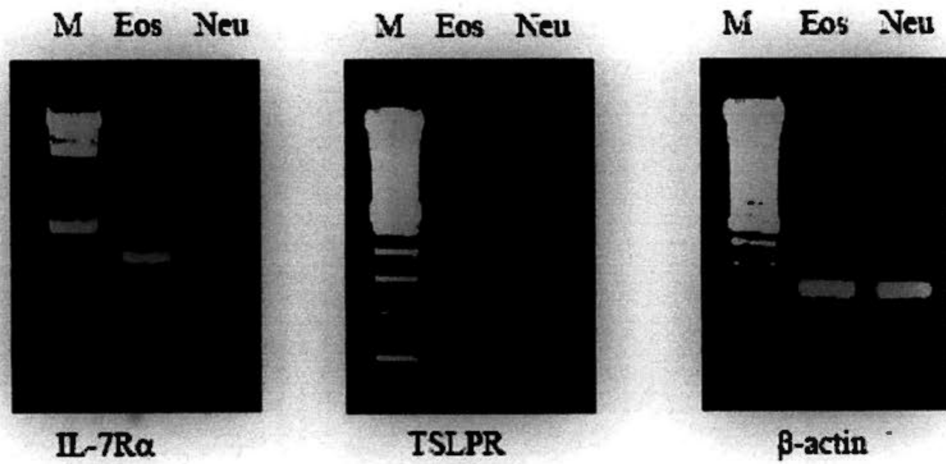
3.2 Results

3.2.1 Human eosinophils express TSLP heterodimeric receptors

Eosinophil purity of 95% or above was used in the present study (Figure 3.1A). Since neutrophils were also involved in allergic reaction (Foley and Hamid, 2007), both eosinophils and neutrophils were examined for TSLP receptor complex expression. IL-7Rα mRNA was highly expressed in freshly isolated eosinophils, and TSLPR mRNA was also expressed in eosinophils though the expression level was lower than that of IL-7Rα. On the other hand, there was no detection of mRNA expression of heterodimeric TSLP receptor complex in neutrophils (Figure 3.1B). Further investigation by flow cytometric analysis showed that both subunits were constitutively expressed on the surface of eosinophils, but not on the surface of neutrophils (Figure 3.1C and 3.1D). In consistent with mRNA level, the expression of IL-7Rα protein was higher compared to TSLPR subunit. We could not detect IL-7Rα expression on neutrophils, which was consistent with a previous report that neutrophils do not express IL-7Rα (Girard and Beaulieu, 1997). Because of the low surface expression of TSLPR, further investigation by intracellular staining using flow cytometry was performed. Results showed that both TSLPR and IL-7Rα have obvious intracellular expression level (Figure 3.1E). Western blot also confirmed that eosinophils but not neutrophils constitutively expressed TSLPR subunit (Figure 3.1F). Moreover, since TSLPR expression could be induced on activated CD4+ and CD8+ T cells but not on freshly isolated CD4+ and CD8+ T cells (Rochman *et al.*, 2007; Rochman and Leonard, 2008), we also examined whether TSLPR and IL-7Rα could be induced to upregulate their expression by IL-5 or GM-CSF, which are hematopoietic cytokines for eosinophil development and/or activation. However, both cytokines had no effects on the expression of TSLPR and IL-7Rα on eosinophils (data not shown). Taken together, the constitutive expression of TSLP receptor complex on eosinophils indicates that TSLP could exert direct effects on eosinophils.

(A)

(B)



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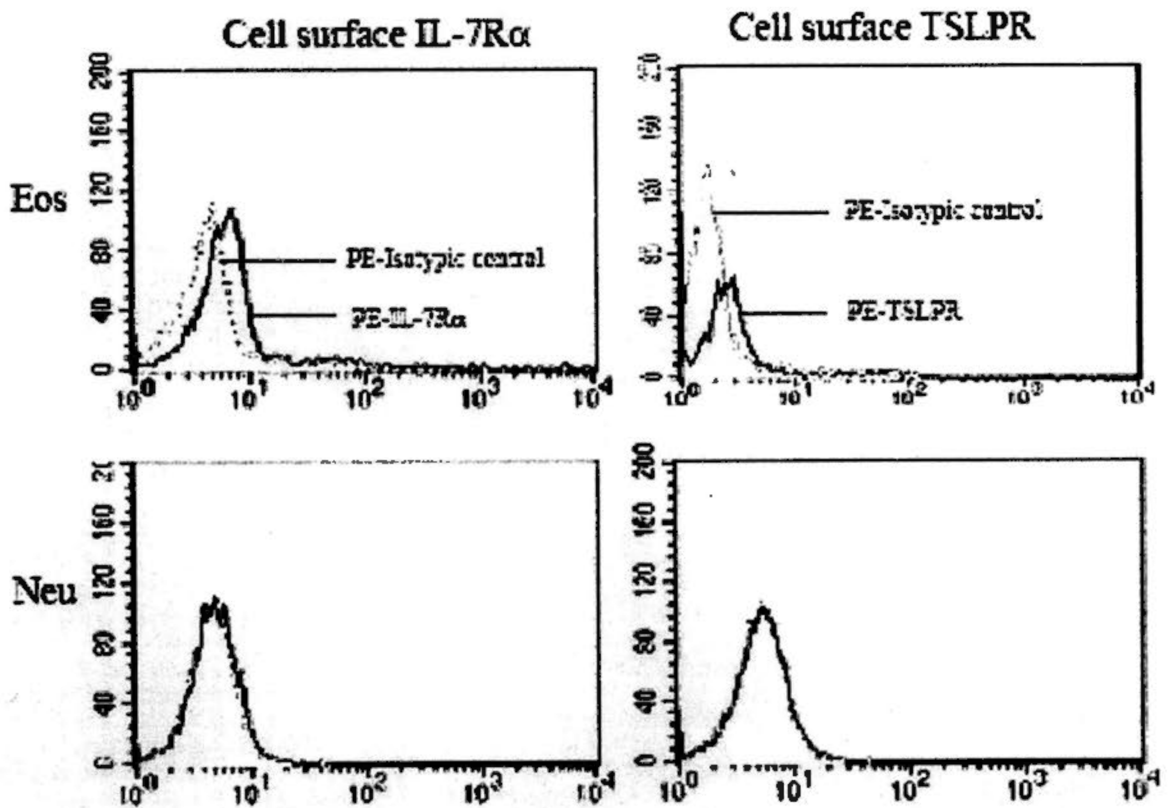

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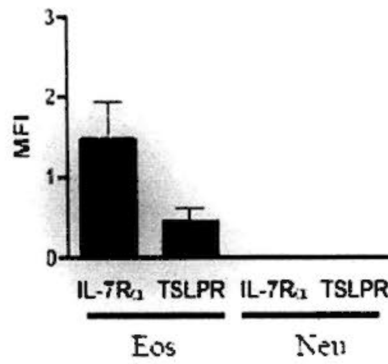

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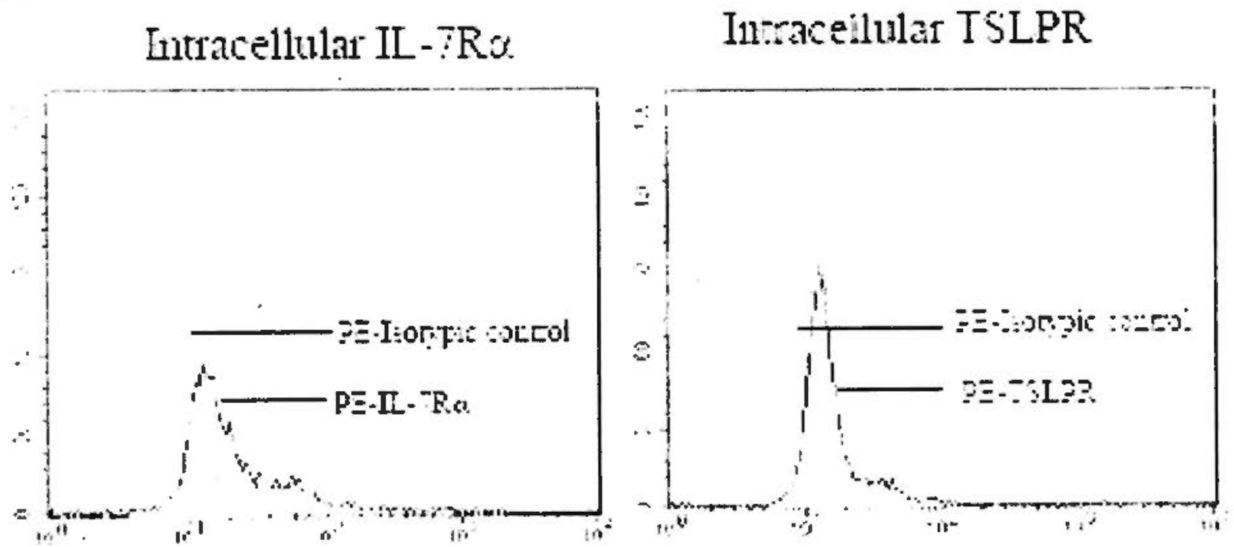

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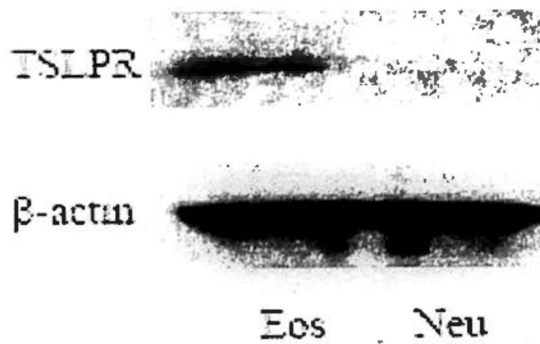


Figure 3.1 Expression of the heterodimeric receptor of TSLP for eosinophils.

(A) The purity of isolated eosinophils was assessed using Harleco hemacolor staining solutions and examined under microscope at 400 × magnification. Left: a heterogenous population of blood cells containing eosinophils and neutrophils before anti-CD16 MACS purification. Right: a population of purified eosinophils. (B) Total RNA was extracted from eosinophils and neutrophils (1×10^6 cells), followed by RT-PCR analysis for IL-7Rα, TSLPR and β-actin (housekeeping gene). (C) Representative histograms of cell surface expression of TSLPR and IL-7Rα on eosinophils and neutrophils were determined by flow cytometry. Triplicate experiments were performed with essentially identical results and representative figure is shown. (D) Quantitative results of flow cytometric analysis of cell surface expression of IL-7Rα and TSLPR on neutrophils and eosinophils are presented with arithmetic mean + SD (MFI) of three independent experiments. Results have been normalized by subtracting appropriate isotypic control. (E) Representative histograms of the intracellular expression of TSLPR and IL-7Rα in eosinophils. Triplicate experiments were performed with essentially identical results and representative figure is shown. (F) Representative Western blot analysis of TSLPR protein expression of human eosinophils and neutrophils is shown from triplicate experiments with essentially identical result. Total proteins were extracted from eosinophils and neutrophils (1×10^6 cells). β-actin was used as control to ensure an equal amount of loaded protein.

Dotted line: isotypic controls, PE-conjugated mouse IgG1 or PE-conjugated mouse IgG2a; solid line: PE-conjugated anti-human IL-7Rα or PE-conjugated anti-human TSLPR antibodies.

M: 100 base-pair molecular size marker; Eos: eosinophils; Neu: neutrophils.

3.2.2 TSLP can enhance eosinophil survival

Reduced eosinophil apoptosis is considered as a central mechanism for allergic inflammation. TSLP could sustain human CD11c+ DCs and human acute myeloid leukemia-derived cell line MUTZ-3 viability and reduce their apoptosis (Quentmeier *et al.*, 2001; Soumelis *et al.*, 2002). However, TSLP showed no significant survival effect on freshly isolated human CD4+ T cells, though TSLP could increase proliferation of T cell receptor (TCR)-activated CD4+ T cell (Rochman *et al.*, 2007). We therefore investigated whether TSLP could influence eosinophil survival. Results showed that about or less 50% untreated eosinophils remained viable after 48 h incubation, while TSLP significantly reduced the apoptosis of eosinophils and increased the percentage of viable cells in a dose-dependent manner (about 80% of viable eosinophils at 50 ng/ml TSLP, all $p < 0.05$) (Figure 3.2A and 3.2B). However, the effect of TSLP on enhancing eosinophil survival was less potent than that of GM-CSF since the viability mediated by 50 ng/ml TSLP was still less than that mediated by 10 ng/ml GM-CSF treatment (Figure 3.2A and 3.2B). The anti-apoptotic effect of TSLP was further confirmed by DNA ladder fragmentation assay, which was regarded as the characteristic of early apoptosis. In agreement with the Annexin V-PI assay result, spontaneous DNA fragmentation was obvious in untreated eosinophils, while eosinophils with TSLP (50 ng/ml) treatment significantly reduced apoptosis-induced DNA-laddering fragmentation (Figure 3.2C). To further demonstrate that the anti-apoptotic effect was specifically mediated by TSLP, anti-human TSLP neutralization antibody was used. The anti-human TSLP neutralization antibody inhibited the enhancing survival effect mediated by TSLP, while purified isotypic control antibody had no significant effect on eosinophil viability (Figure 3.2D). Therefore, TSLP receptor heterodimer expressed on eosinophils were functional for maintaining eosinophil survival.

(A)

PI

FITC-Annexin V

Ctrl

14.2 %

50.5 %

34.8 %

10 ng/ml GM-CSF

2.7 %

95.4 %

1.0 %

10 ng/ml TSLP

7.5 %

69.2 %

22.7 %

20 ng/ml TSLP

11.2 %

71.9 %

16.8 %

50 ng/ml TSLP

7.3 %

78.9 %

13.6 %

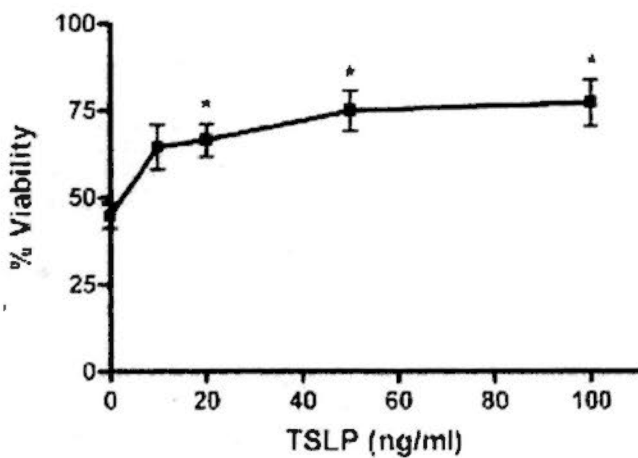
100 ng/ml TSLP

6.3 %

84.6 %

8.8%

(B)



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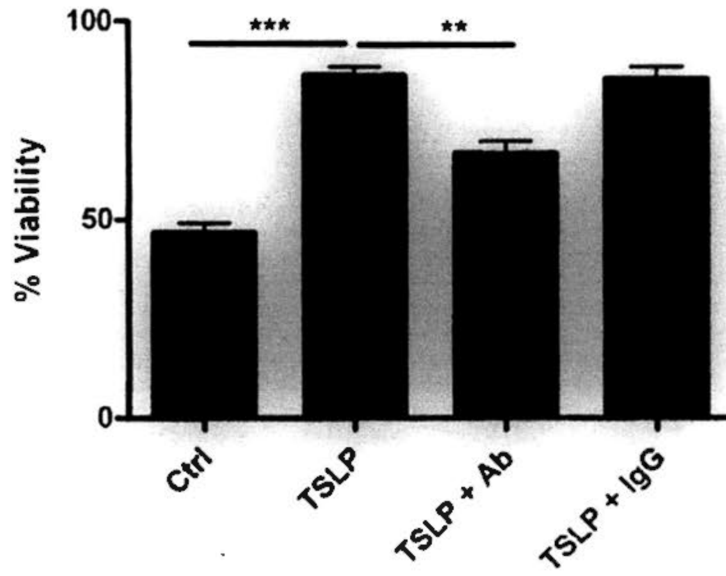


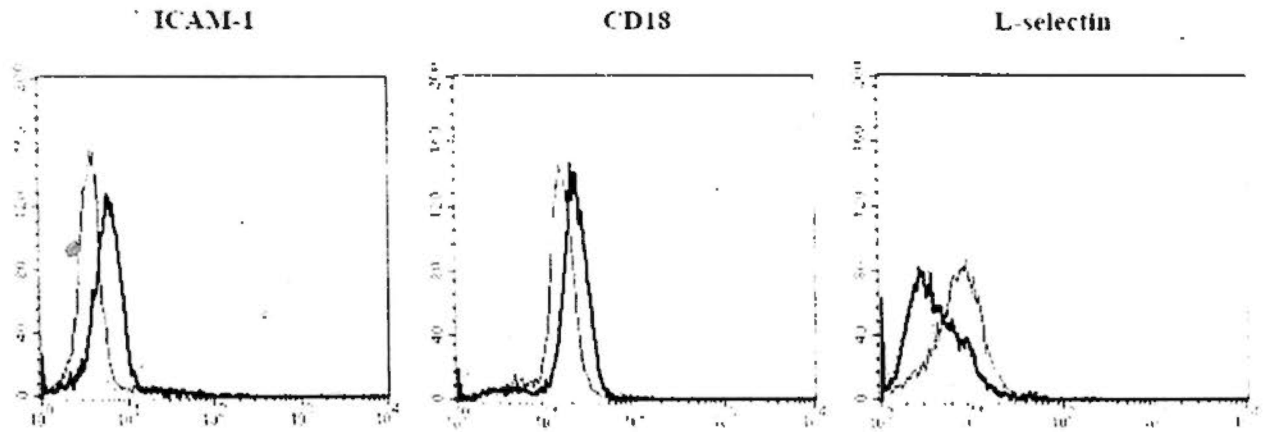
Figure 3.2 Effects of TSLP on the survival of eosinophils.

Eosinophils incubated with TSLP (0 - 100 ng/ml) or GM-CSF (10 ng/ml) for 48 h and then were analysed by flow cytometry using Annexin V-FITC and PI staining or DNA ladder assay. (A) Representative dot plots showed early apoptotic cells in the lower right quadrant (Annexin V-FITC-positive), late apoptotic (necrotic) cells in the upper right quadrant (Annexin V-FITC-positive and PI-positive), and viable cells in the lower left quadrant (double negative). (B) Results of % viability are expressed as the arithmetic mean ± SD from triplicate experiments. * $p < 0.05$ when compared with medium control (Ctrl). (C) DNA ladder assay of eosinophils with or without treatment with TSLP (50 ng/ml) for 24 h. (D) Eosinophils were cultured with or without anti-TSLP neutralization antibody or isotypic control IgG (8 μg/ml) together with TSLP (50 ng/ml) for 48 h. The viability of eosinophils was assessed by Annexin V-FITC and PI staining using flow cytometry from triplicate experiments. Results are expressed as the arithmetic mean + SD. ** $p < 0.01$ and *** $p < 0.001$ when compared between groups denoted by horizontal lines. Ab: anti-TSLP neutralization antibody; IgG: isotypic control IgG

3.2.3 TSLP can modulate the cell surface expression of adhesion molecules on human eosinophils

Tissue eosinophilia is a hallmark for allergic diseases. The trans-endothelial migration of eosinophil to specific tissues involves a variety of adhesion molecules and their counter ligands (Hogan *et al.*, 2008). To investigate the expression profile of cell surface adhesion molecules regulated by TSLP, the effect of TSLP on adhesion molecules on eosinophils was examined. Results showed that TSLP (50 ng/ml) could upregulate the cell surface expression of ICAM-1 and CD18 but reduce the expression of L-selectin (Figure 3.3A). The significant effects of TSLP (0 - 100 ng/ml) modulating eosinophil surface expression of ICAM-1, CD18 and L-selectin was a dose-dependent manner (Figure 3.3B). Changes of eosinophil adhesion molecules modulated by TSLP could be completely reversed by anti-human TSLP neutralization antibody but not by isotypic control antibody (Figure 3.3C). As shown in Figure 3.3D, TSLP could significantly increase the number of eosinophils adhered onto fibronectin-coated wells, with IL-25 treatment serving as the positive control (Wong *et al.*, 2005). Morphological analysis showed that untreated eosinophils maintained the round shape, while TSLP-treated eosinophils processed elongated shape, similar to that of IL-25-treated eosinophils (Figure 3.3E). Therefore, the altered expression profile of adhesion molecules indicated that TSLP may facilitate tissue eosinophilia.

(A)



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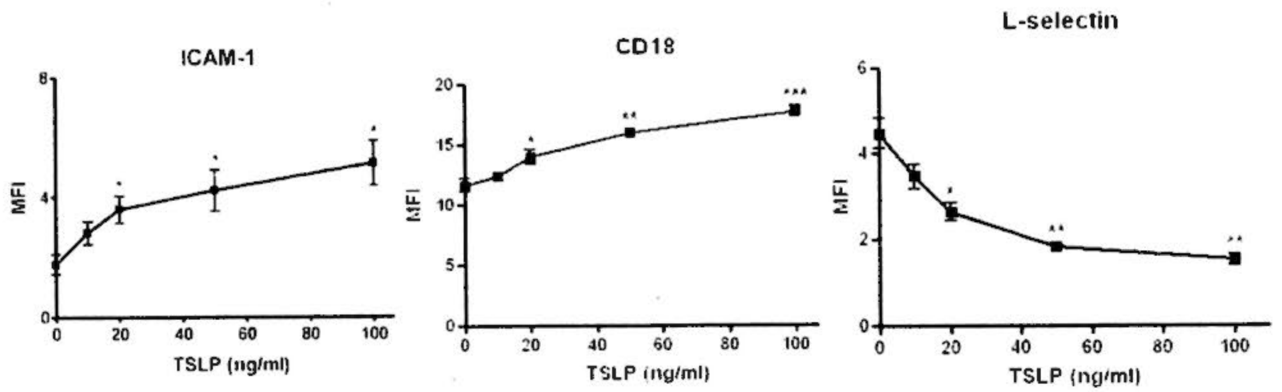

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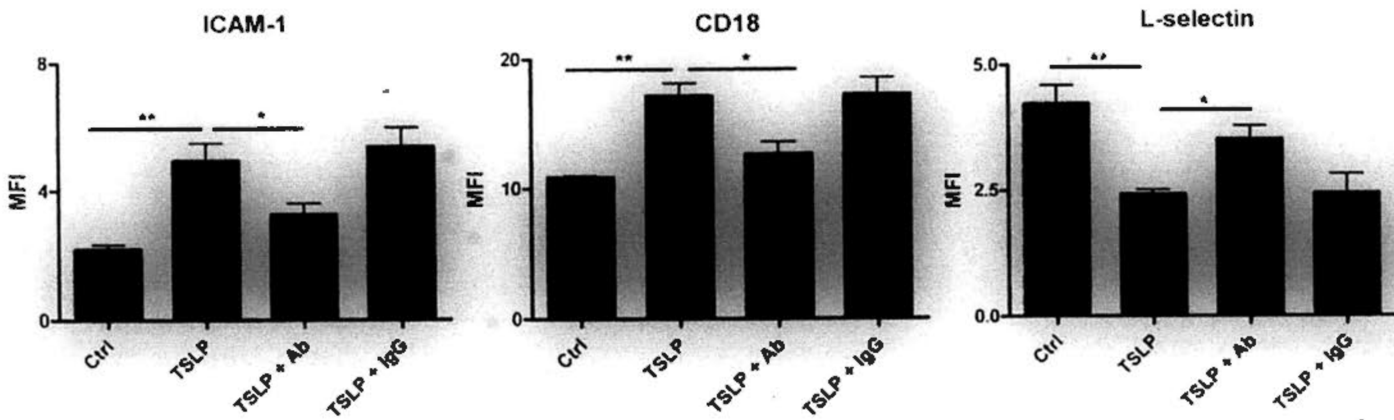

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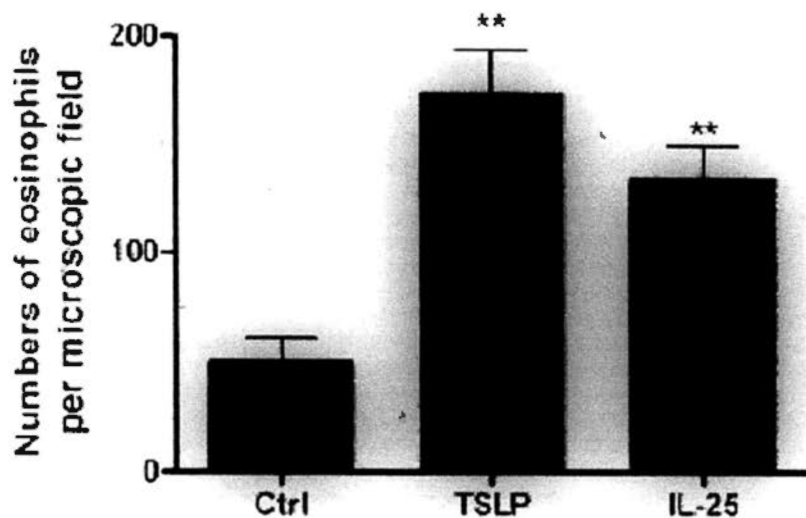


(E)

Ctrl **TSLP** **IL-25**

Figure 3.3 Effects of TSLP on the cell surface expression of adhesion molecules.

(A) Eosinophils (5×10^5/well) were cultured with TSLP (50 ng/ml) for 16 h in a 24-well plate. Surface expression of adhesion molecules of 10,000 cells was analysed by flow cytometry. Representative histograms of cell surface expression of ICAM-1, CD18 and L-selectin on eosinophils are shown. Dotted line: isotypic control; grey line: medium control; black line: TSLP treatment. (B) Surface expression of adhesion molecules are shown as MFI. Results have been normalized by subtracting appropriate isotypic control and are expressed as the arithmetic mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with medium control. (C) Eosinophils were cultured with or without anti-TSLP neutralization antibody or isotypic control IgG (8 µg /ml) together with TSLP (50 ng/ml) for 16 h. Surface expression of adhesion molecules of 10,000 cells was analysed by flow cytometry as MFI and results are expressed as the arithmetic mean + SD of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ when compared between groups denoted by horizontal lines. (D) Eosinophils were stimulated with TSLP (50 ng/ml) and IL-25 (50 ng/ml) for 16 h in fibronectin-coated wells. Results are expressed as the arithmetic mean plus SD of the number of cells adhered onto fibronectin-coated wells in four random 100 × field of quadruplicate experiments. ** $p < 0.01$ when compared with medium control. (E) Eosinophils were incubated with medium, TSLP (50 ng/ml), or IL-25 (50 ng/ml) for 16 hours. Photomicrographs show the morphology of eosinophils with 400 × magnification.

3.2.4 TSLP can potently induce the release of cytokines and chemokines from human eosinophils

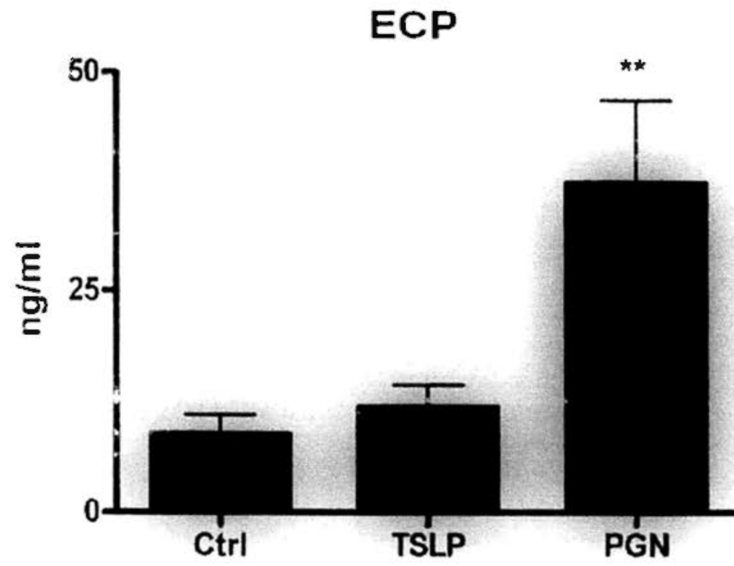
Eosinophils could undergo degranulation to release toxic cationic proteins which cause direct tissue damage involving in the pathophysiology of allergic diseases (Hogan *et al.*, 2008). However, we could not detect any release of ECP from eosinophils upon TSLP stimulation (Figure 3.4A), in which PGN was used as the positive control (Wong *et al.*, 2007). Actually, TSLP also could not induce the release of pre-formed, granule-associated lipid mediators such as β-hexosaminidase, histamine, leukotriene C4, and prostaglandin-D2 from mast cells (Allakhverdi *et al.*, 2007).

Besides degranulation, eosinophils can synthesize and release a number of cytokines and chemokines (Hogan *et al.*, 2008). We first illustrated the cytokine expression profiles of human eosinophils using antibody-based human cytokine protein array as a means of preliminary screening. TSLP could activate eosinophils to prominently induce the release of chemokines CCL2 (3e), CXCL1 (1j) and CXCL8 (2j), and proinflammatory cytokine IL-6 (2h) among the 79 different cytokines being screened after 24 h incubation when compared with that of medium control (Figure 3.4B). The map of the cytokine antibody array on the membrane was listed in Appendix. Results using ELISA confirmed that TSLP directly activated eosinophils to significantly release inflammatory cytokine IL-6, and chemokines CXCL8, CXCL1 and CCL2 dose-dependently (0 - 100 ng/ml) at 12 and 24 h (Figure 3.4C). The most potent induction was found to be CXCL8 with 10,000 fold increase, followed by CCL2, CXCL1 and IL-6. The releases at 12 h of incubation were obviously lower than that at 24 h. All these cytokine and chemokine productions could be suppressed by anti-human TSLP neutralization antibody but not by isotypic control antibody (Figure 3.4D). This indicated that TSLP could specifically induce the release of cytokines and chemokines from human eosinophils. Moreover, transcriptional inhibitor actinomycin D and protein synthesis inhibitor cycloheximide could significantly suppress the production of cytokine and

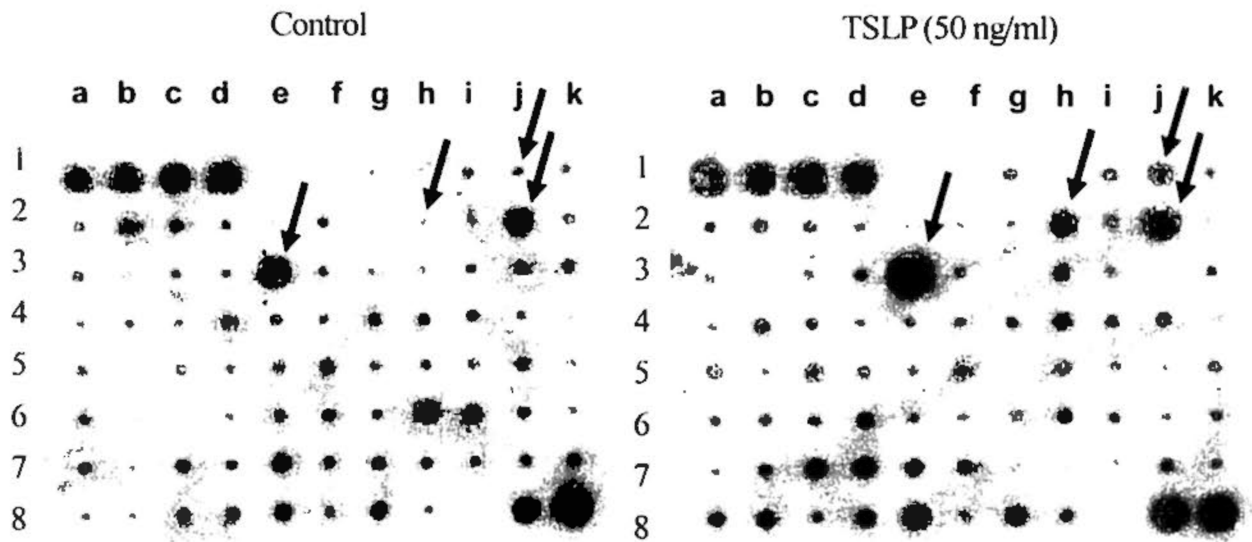
chemokine release induced by TSLP (Figure 3.4E).

Eosinophils are capable of releasing IL-5, GM-CSF, IL-13, IL-1β, IL-4, TNF-α, and CXCL5 upon stimulation (Hogan *et al.*, 2008). However, we found that these cytokines in TSLP-treated eosinophils were either undetectable or the same as the medium control determined by ELISA or cytometric bead array (data not shown).

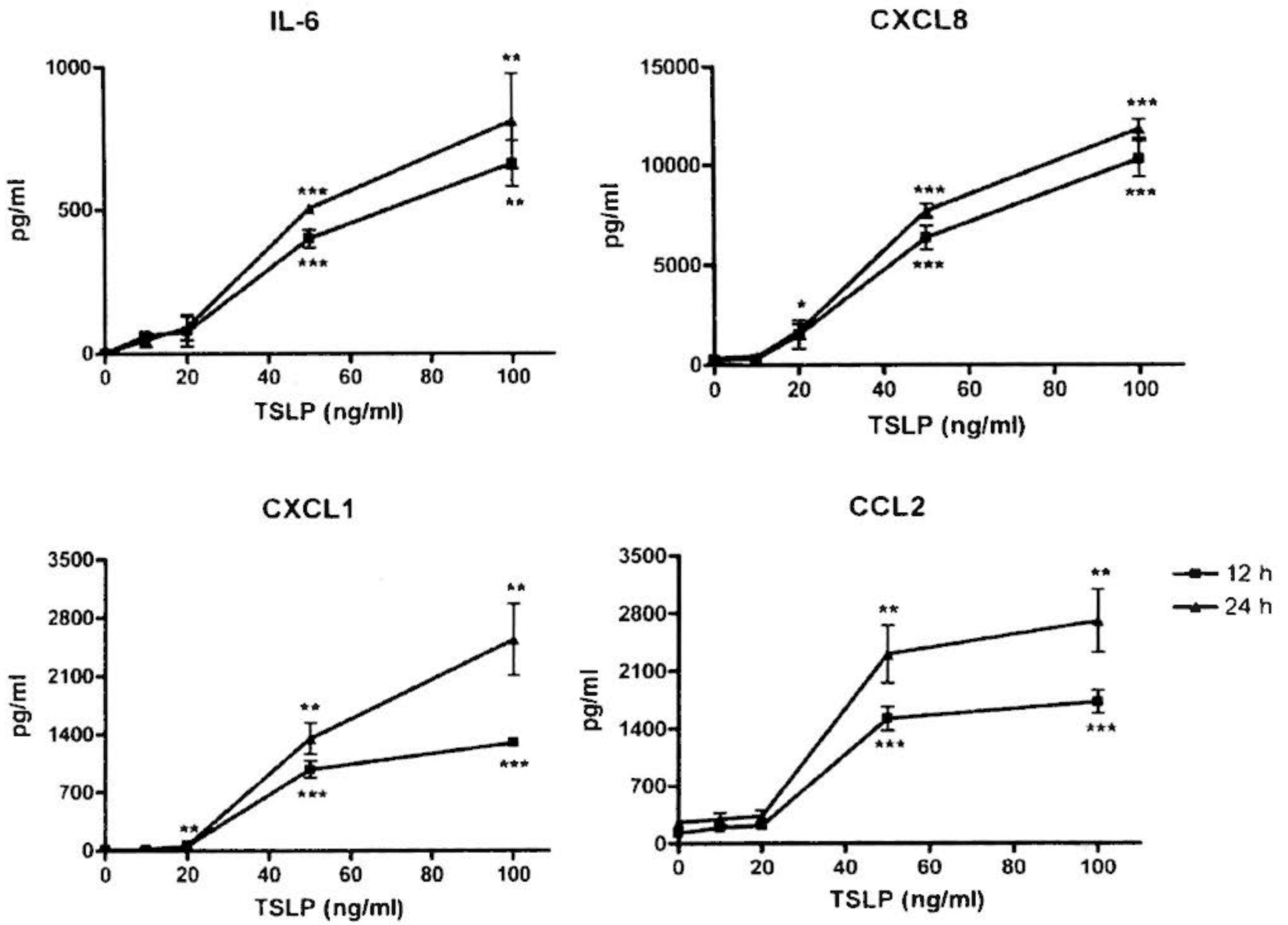
(A)



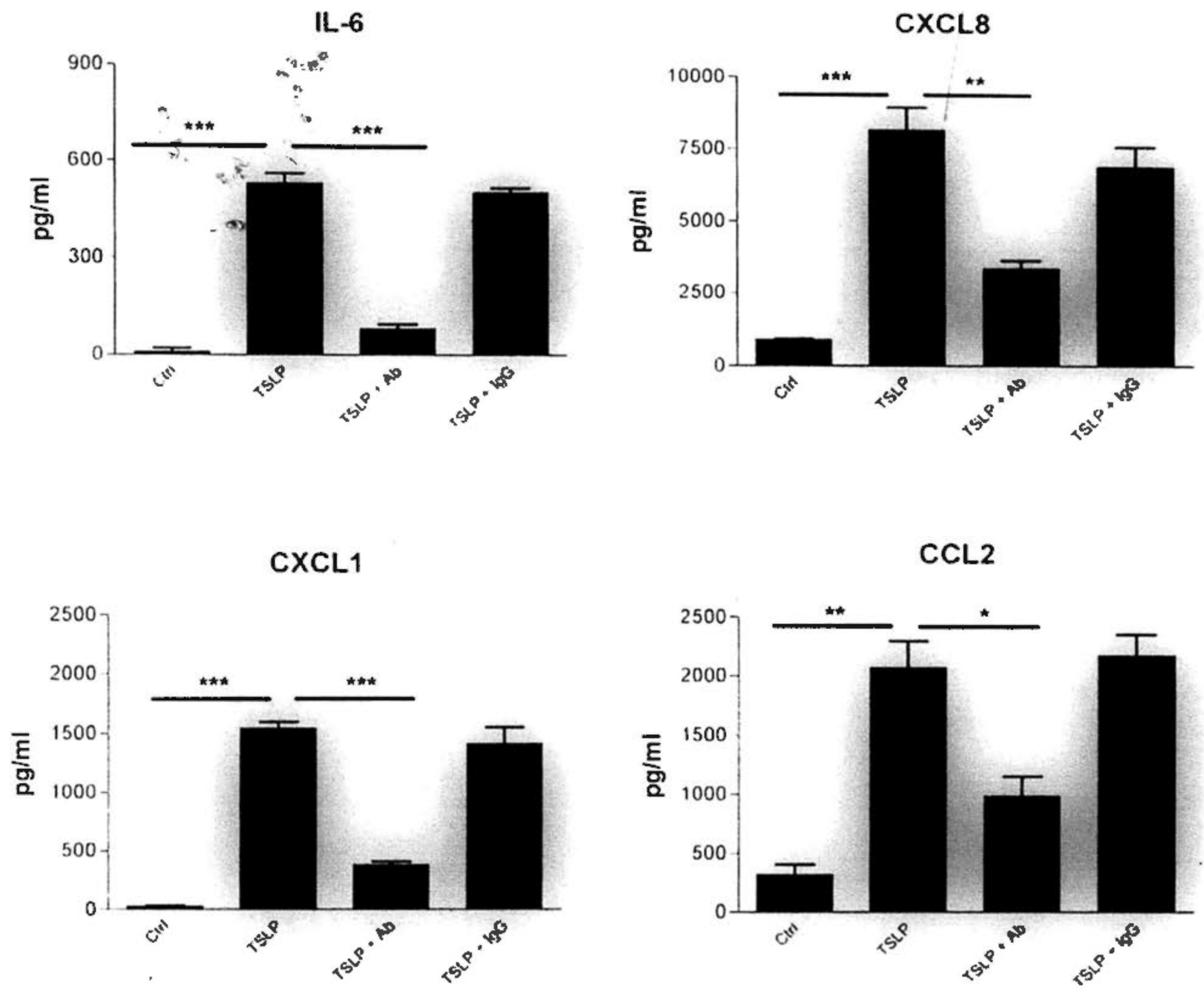
(B)



(C)



(D)



(E)

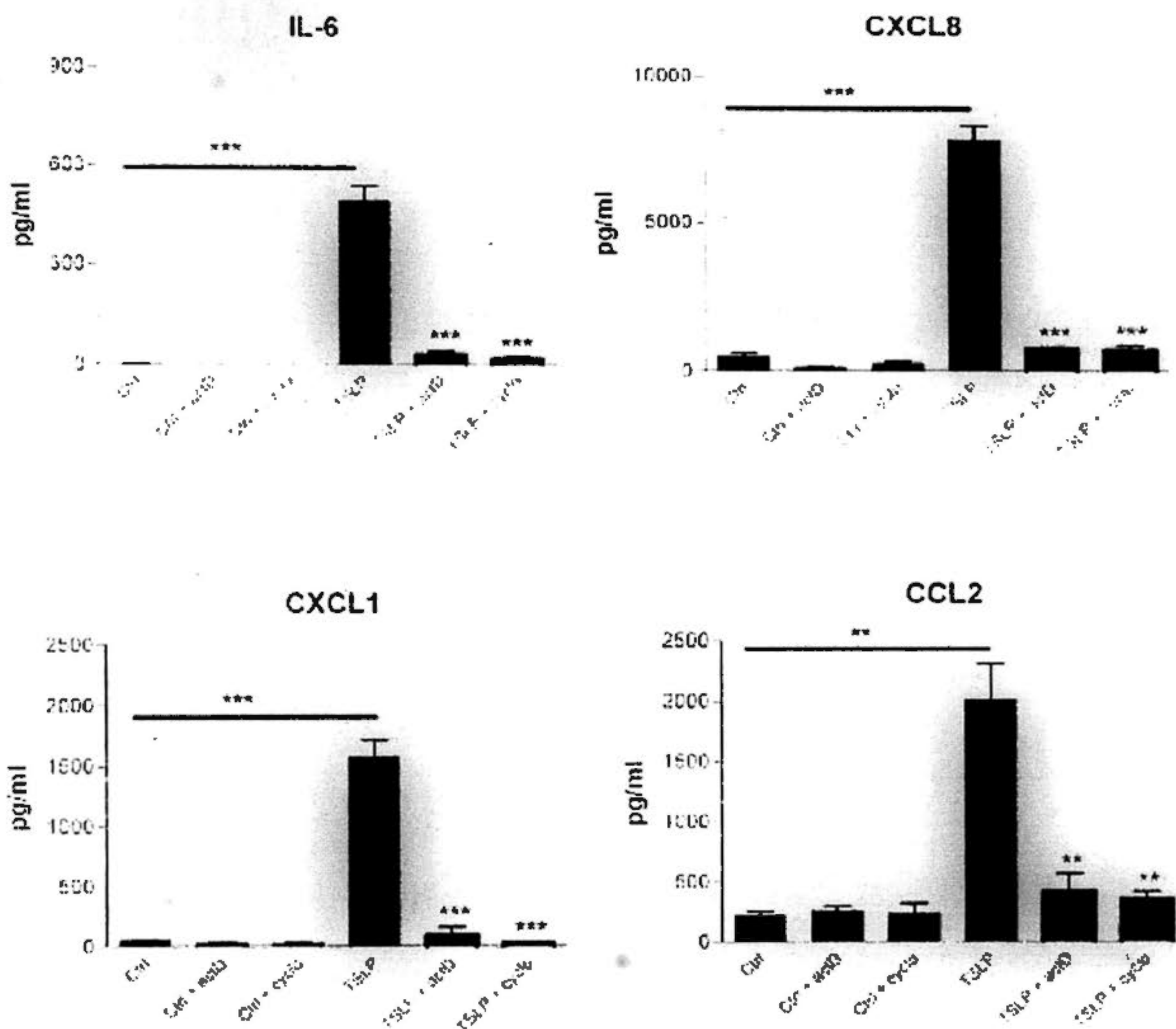


Figure 3.4 Effects of TSLP on ECP, cytokine and chemokine release from eosinophils.

(A) Eosinophils (1×10^6 cells) were cultured with TSLP (50 ng/ml) or PGN (1 μg/ml) for 4 h and the release of ECP was measured by fluorescence enzyme immunoassay. (B) Representative profile of the release of cytokines and chemokines from eosinophils activated by TSLP. Eosinophils (1×10^6 cells) were cultured with (right) or without (left) TSLP (50 ng/ml) for 24 h. Cell free culture supernatant was then collected and 79 different cytokines in culture supernatant were semi-quantitated using antibody based RayBio® human cytokine array V. Positive and negative controls were designated at (1a, 1b, 1c, 1d, 8j, 8k) and (1e, 1f, 8i), respectively. Arrows indicated the spots with obvious differences between the two array membranes. The map of the cytokine antibody array was listed in Appendix in Page 175. (C) Eosinophils (5×10^5/well) were cultured with TSLP (0 - 100 ng/ml) for 12 or 24 h in a 24-well plate. Expression of IL-6, CXCL8, CXCL1 and CCL2 were determined by ELISA. Results are expressed as the arithmetic mean ± SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with medium control. (D) Eosinophils were cultured with or without anti-TSLP neutralization antibody or isotypic control IgG (8 μg/ml) together with TSLP (50 ng/ml) for 24 h. Expression of cytokine and chemokines were determined by ELISA. Results are expressed as the arithmetic mean + SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared between groups denoted by horizontal lines. (E) Eosinophils (5×10^5 /well) were cultured with or without TSLP (50 ng/ml) in the presence or absence of actinomycin D (1 μM) or cycloheximide (10 μM) for 24 h in a 24-well plate. IL-6, CXCL8, CXCL1 and CCL2 released into the culture supernatant were determined by ELISA. Results are expressed as the arithmetic mean + SD from three independent experiments. ** $p < 0.01$ and *** $p < 0.001$ when compared between groups denoted by horizontal lines or groups of inhibitor treatment and TSLP alone. actD: actinomycin D; cyclo: cycloheximide

3.2.5 TSLP could not activate STAT5 and STAT3 signaling pathways

Previous findings showed that STAT5 was activated in TSLP treated pre-B cell line NAG8/7, acute myeloid leukemia (AML) cell line MUTZ-3, and pre-activated human CD4+ and CD8+ T cells (Quentmeier *et al.*, 2001; Reche *et al.*, 2001; Rochman *et al.*, 2007; Rochman and Leonard, 2008). In addition, tyrosine phosphorylation of STAT5 and STAT3 occurred upon TSLP stimulation in pro-B cell line Ba/F3 transfected with TSLP receptor heterodimer (Reche et al., 2001). In our study, TSLP (50 ng/ml) could not obviously induce tyrosine phosphorylation of STAT5 and STAT3 in eosinophils, while the positive control GM-CSF (10 ng/ml) significantly induced tyrosine phosphorylation of STAT5 (Tyr 694) and STAT3 (Tyr 705) within 15 min (Figure 3.5). Another potential phosphorylation site Ser (727) was reported in STAT3 activation (She *et al.*, 2004), however, phosphorylation of STAT3 Ser (727) could not be detected upon both of TSLP and GM-CSF stimulation (Figure 3.5). GM-CSF induced phosphorylation of Ser (727) of STAT3 in eosinophils has actually not been reported before. These results therefore suggested that other signal pathways may involve in TSLP-mediated eosinophil activation.

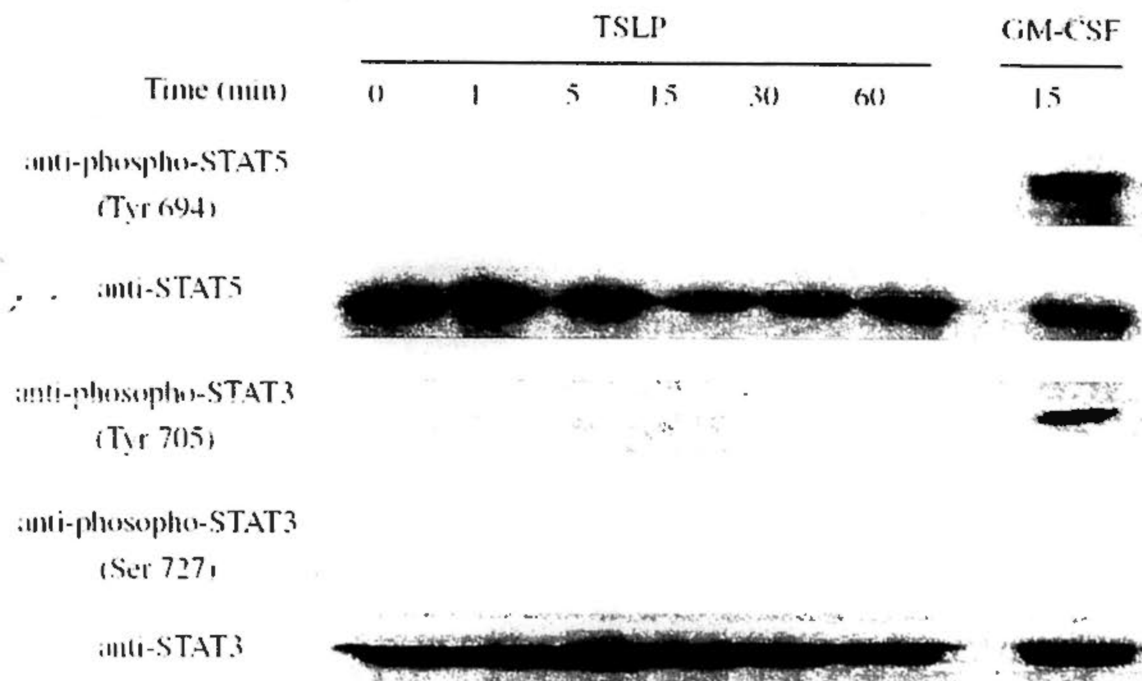


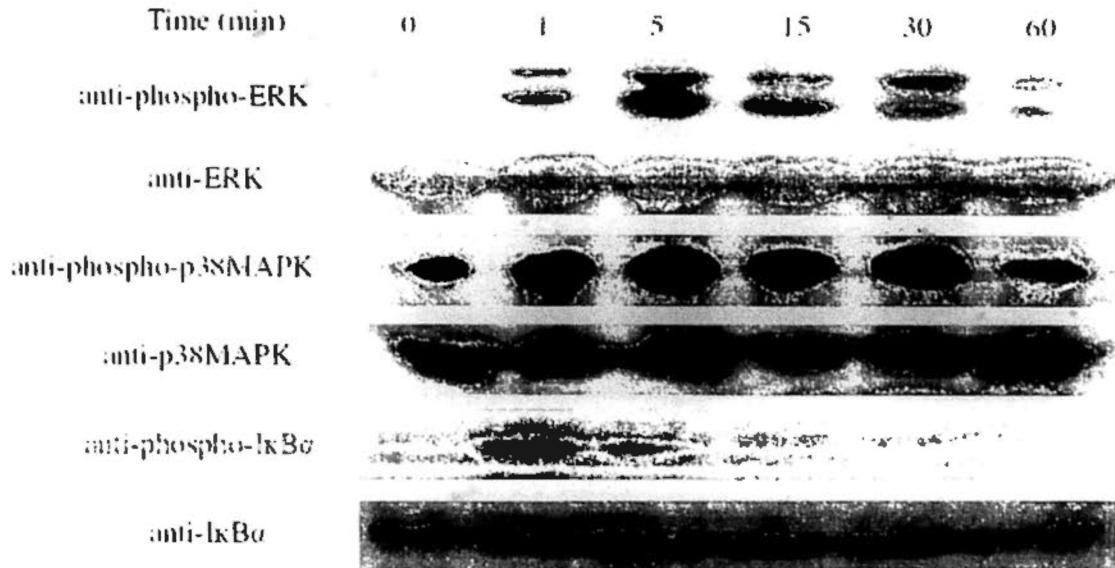
Figure 3.5 Effects of TSLP on STAT3 and STAT5 activities in eosinophils.

Eosinophils (1 × 10^6 cells) were cultured with or without TSLP (50 ng/ml) or GM-CSF (10 ng/ml) for different indicated incubation times. Total cellular proteins were extracted from eosinophils for the detection of phospho-STAT5 (Tyr 694), total STA5, phospho-STAT3 (Tyr 705), phospho-STAT3 (Ser 727) and total STAT3 using indicated antibodies by Western blot analysis. Experiments were performed in three independent replicates with essentially identical results, and representative results are shown.

3.2.6 TSLP activates MAPK and NF-κB signaling pathways

Though TSLP-mediated signaling pathways were rarely reported except STAT5 and STAT3 activation, signal transduction via MAPK and NF-κB play important roles in cellular responses, including cell proliferation, differentiation, and survival (Cheung *et al.*, 2008; O'Neill, 2006; Wong *et al.*, 2007). Western blot analysis showed that phosphorylation of ERK and p38MAPK were detected upon TSLP stimulation, and NF-κB was also activated as demonstrated by IκBα phosphorylation (Figure 3.6A). Results from intracellular staining with flow cytometry further confirmed that ERK, p38MAPK and NF-κB pathways were activated in eosinophils when stimulated by TSLP (Figure 3.6B). For activation kinetics, these three signaling molecules were all rapidly activated upon TSLP stimulation and peaked within 1 or 5 min, and then returned to baseline within 30 or 60 min (Figures 3.6A and 3.6B). Although there was slight difference for the activation kinetic results between Western blot and flow cytometry methods, the signaling responses of eosinophils induced by TSLP were rapid. However, we could not detect JNK, AKT and JAK activity in eosinophils upon TSLP stimulation (data not shown).

(A)



(B)

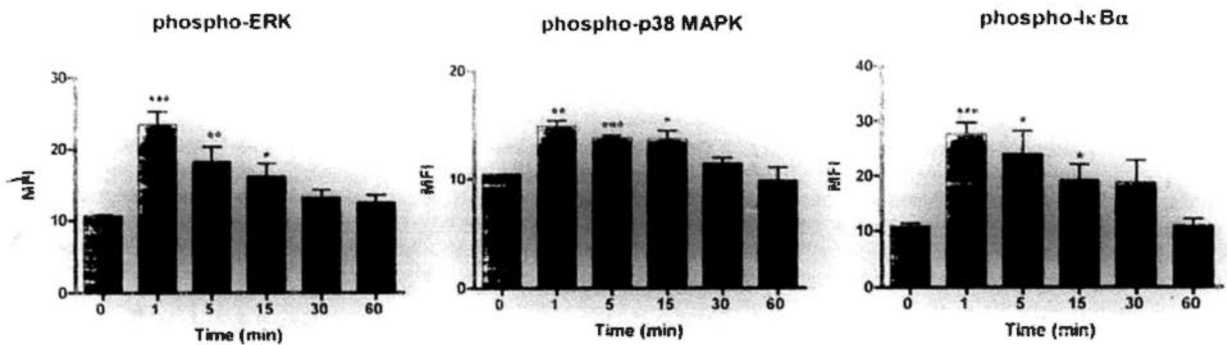
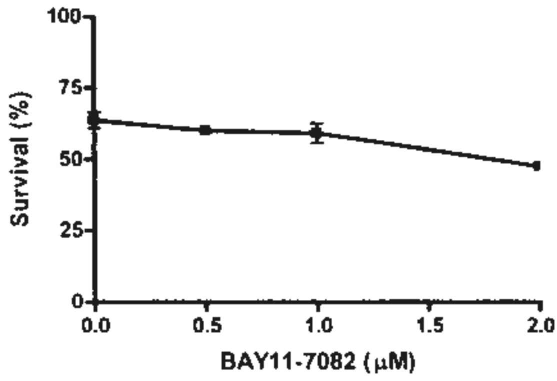


Figure 3.6 Effects of TSLP on intracellular ERK, p38 MAPK and NF-κB activation.

Eosinophils were incubated with or without TSLP (50 ng/ml) for different incubation times. (A) Total cellular proteins were extracted from eosinophils for the detection of phospho-ERK, phospho-p38MAPK and phospho-IκBα using indicated antibodies by Western blot analysis. (B) The amounts of intracellular phosphorylated signaling molecules in 10,000 permeabilized eosinophils were measured by flow cytometry. Results of phospho-ERK, phospho-p38 MAPK and phospho-IκBα are shown in MFI and are expressed as the arithmetic mean + SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with medium control.

3.2.7 Effects of different inhibitors on the viability of eosinophils

We first determined the concentration of different inhibitors for inhibitory assay. The optimal concentration of inhibitors was adopted with the highest inhibitory effects without any cytotoxicity effects. Eosinophils were treated with different dosage of inhibitors for 24 h, and then analysed by flow cytometry using Annexin V-FITC and PI staining. The viability of eosinophils to sustain in different dosage of inhibitors was shown (Figure 3.7). BAY11-7082, SB203580 and LY294002 could suppress the eosinophil viability in a dose dependent manner, while U0126 seemed to have activation effects for eosinophils when concentration higher than 5 μM. For AG490 and SP600125, they did not exert significant cytotoxicity to eosinophils. Therefore, according to the above results and our previous studies (Wong *et al.*, 2005), we adopted the following concentration for eosinophils: BAY11-7082 (1 μM), U0126 (2.5 μM), SB203580 (2.5 μM) and SP600125 (2 μM), AG490 (2 μM), and LY294002 (2 μM). DMSO (0.1%) was used as the vehicle control.


Survival (%)
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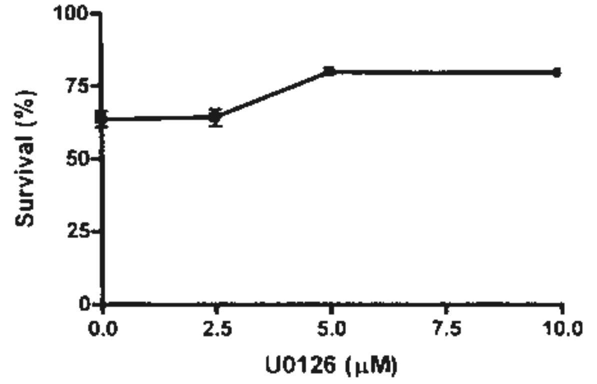
75
50
25
0
0.0
0.5
1.0
1.5
2.0
BAY11-7082 (μM)


Survival (%)
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75
50
25
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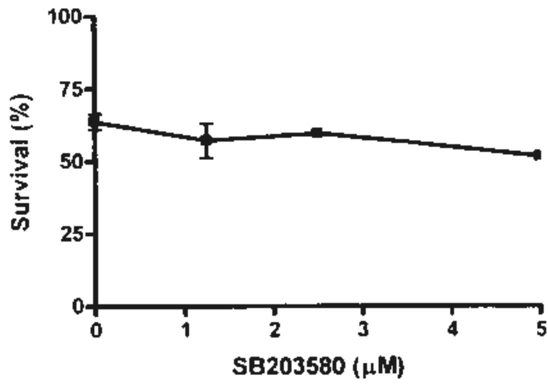
0.0
2.5
5.0
7.5
10.0
U0126 (μM)


Survival (%)
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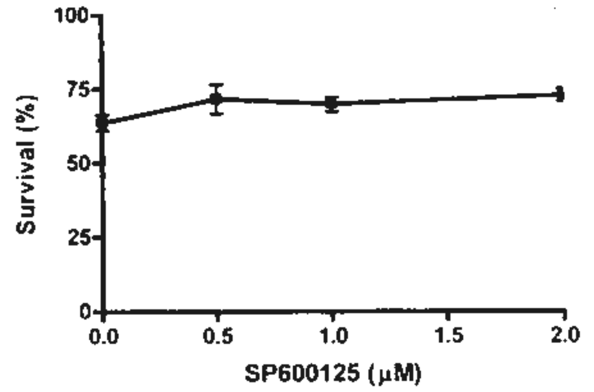
75
50
25
0
0
1
2
3
4
5
SB203580 (μM)


Survival (%)
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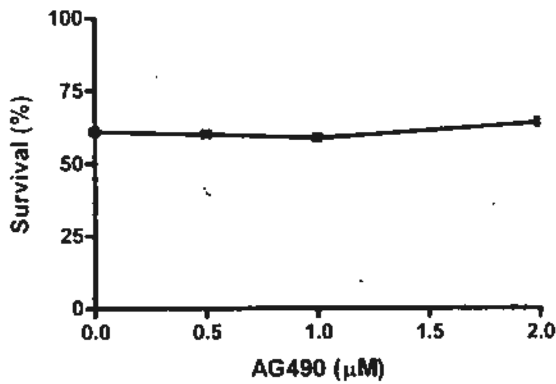
1.0
1.5
2.0
SP600125 (μM)


Survival (%)
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1.0
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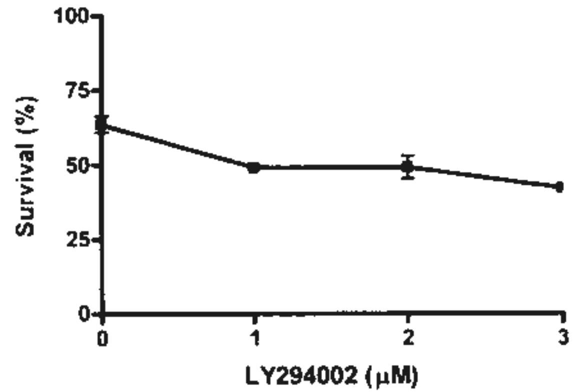
2.0
AG490 (μM)


Survival (%)
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25
0
0
1
2
3
LY294002 (μM)

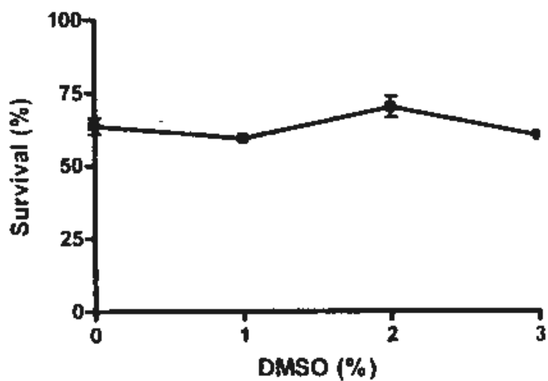

Survival (%)
100
75
50
25
0
0
1
2
3
DMSO (%)

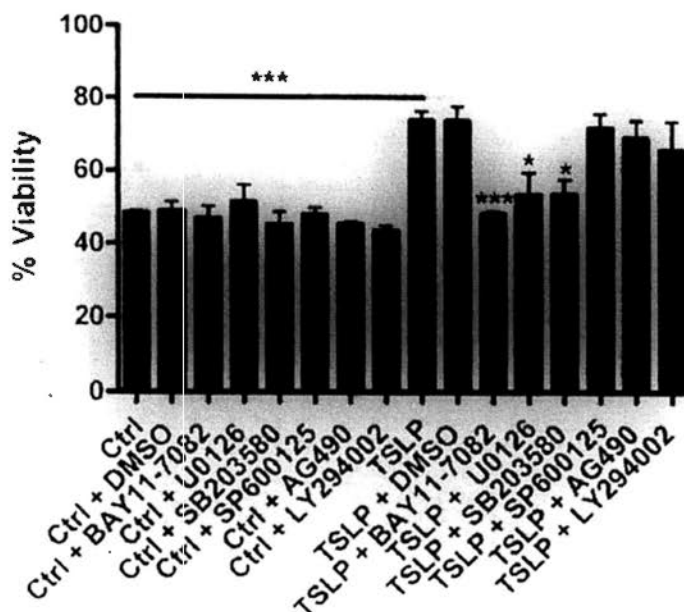
Figure 3.7 Effects of different inhibitors on the viability of eosinophils.

Eosinophils (5×10^5 cells) were treated with inhibitor BAY11-7082, U0126, SB203580, SP600125, AG490, LY294002, or vehicle control DMSO at various concentrations for 24 h. The viability of eosinophils was determined by Annexin V-PI staining assay using flow cytometry. Results are expressed as the arithmetic mean ± SD from three independent experiments.

3.2.8 TSLP-mediated the activation of eosinophils involves MAPK and NF-κB pathways

To elucidate the potential mechanisms by which TSLP promote eosinophil survival, adhesion molecule changes, and cytokine and chemokine release, we evaluated the relative roles of ERK, JNK, p38MAPK, AKT and NF-κB signaling pathways in TSLP-stimulated eosinophils using inhibition assay. The optimal concentration of inhibitors was adopted as the same as the above experiments. As shown in Figure 3.8A, blocking the p38MAPK pathway with inhibitor SB203580, ERK pathway with U0126, and NF-κB pathway with BAY11-7082 significantly reduced TSLP-induced eosinophil survival to the basal level (all $p < 0.05$). For the changes of adhesion molecules, TSLP-induced CD18 and ICAM-1 expressions could be significantly abrogated by all the three inhibitor BAY11-7082, U0126 and SB203580, while TSLP-induced L-selectin down-regulation could only be reversed by inhibitors U0126 and SB203580 (Figure 3.8B, all $p < 0.05$). For cytokine and chemokine release, TSLP-mediated induction of IL-6, CXCL1 and CCL2 could also be significantly suppressed by BAY11-7082, U0126 and SB203580, whereas blocking the NF-κB signaling pathway with BAY11-7082 had no effect on CXCL8 production and only U0126 and SB203580 showed significant inhibition on TSLP-induced CXCL8 (Figure 3.8C, all $p < 0.05$). In addition, JAK inhibitor AG490, PI3K/AKT inhibitor LY294002 or JNK inhibitor SP600125 did not exert any significant effect on eosinophil survival, cytokine and chemokine production induced by TSLP (Figure 3.8). In summary, our results indicated that the three signaling pathways ERK, p38MAPK and NF-κB activation were involved in TSLP-mediated effects on eosinophils.

(A)



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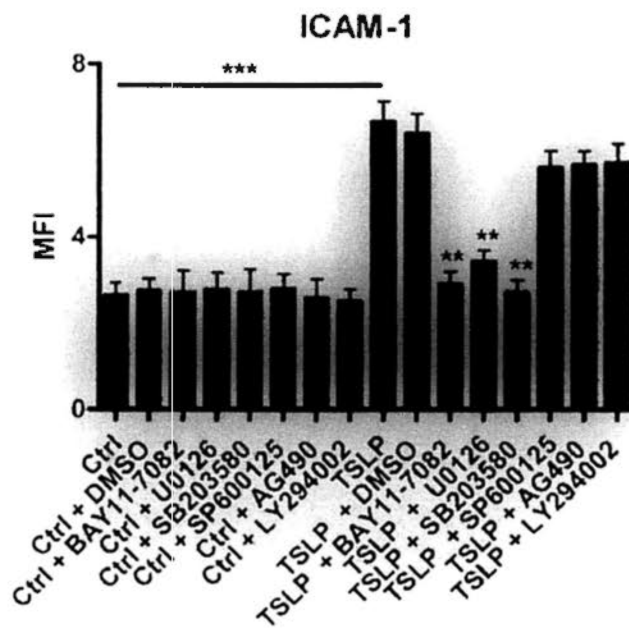
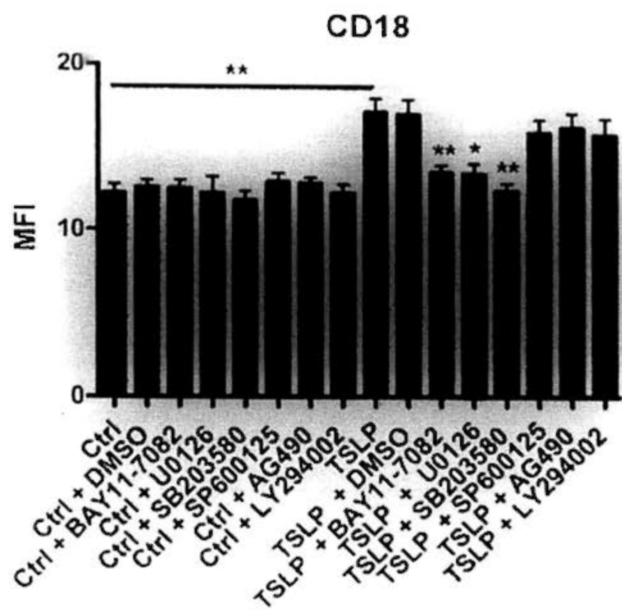
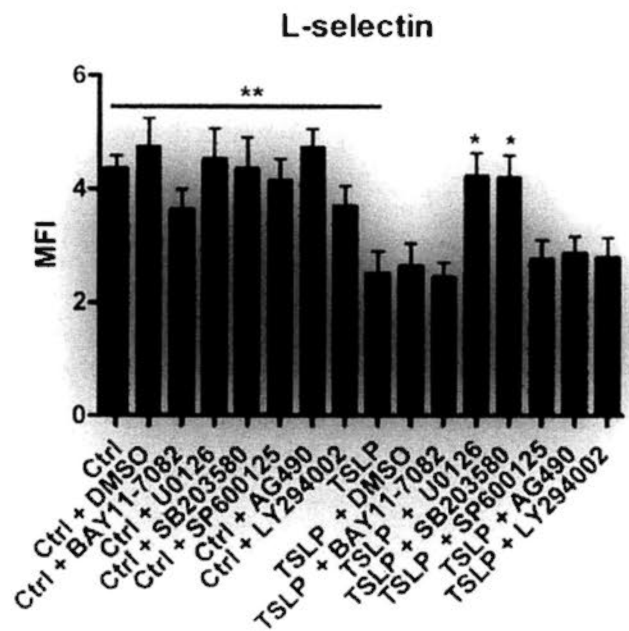



CD18
MFI
20
10
0
**
** *
**
Ctrl
Ctrl + DMSO
Ctrl + BAY11-7082
Ctrl + U0126
Ctrl + SB203580
Ctrl + SP600125
Ctrl + AG490
Ctrl + LY294002
TSLP
TSLP + DMSO
TSLP + BAY11-7082
TSLP + U0126
TSLP + SB203580
TSLP + SP600125
TSLP + AG490
TSLP + LY294002


L-selectin
MFI
6
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* *
Ctrl
Ctrl + DMSO
Ctrl + BAY11-7082
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Ctrl + SP600125
Ctrl + AG490
Ctrl + LY294002
TSLP
TSLP + DMSO
TSLP + BAY11-7082
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TSLP + SB203580
TSLP + SP600125
TSLP + AG490
TSLP + LY294002

(C)

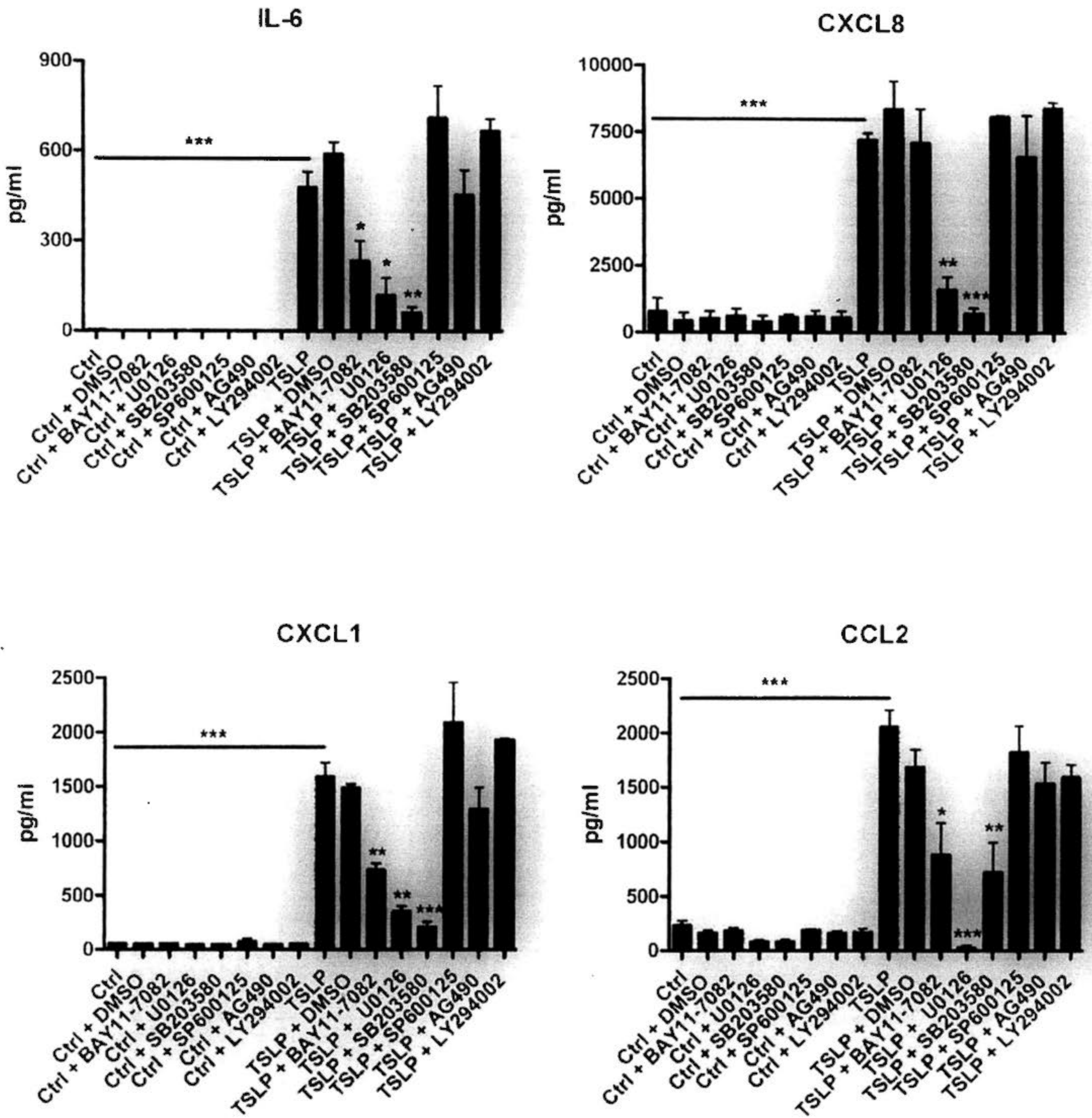


Figure 3.8 Effects of signaling inhibitors on viability, adhesion molecule expression, cytokine and chemokine release of eosinophils.

Eosinophils were pre-treated with BAY11-7082 (1 μM), U0126 (2.5 μM), SB203580 (2.5 μM), SP600125 (2 μM) AG490 (2 μM), and LY294002 (2 μM) for 1 h, followed by incubation with or without TSLP (50 ng/ml) in the presence of inhibitors for further (A) 48 h for viability assay, (B) 16 h for adhesion molecule expression and (C) 24 h for cytokine and chemokine release. Results are expressed as the arithmetic mean + SD from three independent experiments. DMSO (0.1%) was used as the vehicle control. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared between groups denoted by horizontal lines or groups of inhibitor treatment and TSLP alone.

3.2.9 No interaction of TSLP-mediated MAPK and NF-κB signaling pathways

To further explore the relationship among NF-κB, ERK and p38MAPK activation upon TSLP stimulation, selective specific inhibitors were used. BAY11-7082, SB203580 and U0126 specifically inhibit the phosphorylation of IκBα, p38MAPK and MEK1/2 (upstream kinase of ERK), respectively. BAY11-7082 profoundly inhibited TSLP-mediated IκBα phosphorylation, but had no effects on the phosphorylation of ERK or p38MAPK. U0126 specifically inhibited phosphorylation of ERK but had no effects on phosphorylation of p38MAPK or IκBα. Similarly, SB203580 potently inhibited p38MAPK only, but not on phosphorylation of ERK or IκBα (Figure 3.9). These signaling pathways were therefore independent without cross-talk in TSLP-mediated eosinophil activation.

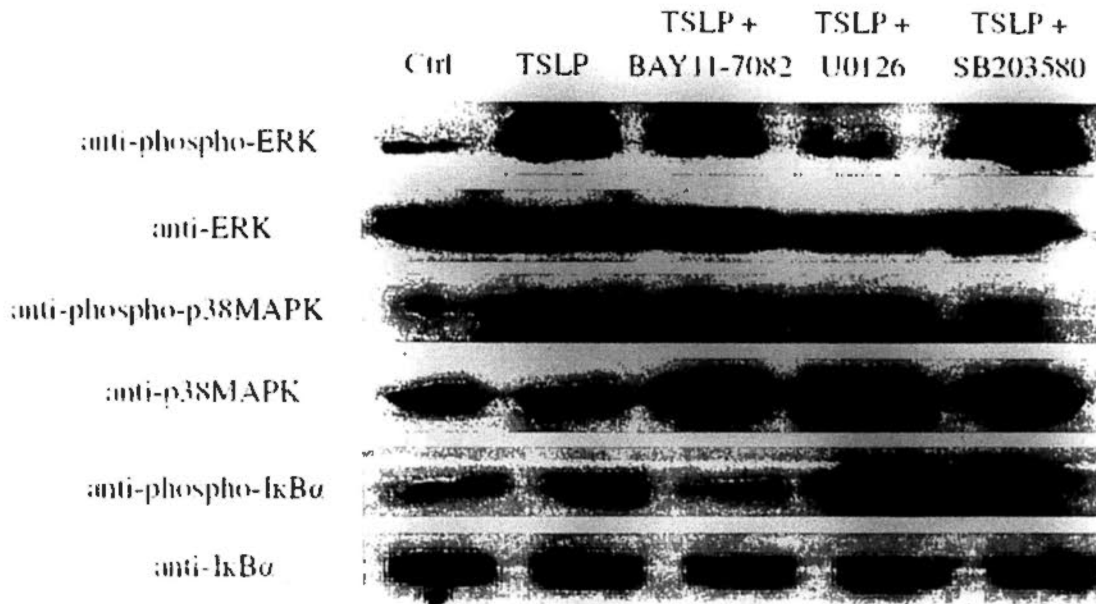


Figure 3.9 Interaction of TSLP-mediated MAPK and NF-κB signaling pathways.

Eosinophils were pre-treated with inhibitor BAY11-7082 (1 μM), U0126 (2.5 μM) and SB203580 (2.5 μM) for 1 h, followed by stimulation with TSLP (50 ng/ml) for further 5 min. Phospho-ERK, phospho-p38MAPK and phospho-IκBα were detected by Western blots. Experiments were performed in three independent replicates with essentially identical results, and representative results are shown.

3.2.10 TSLP could be induced by TLR3 ligand double-stranded RNA in epidermal keratinocytes and bronchial epithelial cells

TSLP has been shown to be involved in the initiation and development of the pathogenesis of allergic diseases (Liu *et al.*, 2007). High expression of TSLP has been found in the skin of patients with acute and chronic atopic dermatitis but not detectable in normal subjects, and the BAL concentration of TSLP was much higher in patients with asthma and chronic obstructive pulmonary disease compared to healthy controls (Soumelis *et al.*, 2002; Ying *et al.*, 2005; Ying *et al.*, 2008). However, the regulation of TSLP expression in keratinocytes and bronchial epithelial cells has not been extensively studied.

Since allergic diseases are associated with severe Th2 immune responses, a variety of Th2-related classical and novel cytokines, including IL-4, IL-5, GM-CSF, IL-31, and IL-33, were investigated to induce the production of TSLP. Because epidermal keratinocytes and bronchial epithelial cells act as the first physical barrier contacting with environment, TLR ligands associated with allergic diseases, including synthetic bacterial lipoprotein PGN (TLR2 ligand), poly I:C (TLR3 ligand), and LPS (TLR4 ligand) were tested for TSLP induction. Quantitative measurement of the culture supernatants showed that keratinocytes and bronchial epithelial cells treated with poly I:C, mimicking viral dsRNA, could release detectable amounts of TSLP (about 20 - 50 ng/ml) (Figure 3.10), which was consistent with other group studies (Kato *et al.*, 2007, Kinoshita *et al.*, 2009). Other TLR ligands PGN and LPS, as well as Th2-related cytokines IL-4, IL-5, IL-31 and IL-33 could not induce the release of TSLP, at least not detectable. However, the above results did not exclude the possibility that TSLP induction was not associated with the tested Th2-related cytokines and allergy-related TLR ligands. It is also possible that besides poly I:C, different combination or synergistic effects of these stimuli are able to induce TSLP release and further aggravate allergic immune responses.

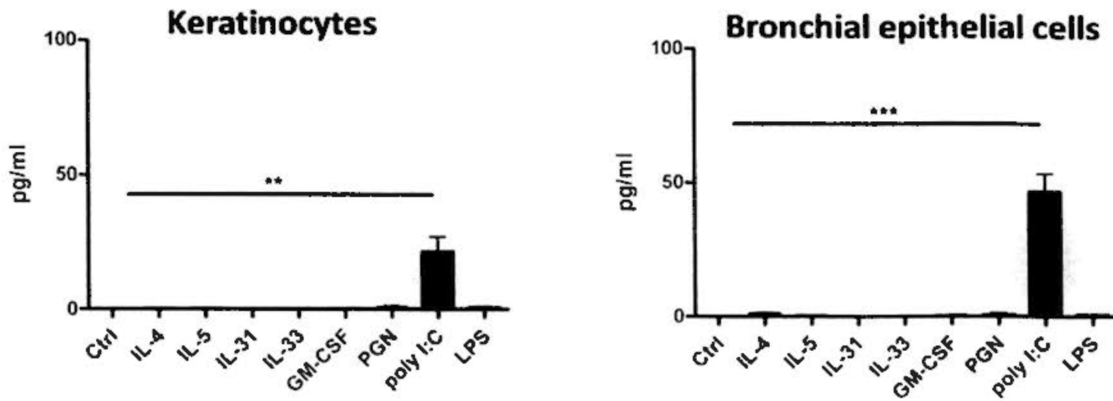


Figure 3.10 Induction of TSLP from primary epidermal keratinocytes and bronchial epithelial cells.

Keratinocytes and bronchial epithelial cells were cultured with medium only, IL-4 (100 ng/ml), IL-5 (100 ng/ml), IL-31 (100 ng/ml), IL-33 (100 ng/ml), GM-CSF (100 ng/ml), PGN (10 μg/ml), poly I:C (25 μg/ml), or LPS (1 μg/ml) for 24 h. Culture supernatant was collected and then subjected to TSLP ELISA assay. ** $p < 0.01$ and *** $p < 0.001$ when compared with medium control treatment.

3.3 Discussion

TSLP has been shown to be necessary and sufficient for the initiation and development of allergic inflammation through the activation of DCs and mast cells, as well as inducing inflammatory Th2 responses (Comeau and Ziegler, 2010). To further characterize the immunological mechanisms of TSLP for sustaining allergic inflammation, we investigated the direct activating effects of TSLP on eosinophils, the principal effector cells in late phase allergic response.

Functional human TSLP receptor heterodimers were demonstrated to express on DCs, mast cells and CD34+ hematopoietic progenitor cells, and in consistent with TSLP receptor heterodimer expression, TSLP has been shown to directly activate these cells (He and Geha, 2010). In this study, we found that eosinophils constitutively express mRNA and protein of functional TSLP receptor heterodimer, TSLPR and IL-7Rα, and the intracellular expression levels of TSLPR were much higher than that of the surface expression. Neutrophils showed negative expression which was consistent with previous study that neutrophils do not express IL-7Rα, though constitutively express common γ chain. Hematopoietic cytokine IL-5 and GM-CSF did not exhibit any significant effects on TSLPR and IL-7Rα expression. Accordingly, cell surface TSLP receptor heterodimer seems to be constitutively expressed rather than regulated via hematopoietic cytokine induction. However, when our paper are preparing, another group reported that the expression of TSLPR subunit on eosinophils was significantly enhanced by IL-3 and/or TNF-α (Hiraguchi *et al.*, 2009). Whether other cytokines and allergic microenvironment could modulate TSLP receptor complex expression on eosinophils requires further investigation.

Reduced eosinophil apoptosis is considered as a central mechanism for allergic inflammation and eosinophilia. TSLP could dose-dependently enhance eosinophil survival by reducing eosinophil apoptosis. This effect is similar to many other allergy-related cytokines, including GM-CSF, IL-5, IL-25 and IL-33, which is highly expressed in allergic

inflammation and maintain eosinophil long-term survival (Chow *et al.*, 2010; Hogan *et al.*, 2008; Wong *et al.*, 2005). Our findings indicate that TSLP receptor heterodimer on eosinophil is functional and TSLP should have other effects or facilitate other mechanisms to activate eosinophil in line with enhancing survival.

Upon stimulation, eosinophils can undergo degranulation to release toxic cationic proteins including ECP, MBP, EDN and EPO at the site of inflammation. This process can cause direct tissue damage involving in the pathophysiology of asthma, atopic dermatitis, and other chronic allergic diseases. However, we could not detect the release of ECP from eosinophils upon different doses and time points of TSLP stimulation. Another group also found that superoxide production and degranulation could not be induced by TSLP (Hiraguchi *et al.*, 2009). This result may indicate that TSLP could not induce eosinophil degranulation. Upon stimulation, however, TSLP could instead induce significantly *in vitro* release of inflammatory cytokine IL-6, CXC chemokines CXCL8 and CXCL1, and CC chemokine CCL2. Moreover, both transcriptional inhibitor actinomycin D and protein synthesis inhibitor cycloheximide significantly suppressed the release of cytokine and chemokines upon TSLP stimulation. The results thereby demonstrated that TSLP could actually induce the release of newly synthesized IL-6, CXCL8, CXCL1 and CCL2 rather than preformed ones from eosinophils. Inflammatory cytokine IL-6 can induce the tissue remodeling and fibrosis in eosinophil-associated diseases (Gomes *et al.*, 2005). IL-6 also induces the synthesis of acute-phase proteins and mediates various inflammatory responses. Furthermore, IL-6 has been reported as a critical inducer for the polarization of a novel subset of T helper lymphocytes, Th17, which has been suggested to play pathogenic role in AD and allergic asthma (Acosta-Rodriguez *et al.*, 2007; Wang and Liu, 2008). For chemokines, both CXCL8 and CXCL1 are potent chemoattractants for neutrophils and basophils (Kikuchi *et al.*, 2006; Rossi and Zlotnik, 2000), while the expression and secretion of CCL2 is well correlated to the infiltration of monocytes, basophils, eosinophils and memory T lymphocytes and

natural killer cells (Rose *et al.*, 2003). TSLP-induced the release of CCL2, CXCL8 and CXCL1 from eosinophils can therefore mediate the recruitment, infiltration and activation of the above immune effector cells in the microenvironment of inflammatory sites, thereby amplifying inflammatory responses in allergic diseases. The result of cytokine and chemokine induction may imply that TSLP-activated eosinophils involved in allergic disorders partly through the release of chemokines recruiting other inflammatory effector cells to aggravate allergic responses. The effects are different from TSLP-DCs-mediated Th2 permissive microenvironment.

In this study, TSLP significantly up-regulated the cell surface expression of ICAM-1 and CD18, but suppress L-selectin dose-dependently. ICAM-1 has been well demonstrated to potentiate inflammatory process in childhood asthma (Marguet *et al.*, 2000). The interaction of ICAM-1 with integrins is essential for the recruitment and trans-endothelial migration of eosinophils (Hogan *et al.*, 2008). The up-regulation of both ICAM-1 and CD18 expression is therefore important for the recruitment and transmigration of eosinophils into inflammatory sites. Our finding of the down regulation of L-selectin in TSLP-treated eosinophils concurs with previous studies showing that activated eosinophils could down-regulate L-selectin (Wong *et al.*, 2004). Unlike most of the other adhesion molecules, the function of the selectin family is uniquely restricted to the interaction of leukocyte with the vascular endothelium. L-selectin mediates the initial attachment of eosinophils onto endothelial cells before their firm adhesion and diapedesis at sites of inflammation (Hogan *et al.*, 2008). As eosinophils pass through the diverse beds of vascular endothelium, a differential utilization of various adhesion molecules is observed. Similar to Th2 cytokine IL-25, TSLP can significantly enhance the adhesion of eosinophils onto fibronectin, one of important components of extracellular matrix. Because of the altered expression profile of adhesion molecules, the enhanced adhesion activity, together with the induced changes in cellular morphology and cytoskeletal arrangement of eosinophils, TSLP may facilitate local site eosinophilia (Cheung

et al., 2006). Large numbers of eosinophils accumulated beneath skin epidermis in AD and bronchial epithelial cells in asthma disease (Rothenberg, 1998; Simon *et al.*, 2004). Though we did not provide direct evidence that TSLP could chemoattract eosinophils, TSLP indeed modulates eosinophil adhesion molecules and thus facilitates eosinophil trans-endothelial migration and tissue eosinophilia. Another important mechanism for TSLP-mediated eosinophilia at skin and bronchial airway is probably due to the local production of eosinophil chemokine eotaxin-2 by TSLP-activated DCs (Comeau and Ziegler, 2010; Soumelis *et al.*, 2002).

According to previous studies, TSLP could activate STAT5 and STAT3 through JAK-STAT independent pathway (Rochman and Leonard, 2008; He and Geha, 2010). However, different from the above studies, TSLP could not activate STAT3 and STAT5 in eosinophils, as demonstrated by the negative tyrosine and serine phosphorylation. The reason may be that eosinophils are terminally differentiated cells and lack of the potential for proliferation and cell division (Rothenberg, 1998). Indeed, STAT5 and STAT3 activation by TSLP stimulation involved in the proliferation of human AML-derived cell line MUTZ-3 and TCR-activated CD4+ T cells (Quentmeier *et al.*, 2001; Reche *et al.*, 2001). Although STAT5 was phosphorylated by TSLP in bone marrow-derived pre-B cell line, TSLP-mediated STAT5 activation is insufficient to induce cellular proliferation (Isaksen *et al.*, 2002). In view of the above findings, this may infer that other signaling cascades should be involved to compensate STAT5 function in mediating cell proliferation or other cell functions. MAPK and NF-κB pathways were involved in regulating adhesion molecule expression, cytokine and chemokine release of activated eosinophils upon exposure to various stimuli, such as Th17 cytokines, IL-25 and TLR ligands (Cheung *et al.*, 2006; Cheung *et al.*, 2008; Wong *et al.*, 2007). In this study, ERK, p38MAPK and NF-κB were also activated in TSLP-treated eosinophils and the activation process is rapid and sustains short time-course. However, these signaling pathways were activated independently without cross-talk. Inhibitor experiments further elucidated that

the intracellular signaling mechanisms regulated the induction of cytokine and chemokine, and maintenance of survival. The consistent results from signaling molecule activation and inhibitor suppression experiments further confirmed that ERK, p38MAPK and NF-κB were activated and involved in TSLP-mediated eosinophil function. Since TSLP could directly activate ERK, p38MAPK and NF-κB intracellular signaling pathways in eosinophils, effect is markedly different from other cell types reported before (Liu *et al.*, 2007). In addition, PI3K/AKT pathway was reported to be activated in mouse CD8+ T cells by murine TSLP (Watanabe *et al.*, 2005). However, we could not detect any AKT activation upon human TSLP stimulation. This may also raise the issue that the mechanisms modulated by TSLP were cell-specific, at least specific for the terminally differentiated eosinophils.

To further elucidate the pathophysiological link between the activated epithelial cells/keratinocytes and eosinophils mediated inflammation in atopic dermatitis and allergic asthma, we examined the induction of TSLP from epidermal keratinocytes and bronchial epithelial cells upon different allergen-related stimulation. TLR3 ligand dsRNA could significantly induce the expression of TSLP, while TLR2 ligand PGN, TLR4 ligand LPS, and Th2-related cytokines alone show little effects on TSLP protein release. Our findings are similar to other group experimental results. Kato et al. found that detectable TSLP protein could be induced by TLR3 ligand poly I:C alone, or the combination of TNF-α and IL-4, and even much higher level by combination of IL-4 and dsRNA, from bronchial epithelial cells (Kato *et al.*, 2007). Bogiatzi et al. found that proinflammatory and Th2 cytokines could act synergistically to induce the release of TSLP from human skin explants obtained from healthy donors (Bogiatzi *et al.*, 2007). Kinoshita et al. have recently reported that dsRNA could also induce the release of TSLP from keratinocytes and the release level of TSLP was enhanced by the addition of IL-4, IL-13, and/or TNF-α (Kinoshita *et al.*, 2009). The induction of TSLP from keratinocytes by dsRNA, with or without the TNF-α/Th2 cytokines, could be upregulated by IFN-α and IFN-β but suppressed by IFN-γ, TGF-β, or IL-17. In addition,

TLR3 activation could strongly induce eosinophil-recruiting chemokines, such as CCL5 and CCL11, in the presence of Th2 cytokines (Niimi *et al.*, 2007; Tsuji *et al.*, 2005). These results together imply viral infection and the recruitment of Th2 cytokine producing cells may amplify Th2 inflammation via the induction of TSLP in atopic skin and asthmatic airway.

Endobronchial secretion of asthma related Th2 cytokine IL-13 at local inflammatory site has been reported to be at least hundred fold higher than in circulation (Kroegel *et al.*, 1996). Moreover, recombinant TSLP expressed by *E. coli* used in this study has a lower activity than native TSLP due to the differences in glycosylation and post-translational modification. Therefore, the recombinant TSLP concentration (50 ng/ml) used in this study may activate eosinophils at sites of inflammation but probably not in the circulation. Actually, the optimal concentration of TSLP (50 ng/ml) used in our experiments is similar to that in previous publications (Rochman *et al.*, 2007; Rochman and Leonard, 2008; He *et al.*, 2008).

We have demonstrated that TSLP can modulate human eosinophils through ERK, p38MAPK and NF-κB dependent but STAT3 and STAT5 independent signaling transduction pathways. Together with previous studies about TSLP, we further elucidated TSLP-mediated immunophysiological mechanisms in allergic inflammation and the underlining signaling transduction (Ziegler and Artis, 2010). Upon the challenge by allergens, virus or bacteria, TSLP is induced from epidermal keratinocytes, bronchial epithelial cells, smooth muscle cells, stromal cells or mast cells. Local immature DCs are immediately activated by TSLP to upregulate cell surface co-stimulatory molecules MHC-II, CD80, CD86, OX40L, which drive inflammatory Th2 cell differentiation (Soumelis *et al.*, 2002). Meanwhile, TSLP-activated DCs release chemokines IL-8 and eotaxin-2, attracting neutrophils and eosinophils, as well as CCL17 and CCL22, attracting inflammatory Th2 cells (Soumelis *et al.*, 2002). In addition, TSLP can directly stimulate locally retained and newly attracted eosinophils by delaying the apoptosis and inducing the release of IL-6, CXCL8, CXCL1 and CCL2, leading to the subsequent infiltration of inflammatory cells, activation of Th17 cells

and aggravation of inflammation. Together with local inflammatory cytokines, TSLP can stimulate mast cells to produce IL-5, IL-13, IL-6 and GM-CSF for triggering IgE production, augmenting Th2 immune response and eosinophilia (Allakhverdi *et al.*, 2007). Regarding intracellular signal transduction, we have elucidated distinct signaling mechanisms in which ERK, p38MAPK and NF-κB are selectively activated by TSLP in eosinophils. In view of recent advances in the application of MAPK and NF-κB inhibitors as potential anti-inflammatory agents in asthma (O'Neill, 2006), our present study should provide new clues for the development of novel treatment for TSLP-mediated allergic diseases. Follow-up studies should be conducted to further investigate whether ERK, p38MAPK and NF-κB are also involved in TSLP-activated DCs, mast cells and CD4+ T cells in allergic inflammation.

Chapter 4
Role of Novel IL-12 Family Cytokine IL-27 in Eosinophil Activation

4.1 Introduction

Interleukin-12, IL-23, and IL-27 are the three known members of the IL-12 cytokine family. IL-12, the first member of this family discovered in 1989, is a heterodimeric cytokine of 70 kDa comprising of two covalently disulfide-linked subunits p40 and p35 (Kobayashi *et al.*, 1989). The expression of the two subunits is independently regulated and only co-expression of both the subunits in one cell could generate biologically active IL-12. When p35 is expressed without p40, free p35 is not secreted. In contrast, in the absence of p35, p40 can be secreted as a monomer or formed a secreted disulfide-linked homodimer in mice but not detected in human, both of which have been proposed as natural inhibitors to IL-12 (Gillessen *et al.*, 1995; Heinzel *et al.*, 1997). The IL-12 receptor is composed of two chains, IL-12Rβ1 and IL-12Rβ2 which respectively interacts with the IL-12 p40 and p35 subunits, and activates the JAK/STAT pathway of signal transduction. IL-12 receptor is expressed mainly by activated T cells and to a lower extent by Natural killer (NK) cells and DCs (Grohmann *et al.*, 1998; Presky *et al.*, 1996). IL-12 is predominately produced by cells of the innate immune system, such as DCs, monocytes, and macrophages, via TLR signaling or CD40/CD40L signaling, that further influences adaptive cell-mediated immunity. IL-12 has a central role in promoting the differentiation of naive CD4+ T cells into Th1 effector cells and is a potent stimulus for NK cells and CD8+ T cells to produce IFN-γ, and elicit cell-mediated immunity against intracellular pathogens.

Recently, an additional IL-12 family member, IL-23, was discovered, which is also a heterodimeric cytokine with many similarities to IL-12. IL-23 comprises of the IL-12p40 subunit and IL-23-specific p19 subunit that associates with and shares approximately 40% of sequence identity to IL-12p35 subunit (Oppmann *et al.*, 2000). Similar to IL-12, formation of biologically active IL-23 requires synthesis of both p19 and p40 subunits within the same cells, and IL-23 is mainly expressed by activated DCs and phagocytic cells in response to microbial stimulation or CD40/CD40L interactions (Oppmann *et al.*, 2000). IL-23 exerts its biological activities through the interaction with a heterodimeric receptor complex composed of IL-12Rβ1 and IL-23R, which is predominantly co-expressed by T cells, NK cells, and to a lower extent by monocytes, macrophages and DCs (Kastelein *et al.*, 2007; Parham *et al.*, 2002). Whereas IL-12 interacts with IL-12Rβ2, IL-23 requires IL-23R as heterodimeric partner to allow JAK/STAT signal transduction. IL-23 was demonstrated to be involved in infectious disease and could stimulate Th17 subset, which produces IL-17 and has an essential role for the development of autoimmune inflammation (Hunter, 2005).

IL-27, a new member of the IL-12 family cytokines, is mainly produced by antigen-presenting cells (APCs), including DCs and macrophages, as well as neutrophils. Similar to IL-12 and IL-23, IL-27 is composed of two subunits, IL-12 p35-related protein p28 and IL-12 p40-analogous protein Epstein-Barr-virus-induced gene 3 (EBI-3) (Pflanz *et al.*, 2002). The IL-27 receptor is a heterodimer, consisting of orphan cytokine receptor WSX-1 (also called IL-27RA, or T cell cytokine receptor, TCCR) and gp130 subunit (Pflanz *et al.*, 2004). The IL-27 receptor heterodimer is simultaneously expressed on DC, CD4+ and CD8+ T cells, NK cells, NKT cells, B cells, monocytes, macrophages, mast cells, langerhans cells, and keratinocytes. In parallel with the receptor expression profile, IL-27 acts directly on the above cells to induce the tyrosine phosphorylation of JAK/STAT family, including JAK1, JAK2, Tyk2, STAT1, STAT2, STAT3, STAT4 and STAT5 (Batten and Ghilardi, 2007; Goriely *et al.*, 2009; Hölscher *et al.*, 2005; Kalliolias and Ivashkiv, 2008; Kanda and Watanabe, 2008;

Kastelein *et al.*, 2007; Larousserie *et al.*, 2006; Owaki *et al.*, 2006; Takeda *et al.*, 2003).

Previous studies of IL-27 have revealed its proinflammatory effects by promoting early stage for the activation of naive CD4+ T cells and Th1 differentiation (Owaki *et al.*, 2005). IL-27 receptor deficient (WSX-1-/-) mice showed defects in Th1 response when infected with *Listeria monocytogenes* or *Leishmania major* (Chen *et al.*, 2000; Yoshida *et al.*, 2001). Neutralizing IL-27 by Abs against IL-27 p28 rapidly suppressed adjuvant-induced arthritis disease and long-lasting experimental autoimmune encephalomyelitis disease (Goldberg *et al.*, 2004; Goldberg *et al.*, 2004). IL-27 could also induce proinflammatory cytokines from human monocytes and mast cells (Kalliolias and Ivashkiv, 2008; Pflanz *et al.*, 2004). Besides, IL-27 has a potential role for Th2 cytokine production. Upon stimulation by α-galactosylceramide, a synthetic glycolipid agonist of NKT cells, EBI3-/- mice showed decrease in Th2 cytokine IL-4 production and were resistant to oxazolone-induced colitis which were primarily mediated by Th2 cytokines from inducible NKT cells (Nieuwenhuis *et al.*, 2002). In addition, IL-27 enhanced IL-1β-induced human β-defensin-2 production by keratinocytes and showed antitumor activity (Kanda and Watanabe, 2008). Moreover, stable expression of IL-27 in tumor cells inhibits tumor development with antiangiogenic effect and induces T cell-dependent antitumor immune memory (Shimizu *et al.*, 2006; Zhu *et al.*, 2010).

On the other hand, recent work has emphasized the immunosuppressive roles for IL-27, such as its inhibition of the development of Th1, Th2 and Th17 cell subsets (Kastelein *et al.*, 2007). WSX-1-/- mice produced high levels of IFN-γ when infected with *Toxoplasma gondii* or *Trypanosoma cruzi* and large amounts of proinflammatory cytokines in the concanavalin A-induced hepatitis model subsets (Hamano *et al.*, 2003; Villarino *et al.*, 2003; Yamanaka *et al.*, 2004). In OVA-induced allergic asthma model, WSX-1-/- mice demonstrated hyperproduction of various cytokines and exhibited progressive asthmatic symptoms. IL-27 was found to inhibit Th2 cell development as well as Th2 cytokines production from polarized Th2 cells by down-regulation of transcription factor GATA-3 but up-regulation of

T-bet expression simultaneously, thereby suppressing allergic responses (Artis *et al.*, 2004; Fujita *et al.*, 2009; Miyazaki *et al.*, 2005; Yoshimoto *et al.*, 2007). In addition, IL-27 directly block Retinoic acid receptor (RAR)-related orphan receptor C (RORc) expression, repress the development of Th17 cells and limit IL-17-driven inflammation in the central nervous system. IL-27 also limits Th17 cell-mediated uveitis and scleritis (Amadi-Obi *et al.*, 2007). More convincing evidence about the suppressive role comes from IL-27-induced IL-10 production from T cells (Awasthi *et al.*, 2007; Batten *et al.*, 2006; Fitzgerald *et al.*, 2007; Ilarregui *et al.*, 2009; Murugaiyan *et al.*, 2009; Stumhofer *et al.*, 2006; Stumhofer *et al.*, 2007; Yang *et al.*, 2008). IL-27 has also been identified as a key negative regulator of innate immune cell function in septic peritonitis. Moreover, IL-27 could inhibit the function of murine DCs, mast cells and macrophages (Artis *et al.*, 2004; Yoshimoto *et al.*, 2007). Therefore, the inhibitory effects of IL-27 on murine innate immune cells are in contrast to the activation effects of IL-27 for human studies (Kalliolias and Ivashkiv, 2008; Pflanz *et al.*, 2004).

Though IL-27 has been shown to exhibit multiple effects on various immune cells, little information is known about the effects of IL-27 on eosinophils, one of the most important innate immune cells that generate in bone marrow, circulate in peripheral blood, migrate into and ordinarily reside in different tissues (Hogan *et al.*, 2008). Increased circulating number and infiltration of eosinophils at local tissues have been associated with allergic diseases (Simon *et al.*, 2004). The prominent detrimental effects of eosinophils are also involved in allergic inflammation by the release of toxic granule proteins, lipid mediators, cytokines and chemokines (Hogan *et al.*, 2008). In addition, the other two IL-12 family cytokines IL-12 and IL-23 seemed to exert opposite effects on eosinophils (Cheung *et al.*, 2008; Nutku *et al.*, 2001). In an attempt to further elaborate the role of IL-12 family members in allergic responses, we investigated the biological effects of IL-27 on human peripheral blood eosinophils and explored the underlying mechanisms.

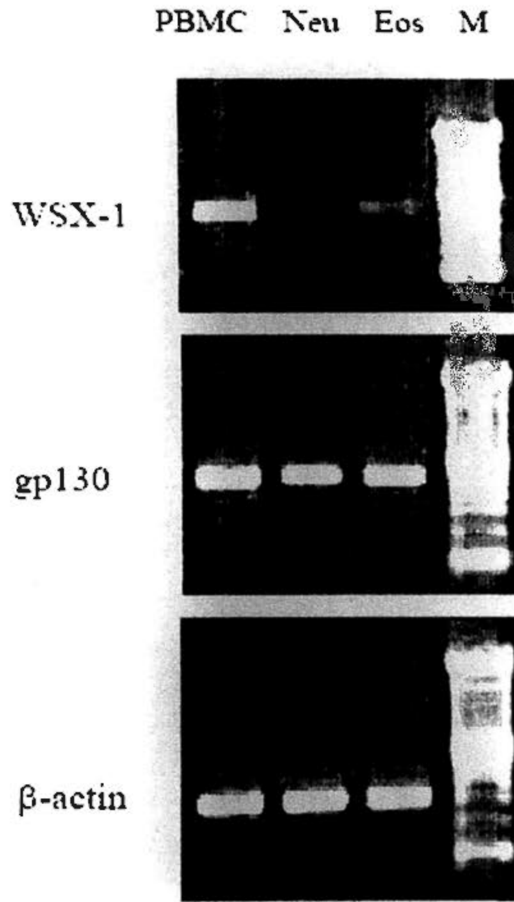
4.2 Results

4.2.1 Human eosinophils express functional IL-27 heterodimeric receptors

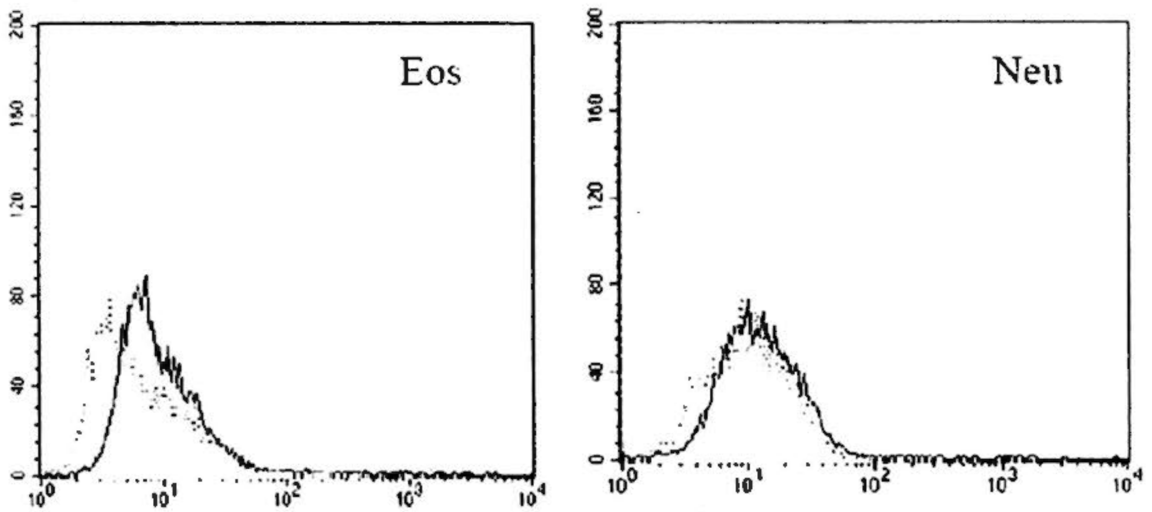
We first examined the expression of IL-27 receptor complex, WSX-1 and gp130, on freshly isolated human peripheral blood eosinophils, while neutrophils and PBMC were used as cell controls. RT-PCR analysis showed that gp130 mRNA was highly expressed on all the three types of cells (Figure 4.1A). For the subunit WSX-1, though high level expression was found on PBMC, the expression level was low for eosinophils and hardly detectable for neutrophils (Figure 4.1A). In consistent with mRNA expression level, flow cytometric analysis showed that gp130 subunit constitutively expressed on the surface of eosinophils but low expression level on neutrophils (Figure 4.1B). Western blot analysis also further confirmed that eosinophils expressed higher protein level of gp130 compared to neutrophils which showed relatively low level of protein expression. PBMC was used as the positive control (Figure 4.1C). Because of the lack of commercial available anti-human WSX-1 antibody for flow cytometry, we confirmed the expression of WSX-1 on eosinophils and PBMC but not on neutrophils using Western blot (Figure 4.1D).

IL-27 stimulation could lead to receptor-mediated tyrosine phosphorylation of STAT family (Kastelein *et al.*, 2007). To verify whether IL-27 receptor expressed on eosinophils was functional, activation of STATs upon IL-27 stimulation was examined. Incubation with IL-27 (50 ng/ml) resulted in potent tyrosine phosphorylation of STAT1 within one minute and maintained for over thirty minutes in eosinophils (Figure 4.1E). However, the phosphorylation of STAT3 and STAT5 was not detected, even up to one hour incubation with IL-27 (Figure 4.1E). Hematopoietic cytokine GM-CSF (10 ng/ml) stimulation as positive control could lead to significant phosphorylation of STAT1, STAT3, and STAT5. All the above results here showed that eosinophils constitutively express functional IL-27 receptor heterodimer.

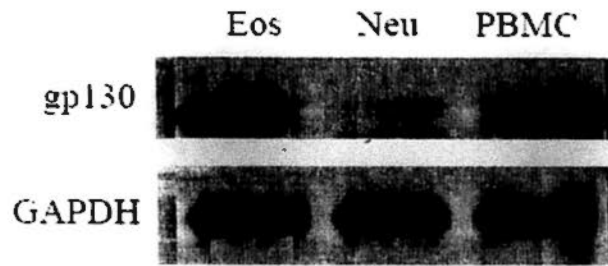
(A)



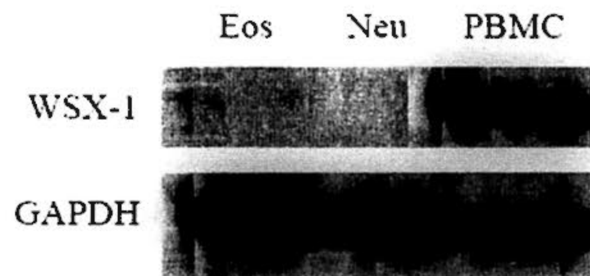
(B)



(C)



(D)



(E)

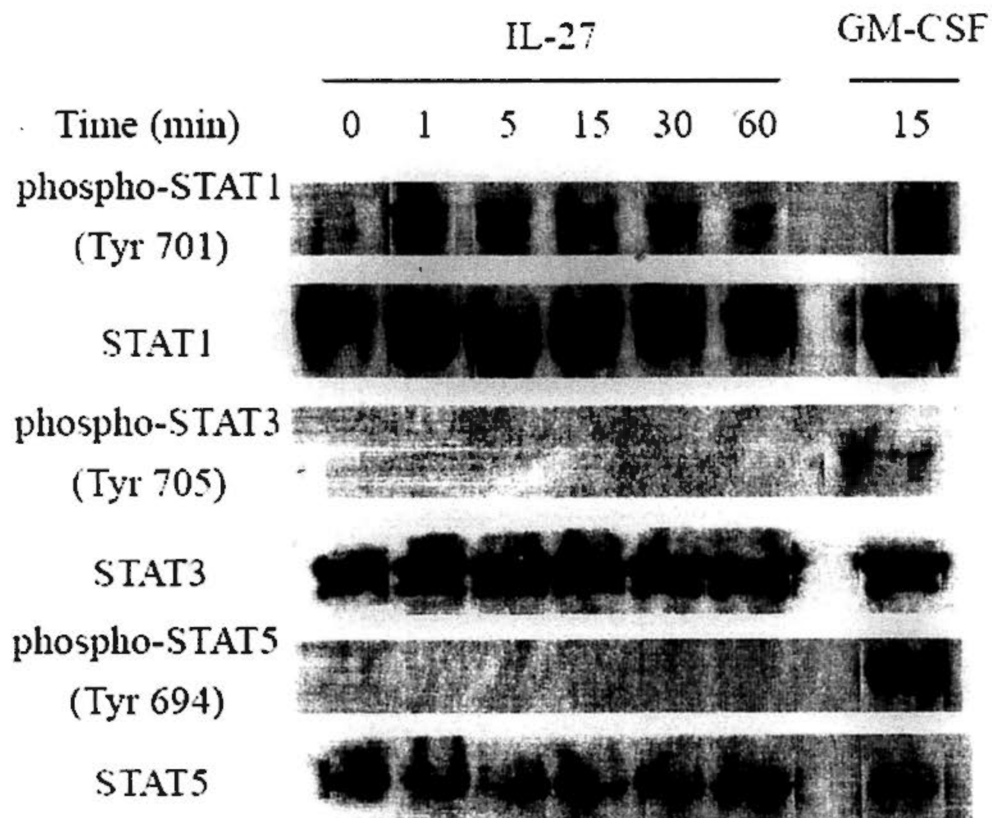


Figure 4.1 Expression of functional IL-27 heterodimeric receptor on eosinophils.

(A) Total RNA was extracted from eosinophils, neutrophils and PBMC, followed by RT-PCR analysis for WSX-1, gp130 and β-actin (house keeping gene) expression. (B) Representative histograms of cell surface expression of gp130 on eosinophils and neutrophils determined by flow cytometry. Dotted line: isotypic control; solid line: anti-human gp130. (C) Representative Western blot analysis of gp130 protein expression of human eosinophils, neutrophils and PBMC. GAPDH was used as protein control to ensure an equal amount of loaded protein. (D) Representative Western blot analysis of WSX-1 protein expression of human eosinophils, neutrophils and PBMC. GAPDH was used as protein control to ensure an equal amount of loaded protein. (E) Eosinophils were stimulated with IL-27 (50 ng/ml) or GM-CSF (10 ng/ml) for indicated incubation time. Total cellular proteins were extracted and STAT1, STAT3 and STAT5 tyrosine phosphorylation were detected by Western blot.

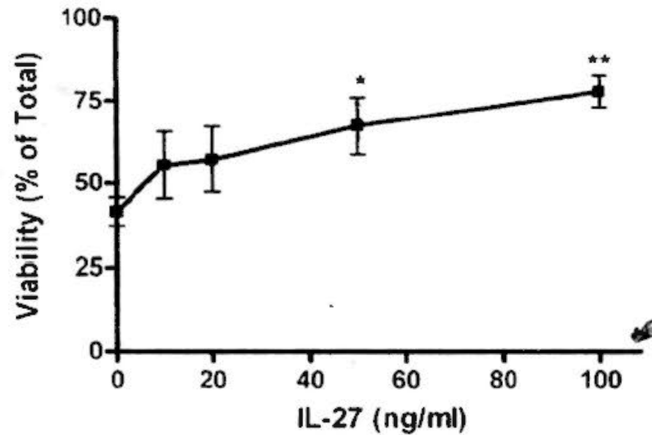
All the experiments were performed in three independent replicates with essentially identical results.

M: 100 base-pair molecular size marker; Eos: eosinophils; Neu: neutrophils; PBMC: peripheral blood mononuclear cells.

4.2.2 IL-27 can enhance eosinophil survival

Eosinophils have a very short life-span and delayed eosinophil apoptosis is a key mechanism for eosinophilia (Simon *et al.*, 2004). Less than half of untreated eosinophils remained viable after 48 h incubation (Figure 4.2). After incubation with IL-27, the percentage of viable eosinophils significantly enhanced and the effect was concentration-dependent (about 75% of viable eosinophils at 50 ng/ml IL-27, all $p < 0.05$), while Annexin V positive population was significantly reduced, which indicated that IL-27 maintained eosinophil survival through the suppression of apoptosis (Figure 4.2).

(A)



(B)

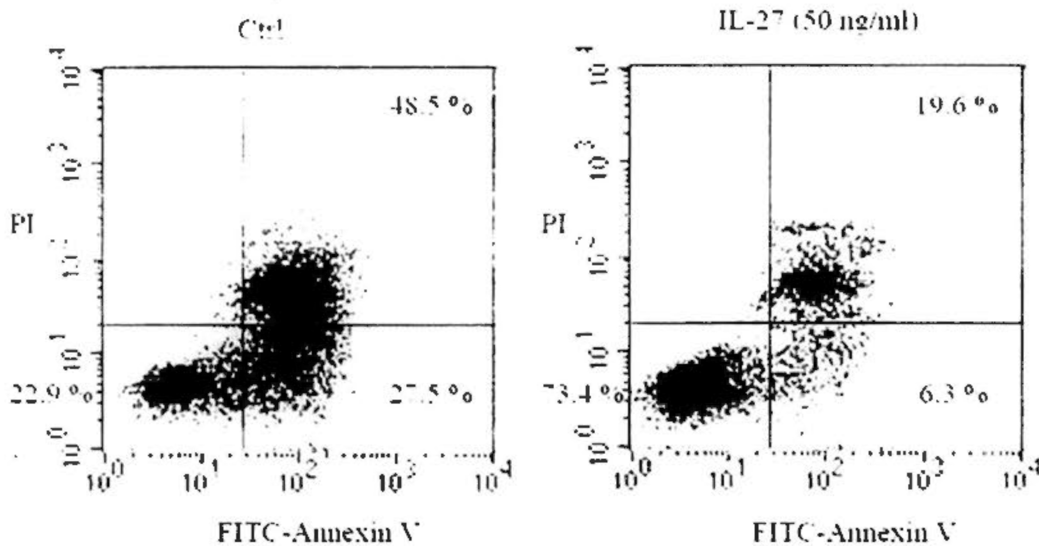


Figure 4.2 Effects of IL-27 on the survival of eosinophils.

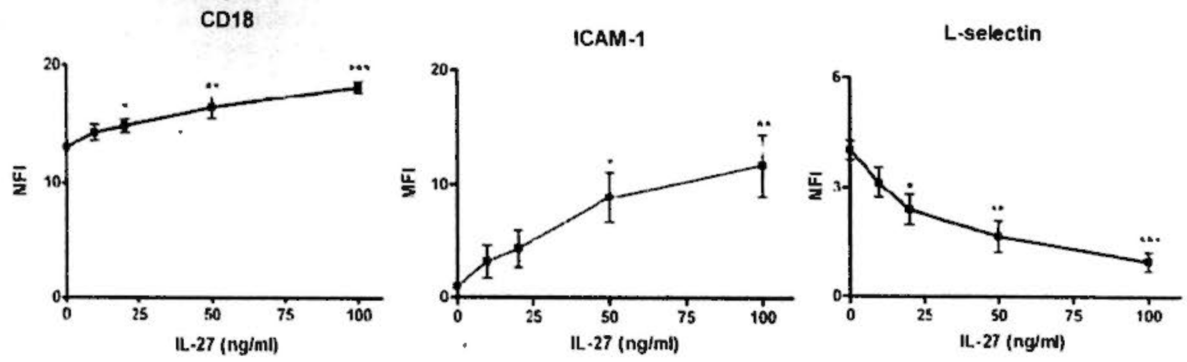
Eosinophils were incubated with IL-27 (0 - 100 ng/ml) for 48 h and then were analysed by flow cytometry using Annexin V-FITC and PI staining. (A) Results of % viability are expressed as the arithmetic mean ± SD from triplicate experiments. (B) Representative dot plots showed early apoptotic cells in the lower right quadrant (Annexin V-FITC-positive), late apoptotic (necrotic) cells in the upper right quadrant (Annexin V-FITC-positive and PI-positive), and viable cells in the lower left quadrant (double negative).

*$p < 0.05$ and **$p < 0.01$ when compared with medium control. Ctrl: control.

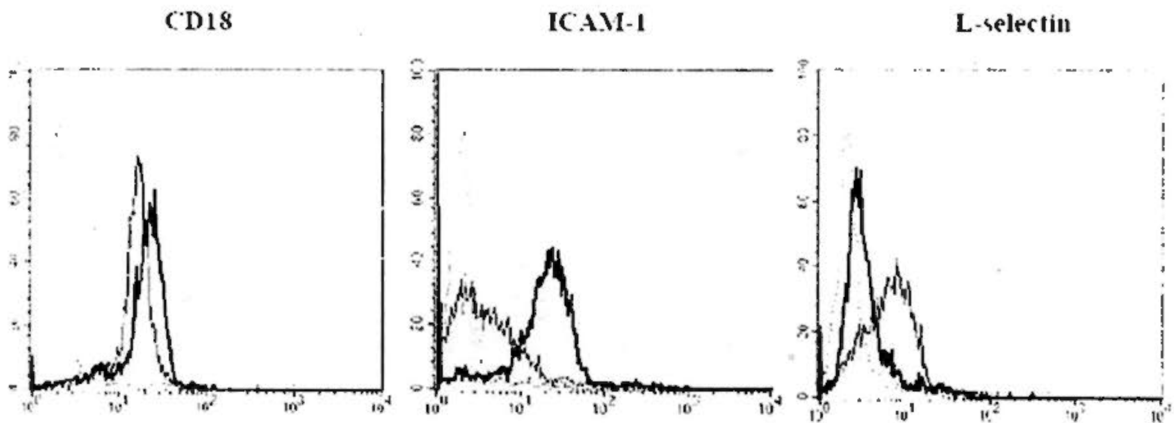
4.2.3 IL-27 can modulate the cell surface expression of adhesion molecules on human eosinophils

Eosinophil migration and recruitment into specific tissues is finely regulated by the interaction between adhesion molecules and their counter ligands (Hogan *et al.*, 2008). Eosinophils generally maintain basal level expression of ICAM-1 and L-selectin, and moderate expression of integrin CD18 (Cheung *et al.*, 2006; Cheung *et al.*, 2008; Wong *et al.*, 2007). IL-27 significantly up-regulated the surface expression of CD18 and ICAM-1, but down-regulated the expression of L-selectin in a dose-dependent manner at 16 h incubation time (Figure 4.3A and 4.3B). In addition, IL-27 could significantly increase the number of eosinophils adhered to fibronectin-coated wells, with IL-25 treatment serving as the positive control (Figure 4.3C) (Cheung *et al.*, 2006). Morphological analysis showed that untreated eosinophils maintained the round shape, while IL-27-treated eosinophils processed elongated shape and aggregated together which is similar to that of GM-CSF-treated eosinophils (Figure 4.3D) (Takashi *et al.*, 2001).

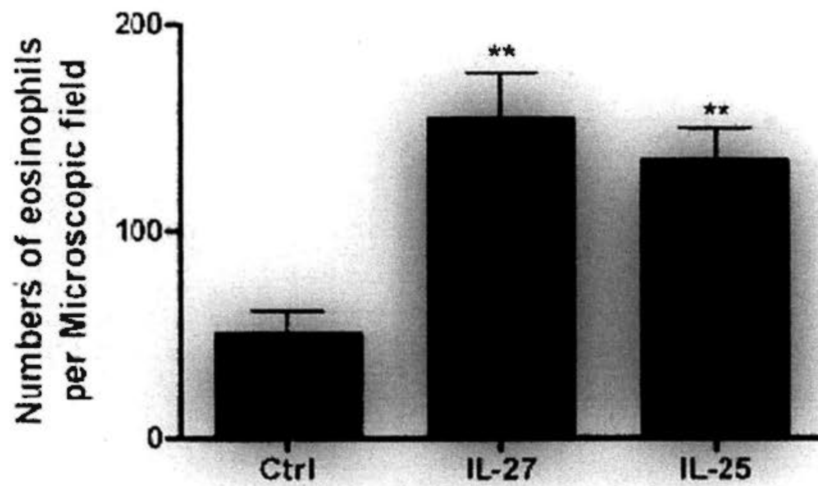
(A)



(B)



(C)



(D)

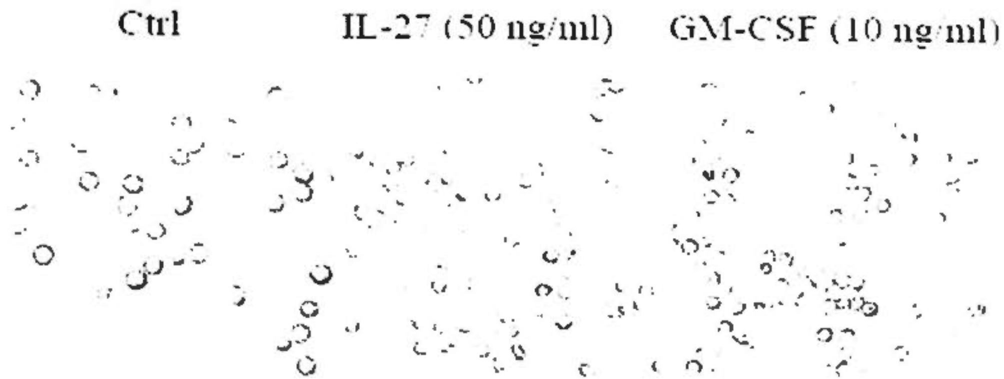


Figure 4.3 Effects of IL-27 on the cell surface expression of adhesion molecules.

Eosinophils were cultured with IL-27 (0 - 100 ng/ml) for 16 h and then the surface expression of adhesion molecules was analysed by flow cytometry. (A) The modulation of surface expression of adhesion molecules are shown as MFI. Results have been normalized by subtracting appropriate isotypic control and are expressed as the arithmetic mean ± SD of three independent experiments. (B) Representative histograms of cell surface expression of CD18, ICAM-1 and L-selectin on eosinophils are shown. Dotted line: isotypic control; grey line: medium control; black line: IL-27 treatment. (C) Eosinophils were stimulated with IL-27 (50 ng/ml) and IL-25 (50 ng/ml) for 16 h in fibronectin-coated wells. Results are expressed as the arithmetic mean of the number of cells adhered onto fibronectin-coated wells plus SD of quadruplicate experiments in four random 100 × field. (D) Eosinophils were incubated with medium, IL-27 (50 ng/ml) or GM-CSF (10 ng/ml) for 16 hours. Photomicrographs show the morphology of eosinophils with 400 × magnification.

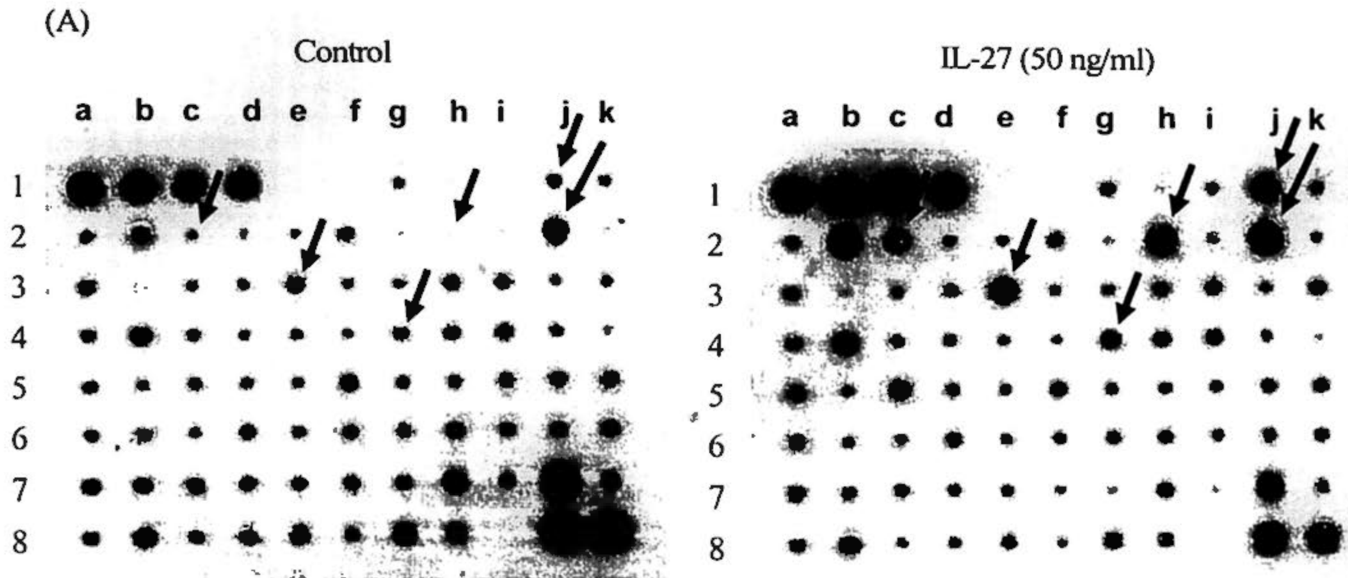
*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ when compared with medium control.

4.2.4 IL-27 can potently induce the release of cytokines and chemokines from human eosinophils

Eosinophils produce a variety of cytokines and chemokines aggravating immune responses (Hogan *et al.*, 2008). We first illustrated the cytokine expression profiles of human eosinophils using antibody-based human cytokine protein array as a means of preliminary screening. IL-27 could activate eosinophils to prominently induce the release of proinflammatory cytokine IL-6 (2h), TNF-α (4g), IL-1β (2c) and chemokines CCL2 (3e), CXCL1 (1j) and CXCL8 (2j), among the 79 different cytokines being screened after 24 h incubation when compared with that of medium control (Figure 4.4A). The map of the cytokine antibody array on the membrane was listed in Appendix. We then further confirmed that IL-27 treatment significantly induced the release of inflammatory cytokines IL-6, TNF-α, IL-1β and chemokines CCL2, CXCL8 and CXCL1 at 12 and 24 h in a dose-dependent manner by quantitation of either CBA or ELISA. The release of chemokines CCL2, CXCL8 and CXCL1 at 12 h was much lower than that at 24 h (Figure 4.4B). However, the induction of inflammatory cytokines IL-6 and IL-1β at 12 h and 24 h were similar and the amount of early response cytokine TNF-α was even higher at 12 h than that at 24 h (Figure 4.4B).

Since IL-27 (50 ng/ml) significantly enhanced eosinophil survival, modulated adhesion molecules and induced cytokine and chemokine release, the optimal concentration (50 ng/ml) was used in the following mechanistic studies.

(A)



(B)

IL-6
pg/ml
IL-27 (ng/ml)

IL-1β
pg/ml
IL-27 (ng/ml)

TNF-α
pg/ml
IL-27 (ng/ml)

CXCL8
pg/ml
IL-27 (ng/ml)

CXCL1
pg/ml
IL-27 (ng/ml)

CCL2
pg/ml
IL-27 (ng/ml)

12 h
24 h

Figure 4.4 Effects of IL-27 on cytokine and chemokine release from eosinophils.

(A) Representative profile of the release of cytokines and chemokines from eosinophils activated by IL-27. Eosinophils (1×10^6 cells) were cultured with or without different IL-27 (50 ng/ml) for 24 h in a 24-well plate. Cell free culture supernatant was then collected and 79 different cytokines in culture supernatant were semi-quantitated using antibody based RayBio® human cytokine array V. Positive and negative controls were designated at (1a, 1b, 1c, 1d, 8j, 8k) and (1e, 1f, 8i), respectively. Arrows indicated the spots with obvious differences between the two array membranes. The map of the cytokine antibody array was listed in Appendix in Page 175. (B) Eosinophils were cultured with IL-27 (0 - 100 ng/ml) for 12 or 24 h and the release of IL-6, TNF-α, IL-1β and CXCL8 in culture supernatant were determined by CBA while CXCL1 and CCL2 were measured by ELISA. Results are expressed as the arithmetic mean ± SD from three independent experiments.

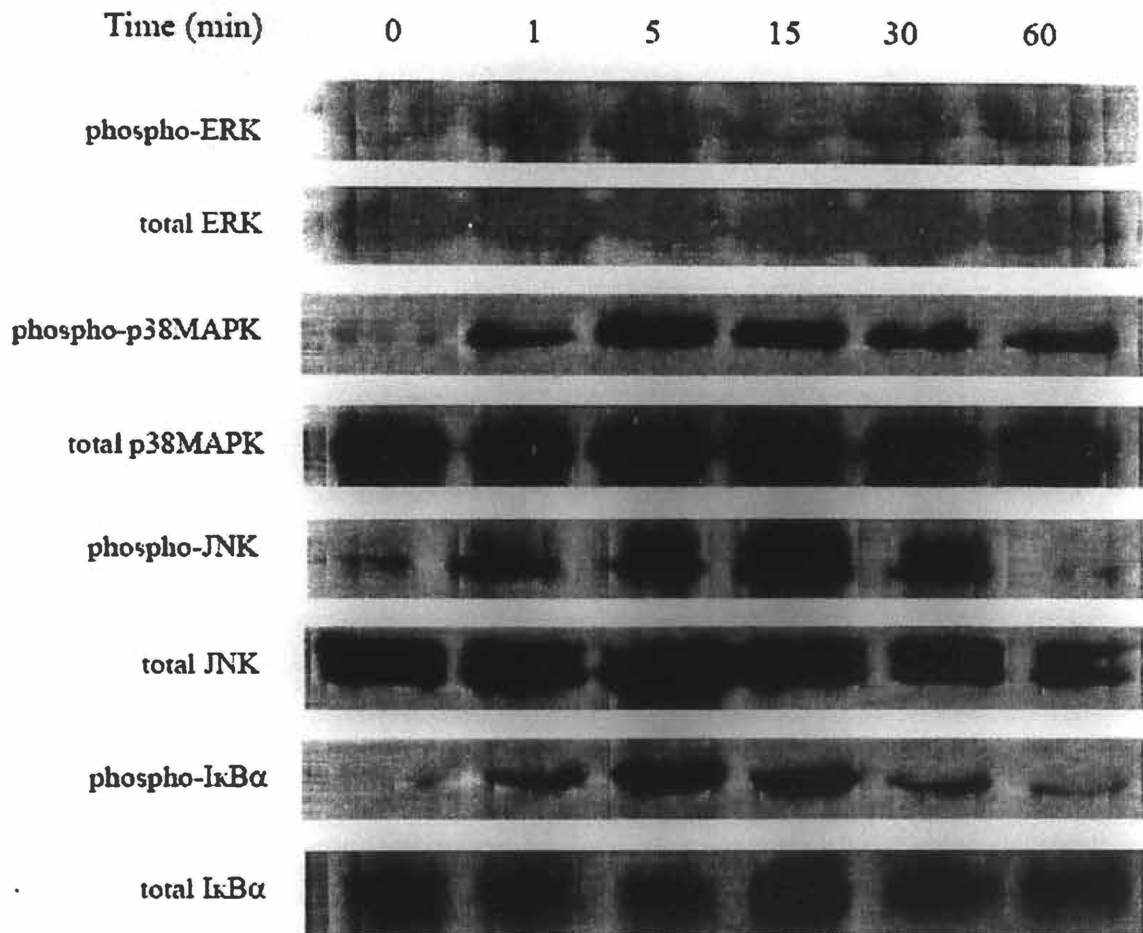
$*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ when compared with medium control.

4.2.5 IL-27 activates intracellular MAPK and NF-κB signaling pathways

MAPKs and NF-κB signaling pathways have been demonstrated to play important roles in eosinophil activation (Cheung *et al.*, 2006; Cheung *et al.*, 2008; Wong *et al.*, 2007). Upon IL-27 stimulation, all the three MAPK members, ERK, JNK and p38MAPK were phosphorylated in eosinophils as determined by Western blot (Figure 4.5A). The activation of the transcription factor NF-κB is due to the rapid degradation of IκBα. In resting state, NF-κB is present in the cytosol interacting with inhibitory IκBα proteins. Upon activation, IκBα was phosphorylated and degraded by proteasome-mediated proteolysis, resulting in the release and nuclear translocation of active NF-κB (Baldwin, 1996). We therefore examined IκBα phosphorylation as the indicator for NF-κB activation. Similar to MAPK activation, NF-κB was also activated as demonstrated by IκBα phosphorylation (Figure 4.5A). The kinetic studies showed that all the signaling activation appeared within 1 min, peaked at 5 min or 15 min, maintained the activation status for 30 min and then down-regulated to the basal level within 60 min. However, PI3K/Akt activation in IL-27-treated eosinophils could not be detected (data not shown).

To further confirm the activation and nuclear translocation of NF-κB upon IL-27 stimulation, EMSA was performed with nuclear extracts from untreated and IL-27-treated eosinophils. Untreated eosinophils showed a very faint shifted band (Figure 4.5B, Lane 2), while eosinophils treated with IL-27 (50 ng/ml) for 2 h showed significant increase in band shift (Figure 4.5B, Lane 3), indicating that IL-27 could potently induce the activation of NF-κB-DNA binding activity. Competitive control using excessive unlabeled NF-κB binding DNA could totally suppress the IL-27-induced band shift, which confirmed the specificity of NF-κB-DNA interaction (Figure 4.5B, Lane 4). Collectively, the above results demonstrated that IL-27 could stimulate NF-κB and MAPK activation in eosinophils.

(A)



(B)

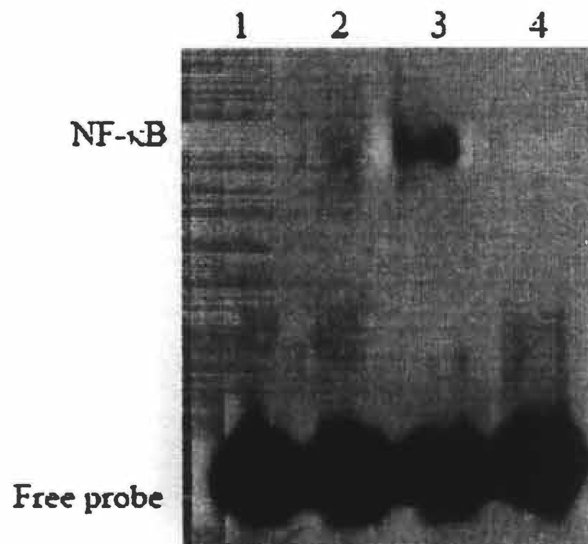


Figure 4.5 Effects of IL-27 on intracellular MAPK and NF-κB activation.

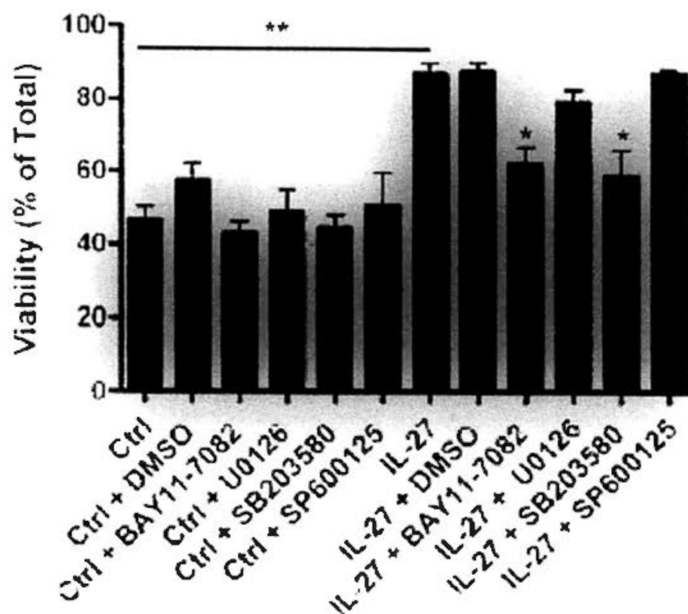
(A) Eosinophils (1 × 10^6 cells) were stimulated with IL-27 (50 ng/ml) for indicated incubation time and total cellular proteins were extracted. Total and phospho-ERK, total and phospho-JNK, total and phospho-p38MAPK, and total and phospho-IκBα were assessed by Western blot. Unphosphorylated protein was used to ensure an equal amount of loaded protein. (B) Eosinophils were stimulated with IL-27 (50 ng/ml) for 2 h. Nuclear proteins were extracted and then subjected to EMSA. Lane 1: labeled probe only without sample; Lane 2: labeled probe with untreated eosinophil nuclear proteins; Lane 3: labeled probe with IL-27-treated eosinophil nuclear proteins; Lane 4: IL-27-treated eosinophil nuclear proteins with excessive unlabeled cold and labeled probes.

Experiments were performed in three independent replicates with essentially identical results and representative results are shown.

4.2.6 Roles of MAPK and NF-κB pathways in IL-27-mediated eosinophil activation

To further address the relation between intracellular signaling pathways and IL-27-induced eosinophil activation, eosinophils were pretreated with specific signaling molecule inhibitors and then stimulated with IL-27. The optimal concentration of these inhibitors was tested in Chapter 3 with the highest inhibition without any cytotoxicity. IκBα kinase inhibitor BAY11-7082 and p38MAPK inhibitor SB203580 could significantly suppress IL-27-enhanced eosinophil survival (Figure 4.6A). Pre-treatment of eosinophils with ERK inhibitor U0126, p38MAPK inhibitor SB203580 and JNK inhibitor SP600125 could significantly suppress IL-27-induced up-regulation of CD18 and ICAM-1 but restore IL-27-induced down-regulation of L-selectin (Figure 4.6B). In addition, IκBα kinase inhibitor BAY11-7082 could also reverse IL-27-induced up-regulation of CD18 and ICAM-1 but not for down-regulation of L-selectin (Figure 4.6B). For cytokine and chemokine release, IL-27-induced release of IL-6, TNF-α and CXCL8 was partially abolished by inhibitor BAY11-7082, U0126 and SB203580, but not by SP600125. The release of IL-1β and CXCL1 was partially suppressed by inhibitor BAY11-7082 and SB203580, while CCL2 was only inhibited by BAY11-7082 and U0126 (Figure 4.6C). Collectively, the results demonstrated that NF-κB and MAPK pathways were differentially involved in IL-27-mediated diverse activation on eosinophils.

(A)



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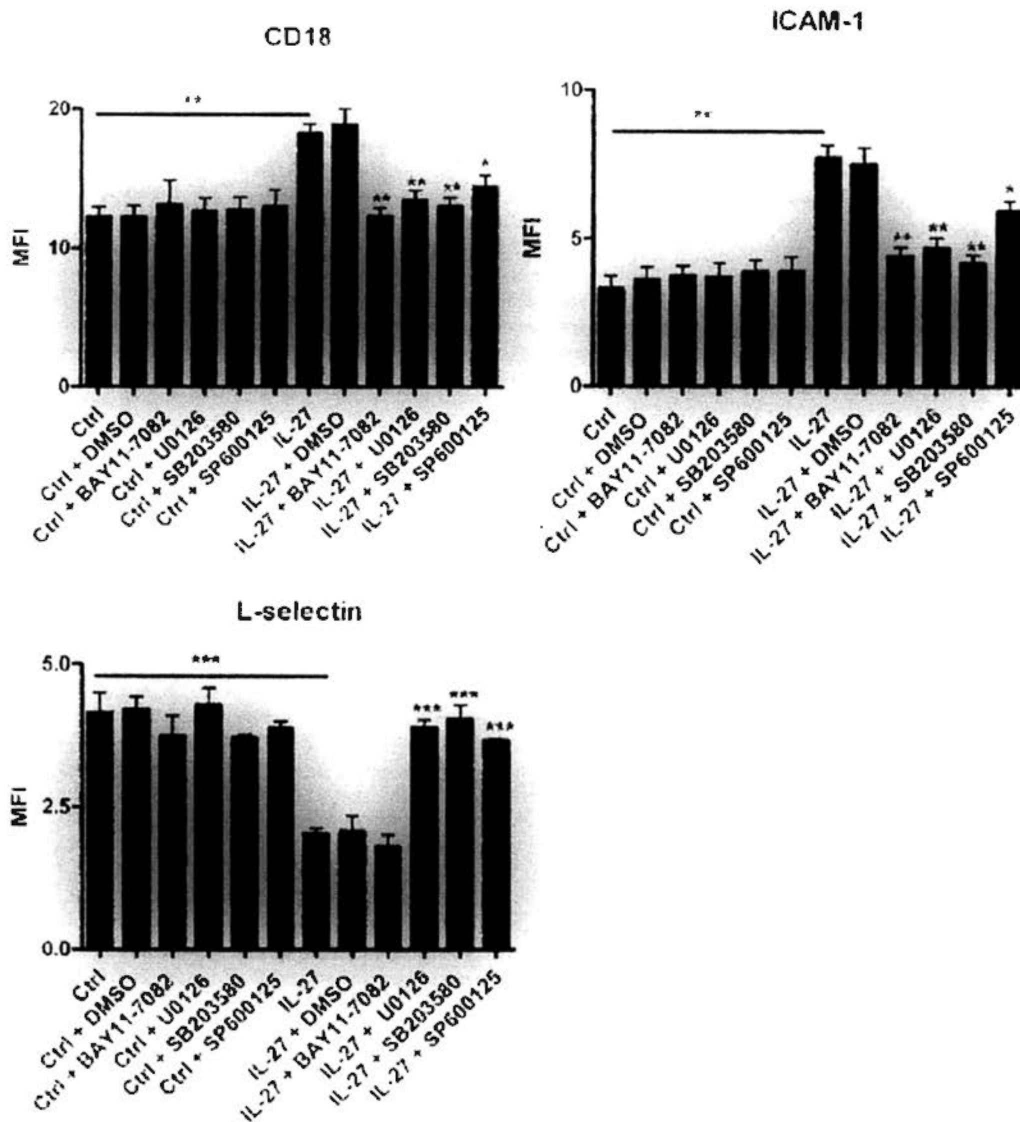


(C)

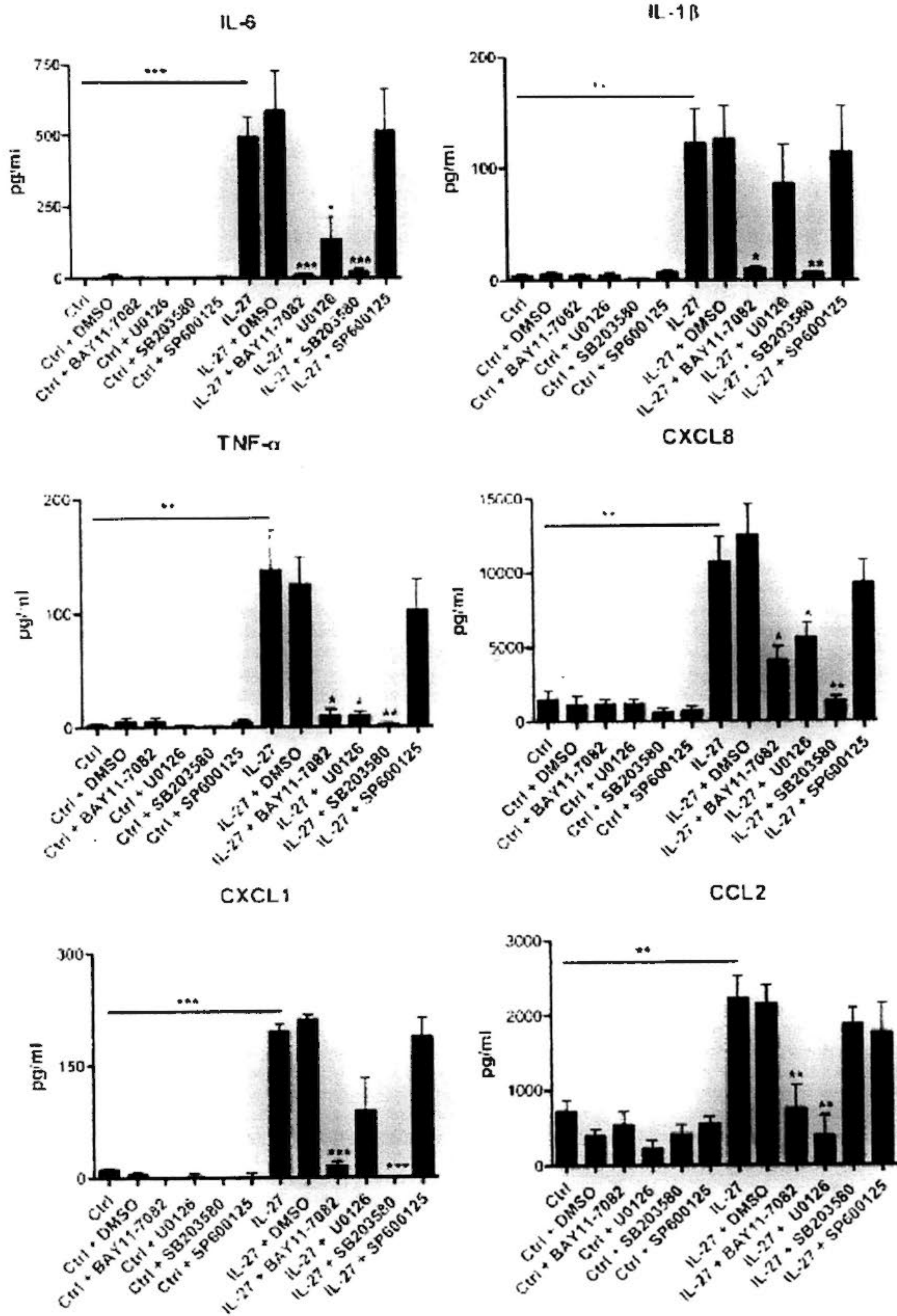


Figure 4.6 Effects of signaling inhibitors on viability, adhesion molecule expression, and cytokine and chemokine release of eosinophils upon IL-27 stimulation.

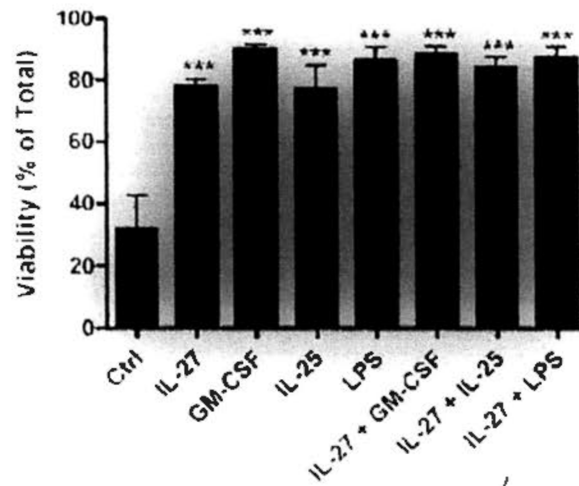
Eosinophils were pre-treated with BAY11-7082 (1 μM), U0126 (2.5 μM), SB203580 (2.5 μM) and SP600125 (2 μM) for 1 h, followed by incubation with or without IL-27 (50 ng/ml) in the presence of inhibitors for further (A) 48 h for viability assay, (B) 16 h for adhesion molecule expression, and (C) 24 h for cytokine and chemokine release. Results are expressed as the arithmetic mean + SD from three independent experiments. DMSO (0.1%) was used as the vehicle control.

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ when compared between groups denoted by horizontal lines or groups of IL-27 with or without inhibitor treatment. Ctrl: control treatment.

4.2.7 GM-CSF, IL-25 and LPS could not abolish IL-27-mediated eosinophil activation, and vice versa

IL-27 or TLR4 ligand LPS alone could potently activate human monocytes, however, the inflammatory functions of IL-27 for monocytes could be abrogated by LPS (Kalliolias and Ivashkiv, 2008). As the effects for monocytes, IL-27, GM-CSF, IL-25 or LPS alone could potently activate eosinophils by increasing the viability and release of CXCL8 (Figure 4.7). However, in contrast to the results for monocytes, all of these pre-stimulations (GM-CSF, IL-25 and LPS) could not abrogate IL-27-mediated eosinophil activation, as demonstrated by the unaltered levels of eosinophil survival analyzed by Annexin V/PI staining, and even exhibited additive effect for the secretion of chemokine CXCL8 determined by ELISA (Figure 4.7). In addition, similar to the above effects, IL-27 pretreatment or treatment at the same time also could not affect GM-CSF, IL-25 or LPS-enhanced eosinophil survival and secretion of CXCL8 (data not shown).

(A)



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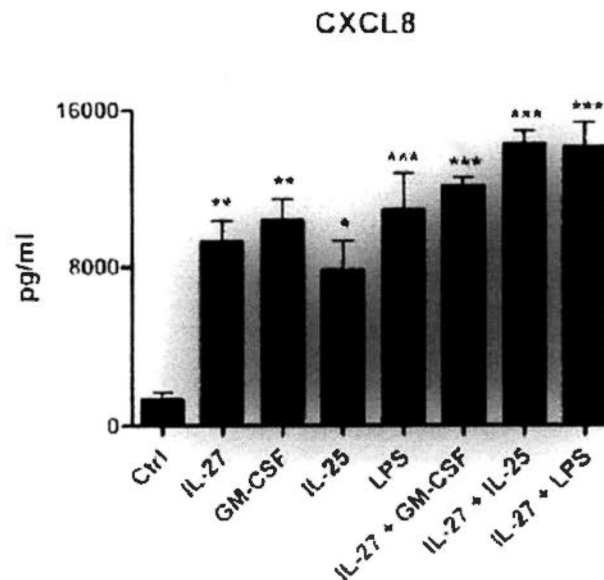


Figure 4.7 Effects of GM-CSF, IL-25 and LPS on IL-27-mediated activation of eosinophils.

Eosinophils were cultured in the presence or absence of GM-CSF (10 ng/ml), IL-25 (50 ng/ml) or LPS (100 μg/ml) for 30 min and then stimulated with IL-27 (50 ng/ml) for (A) 48 h for viability assay, (B) 24 h for chemokine CXCL8 release. Results are expressed as the arithmetic mean + SD from three independent experiments.

$*p < 0.05$, $^{**}p < 0.01$ and $***p < 0.001$ when compared with medium control.

4.3 Discussion

IL-12 family members have been shown to be involved in regulating eosinophil activation. IL-23 could enhance the survival and activation of eosinophils in Th17 cell-mediated allergic inflammation, while another member IL-12 could induce eosinophil apoptosis and decrease subsequent tissue eosinophilia (Cheung *et al.*, 2008; Nutku *et al.*, 2001). Although IL-27, the new member of IL-12 family, could play both pro- and anti-inflammatory function (Kastelein *et al.*, 2007), the present study demonstrated the *in vitro* stimulation effects of IL-27 on human eosinophils.

Human eosinophils constitutively expressed functional IL-27 receptor complex, and STAT1 but not STAT3 was phosphorylated upon IL-27 stimulation. IL-27-mediated STAT1 phosphorylation is mainly required for cell activation (Kastelein *et al.*, 2007). For example, IL-27 could activate STAT1 to induce IL-12Rβ2 expression in naive CD4+ T cells and contribute to Th1 differentiation, whereas STAT1 activation was decreased in IL-27-activated CD4+ T cells (Batten *et al.*, 2006; Takeda *et al.*, 2003). IL-27-induced STAT1 activation was associated with proinflammatory effects in human monocytes and keratinocytes (Kalliolias and Ivashkiv, 2008; Kanda and Watanabe, 2008). However, the inhibition of Th17 development by IL-27 is also dependent on STAT1 activity (Amadi-Obi *et al.*, 2007; Neufert *et al.*, 2007). To elucidate effects of IL-27 on eosinophils, we further investigated the responses of eosinophils and the underlying mechanisms.

IL-27 induced proliferation of human naïve CD4+ T cell and anti-Ig-stimulated naïve B cells (Kastelein *et al.*, 2007; Larousserie *et al.*, 2006). However, as the terminally differentiated cells, eosinophils could not further proliferate but undergo apoptosis. IL-27 indeed enhanced eosinophil survival through reducing apoptosis. Belonging to IL-12 family cytokines, IL-23 and IL-27 enhanced eosinophil survival (Cheung *et al.*, 2008). On the other hand, IL-12 increased *in vitro* apoptosis of human eosinophils (Nutku *et al.*, 2001). The discrepancy of the effects of IL-12 family members was also observed in the differentiation of

naïve CD4+ T cells into effector populations (Kastelein *et al.*, 2007). IL-12 is well-known as a factor that drives and enhances the development of IFN-γ-producing Th1 effector cells, which have been associated with inflammatory conditions. Another IL-12 family member IL-23 is mainly responsible for Th17 differentiation and the progression of Th17 cell-dependent inflammatory diseases. However, a definitive function of IL-27 has remained more elusive and complicated, involving in early steps of Th1 differentiation and the subsequent suppression of Th1, Th2 and Th17 differentiation (Kastelein *et al.*, 2007). The reasons for the diverse effects of IL-12 family members may be due to the different intracellular signaling mechanisms which require further detailed investigation.

In the present study, IL-27-induced expression profile of adhesion molecules, enhanced expression of CD18 and ICAM-1, as well as reduced the expression of L-selectin, is similar to that of other eosinophil stimulator such as Th2 cytokine IL-25 and adipokine Leptin (Cheung *et al.*, 2006; Wong *et al.*, 2007). The modulated expression profile of adhesion molecules could facilitate eosinophil adhesion and accumulation (Cheung *et al.*, 2006). Actually, IL-27 has been shown to up-regulate ICAM-1 expression on CD4+ T cells and anti-Ig-stimulated B cells (Kastelein *et al.*, 2007; Larousserie *et al.*, 2006). The induction of ICAM-1 expression indicated that ICAM-1 might be a target gene regulated by IL-27 and IL-27-mediated STAT1 activation was demonstrated to be essential for the up-regulation of ICAM-1 expression (Owaki *et al.*, 2005). In addition, it was found that ICAM-1 promoter contained a large number of binding sites for inducible transcription factors, including NF-κB and STAT1, which were also activated by IL-27 in eosinophils (Owaki *et al.*, 2005; Roebuck and Finnegan, 1999). As a ligand of β2-integrin, ICAM-1 might facilitate the adhesion of leukocytes because microscopic examination found that IL-27-activated eosinophils aggregated together, which was similar to that of GM-CSF stimulation (Takashi *et al.*, 2001).

The activation effects of IL-27 on eosinophils were further demonstrated by the induction of inflammatory cytokines and chemokines. Similar to human mast cells and

monocytes, early response proinflammatory cytokines TNF-α, IL-1β and IL-6 were promptly induced by IL-27 and reached plateau within 12 h. Chemokines CCL2, CXCL8 and CXCL1 for chemotactic migration of macrophages and neutrophils were also induced in a dose- and time-dependent manner. These chemokines have proinflammatory effects such as the up-regulation of adhesion molecules, regulation of vascular permeability, mucus secretion and smooth muscle constriction (Hogan *et al.*, 2008). Eosinophils can release IL-10 upon IL-12 stimulation and IL-27 has been shown to induce IL-10 from CD4+ T cells, however, the induction of IL-10 by IL-27 from eosinophils could not be detected by CBA analysis (detection limit: 3.3 pg/ml), which further confirmed the activation but not suppressive effects of IL-27 for eosinophils (Awasthi *et al.*, 2007; Fitzgerald *et al.*, 2007; Ilarregui *et al.*, 2009; Spencer *et al.*, 2009; Stumhofer *et al.*, 2007). The induction of IL-10 from eosinophils by IL-12 but not IL-27 also supported the diverse effects of IL-12 family members.

The intracellular mechanistic study indicated that IL-27 activated all the three MAPK members, ERK, JNK and p38MAPK as well as NF-κB. These pathways are crucial cascades for regulating multiple cellular responses, including growth, proliferation, survival, and expression of proinflammatory cytokines and chemokines (Ho *et al.*, 2008; Cheung *et al.*, 2006). In addition, previous studies showed that IL-25, GM-CSF and LPS could also activate MAPK and NF-κB in eosinophils (Cheung *et al.*, 2006; Plötz *et al.*, 2001; Wong *et al.*, 2003). The similar activation profile of intracellular signaling transduction pathways of IL-27, IL-25, GM-CSF and LPS may be the underlying mechanism, by which IL-25, GM-CSF and LPS could not abrogate IL-27-mediated eosinophil activation. The effect was different from monocytes in which inflammatory activation of IL-27 could be abrogated by LPS (Kalliolias and Ivashkiv, 2008). Another reason for the discrepancy between IL-27 and LPS for the activation of monocytes and eosinophils may be due to the fact that eosinophils are terminally differentiated cells and can only maintain for short life-span without proliferation. Our results also indicated that IL-27-mediated intracellular signaling transduction was different between

eosinophils and other cell types, such as T cells, macrophages and keratinocytes, because JNK and NF-κB pathways activated by IL-27 have not been previously reported (Kastelein *et al.*, 2007).

However, there are still questions we need to address. Eosinophils generally involved in Th2-related diseases such as allergic diseases and parasite infection, while IL-27 was reported to suppress Th2 responses in animal studies (Kastelein *et al.*, 2007). Our findings regarding *in vitro* stimulation effects of IL-27 on human eosinophils seemed to be contradictory to IL-27-suppressed Th2 immunity. One reason for the contradiction may be due to the differences between human and mouse studies. IL-27 leads to the production of pro-inflammatory cytokines from human mast cells and monocytes (Kalliolias and Ivashkiv, 2008; Pflanz *et al.*, 2004), however, IL-27 negatively regulate murine mast cells and activated macrophages (Artis *et al.*, 2004; Hölscher *et al.*, 2005). The contradiction may also be due to the *in vitro* and *in vivo* assay designs. *In vivo* studies are complicated and involve multi-factorial interaction, while *in vitro* studies simplify the direct effects of IL-27 on targeted cells. IL-27 inhibition of Th2 related diseases, such as *Trichuris muris* infection and OVA-induced asthma were all derived from *in vivo* mouse models (Artis *et al.*, 2004; Fujita *et al.*, 2009; Miyazaki *et al.*, 2005). However, no *in vivo* human studies of IL-27 have been reported and the stimulation effects of IL-27 on human eosinophils, monocytes and mast cells have all been derived from *in vitro* studies. The third reason may be due to the different signal transduction pathways. In human eosinophils, IL-27 could not activate STAT3 which is an important transcription factor for IL-27-mediated suppression (Kastelein *et al.*, 2007), while MAPKs were involved in IL-27-mediated activation effects. Moreover, IL-27 inhibits acquired immune responses mediated by Th1, Th2 and Th17 cells, but not innate immune responses, while eosinophils are considered to be involved in innate immunity (Hogan *et al.*, 2008; Kalliolias and Ivashkiv, 2008). The adaptive immune system initiated by innate immune responses can be subsequently activated. We postulated that IL-27 may be served as

an immunomodulatory cytokine in allergic diseases. Human IL-27 could activate innate immune responses through the stimulation of mast cells and eosinophils upon allergen stimulation, and then IL-27 inhibited acquired-Th2 immune responses. This regulatory mechanism may be helpful to react with foreign stimuli and limit the allergic inflammation.

In conclusion, our studies have demonstrated that APC-released IL-27 can stimulate eosinophils, thereby extending the novel IL-27 activation effects on human innate immune system and further elaborating the roles of IL-12 family cytokines on eosinophils in allergic inflammation. Since single nucleotide polymorphisms of IL-27p28 were related to human asthma (Chae *et al.*, 2007), the pleiotropic roles of IL-27 in human allergic responses need further exploration.

Chapter 5

Co-culture of Eosinophils and Bronchial Epithelial Cells Upon NOD-like Receptor Ligand Stimulation: Innate Immune Responses and Allergic Asthma

5.1 Introduction

Allergic asthma is a chronically relapsing inflammatory disease whose prevalence is increasing all over the world. Respiratory tract infection, such as rhinovirus, respiratory syncytial virus (RSV), influenza A and *Chlamydia pneumoniae* infection, can cause allergen sensitization, and subsequently amplify and sustain airway inflammation in allergic asthma (Johnston, 2007). Epidemiological studies also suggest that severe asthma exacerbation is characterized by the association of bacterial and viral infection (Busse and Gern, 1997; Hashimoto *et al.*, 2008). Innate immunity is important for host defence against microbial infections, including respiratory infection. Host innate immune system have developed a limited number of germline-encoded receptors, pattern-recognition receptors (PRRs), which could recognize a variety of conserved microbial molecular signatures, thereby allowing for efficient and rapid discrimination between pathogens and self. The best-characterized PRRs are TLRs, which localize either at the cell surface or within endosomes and/or lysosomes, and are characterized by an intracellular Toll/Interleukin-1 receptor homology (TIR) signaling domain and an extracellular leucine-rich repeat (LRR) domain for antigen recognition (Akira and Takeda, 2004). Activation of TLRs leads to NF-κB and MAPK activation, resulting in

the expression of inflammatory cytokines and costimulatory molecules. Moreover, ligands for TLR3, TLR4, TLR7 and TLR9 could induce type I IFN production (e.g. IFN-α and IFN-β) by activation of IFN regulatory factors (IRF) (Akira and Takeda, 2004). TLRs have been involved in infectious diseases, autoimmune diseases, cancers, and as adjuvants for potent new vaccines. TLRs have also been shown to be involved in allergic diseases. Infants raised in homes with high levels of endotoxin have been found to be at relative low risk for the development of allergic hypersensitivities (Braun-Fahrländer *et al.*, 2002; Gereda *et al.*, 2000). One of the characteristic of allergic asthma is the unbalanced Th1/Th2 shifting to Th2 differentiation, and the aim of treating asthma with immunomodulators would be to try to change the Th-cell balance. It was shown that the Th1/Th2 polarizing effect by LPS from *Escherichia coli* via TLR4 was concentration-dependent, at low concentrations for Th2 induction and higher concentrations for Th1 differentiation (Eisenbarth *et al.*, 2002). Besides, polymorphisms in the genes of TLR4 and TLR2 have furthermore been shown to interact with the environment to modulate the allergic protective effects (Eder *et al.*, 2004). When used as adjuvant, Pam3Cys dependent on TLR2 induced Th2 polarization (Redecke *et al.*, 2004), whereas CpG oligodeoxynuleotides (ODN)-based therapies through TLR9 induced Th1 polarization both in murine allergic disease models and also being developed for the treatment of human allergic diseases (Vollmer and Krieg, 2009). Ligands for TLR7 and TLR8 (e.g. R848 and its derivatives) have consistently been described as Th1-polarizing adjuvant and inhibit Th2 cytokine production (Brugnolo *et al.*, 2003). Together, these investigations demonstrated that the activation of TLRs could modulate asthmatic immune responses.

Though TLRs as one of PRRs play important roles in defence against microbial infections, however, several pathogenic bacteria and virus hidden inside the cells could avoid TLR-mediated detection, which indicated other possible PRRs may exist. Recent studies using computational analysis of the genome, two members of the NLR proteins, NOD1 (CARD4) and NOD2 (CARD15), were identified. NLRs resemble plant disease resistance (R)

genes and shed a new light on innate recognition of microbes and induction of inflammatory responses (Bertin *et al.*, 1999; Inohara *et al.*, 1999; Ogura *et al.*, 2001). Besides NOD1 and NOD2, NLR family also contain other members, including IPAF (ice-protease activating factor), CIITA (MHC class II transactivator), NAIP (neuronal apoptosis inhibitory protein) and NALP (NACHT, LRR and PYD containing protein) (Ting *et al.*, 2008).

Indeed, the characterization of NLRs has greatly advanced in recent years and underlined their essential roles in innate immunity. NOD1 and NOD2 are both cytosolic proteins function as intracellular PAMP receptors, and biochemical and functional analyses identified that these proteins recognize different moieties of bacterial PGN. NOD1 recognizes the iE-DAP, which is produced by most Gram-negative and certain Gram-positive bacteria (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003; Hasegawa *et al.*, 2006). In contrast, NOD2 is activated by MDP, the essential structure of virtually all types of PGN present in the cell walls of all bacteria, making NOD2 a general bacterial PRR (Girardin *et al.*, 2003). In addition, NOD2 recently was also found to function as a cytoplasmic viral PRR that recognize viral ssRNA genome (Sabbah *et al.*, 2009).

The NOD proteins are comprised of three distinct functional domains: an amino-terminal effector-binding domain (EBD) involved in signaling transduction and biological functions, a centrally regulatory NOD domain that mediates self-oligomerization, and carboxyl-terminal LRRs that serve as a ligand-recognition domain (LRD) (Inohara *et al.*, 2005; Strober *et al.*, 2006). Binding of the ligands, the effector domains of NOD proteins are involved in homophilic interactions with downstream signaling partners with a caspase-recruitment domain (CARD). The diversity of the effector domains allows NOD proteins to interact with a variety of binding partners to activate multiple signaling pathways. Upon activation, NOD1 and NOD2 rapidly form oligomers and then physically associate with the CARD-containing protein kinase RICK (RIPK2/RIP2/CARDIAK) through homophilic CARD-CARD interactions (Inohara *et al.*, 1999; Ogura *et al.*, 2001). RICK then

interacts with IKKγ/NEMO, the regulatory subunit of the IKK complex, and further leads to the subsequent phosphorylation and degradation of IκBα by the proteasome (Inohara *et al.*, 2000). NF-κB is then released and translocated to the nucleus, and mediates the transcription of target genes. Importantly, NOD proteins-mediated signaling pathway is independent of myeloid differentiation factor 88 (MyD88), a key adaptor molecule involved in TLR signaling pathways. NOD2 might also regulate the non-canonical NF-κB pathway, which exhibits much slower kinetics and is totally dependent on the NF-κB-inducing kinase (NIK) (Pan *et al.*, 2006). NIK associates with the p100 subunit of NF-κB and induces its cleavage to active form p52, which causes the expression of a distinct subset of inflammatory genes (Pan *et al.*, 2006). NIK is required for MDP-induced transcription of the chemokine CXCL13. In addition to NF-κB pathway, NOD1 and NOD2 stimulation results in the activation of MAPK, including p38, ERK, and JNK, through the CARD-containing adaptor protein CARD9 (Girardin *et al.*, 2001; Hsu *et al.*, 2007; Kobayashi *et al.*, 2002; Navas *et al.*, 1999; Pauleau and Murray, 2003). However, when recognizing viral ssRNA genome, NOD2 could trigger the activation of IRF3 and the production of IFN-β (Sabbah *et al.*, 2009). Administration of MDP and iE-DAP, as well as their derivatives have been shown to induce broad activity against multiple pathogens, including secretion of proinflammatory cytokines and chemokines, synthesis of nitric oxide synthase, and also the expression of adhesion molecules, all of which are critical for the innate immune response and potentiate adaptive immune response against pathogens (Inohara *et al.*, 2002; Inohara *et al.*, 2005).

The importance of NOD1 and NOD2 is further confirmed by their genetic association with human inflammatory diseases. The most common frame-shift mutations in NOD2, which result in the loss of the terminal LRR and could not detect MDP, are associated with Crohn's disease, the chronic inflammatory disease of the intestine and gastrointestinal tract (Hugot *et al.*, 2001, Hugot, 2006; Ogura *et al.*, 2001). NOD2 is also implicated in the Blau syndrome, a rare long-life disorder starting in childhood and characterized by skin rashes,

uveitis and recurrent arthritis, which can evolve toward camptodactyly (Miceli-Richard *et al.*, 2001). Genetic variants and single nucleotide polymorphisms of NOD2 that might result in inappropriate immune responses are also associated with atopic disorders. It was shown that the Crohn's disease-associated polymorphisms in NOD2 gene are also significantly associated with an increased risk for atopic diseases and hyper IgE syndrome (Kabesch *et al.*, 2003; Reijmerink *et al.*, 2010; Weidinger *et al.*, 2005). In addition, NOD1 polymorphisms are also associated with the development of atopic eczema, asthma, and increased serum IgE concentrations (Hysi *et al.*, 2005).

Epithelial cells are the first barrier of defence against invading microbial pathogens. Various PRRs are expressed by epithelial cells to recognize conserved microbial patterns and mediate inducible activation of innate immunity. Because of the gas exchange with the outside environment, airway epithelial cells, the body's largest epithelial surface, are almost prone to contacting with airborne microbes (Buc *et al.*, 2009). Bronchial epithelial cells and derived cell line BEAS-2B have been shown to equipped with a variety of PRRs, including almost all the TLR member, NOD1 and NOD2 (Barton *et al.*, 2007; Bérubé *et al.*, 2009; Farkas *et al.*, 2008; Mayer *et al.*, 2007; Sha *et al.*, 2004). In addition, airway epithelial cells could produce various antimicrobial substances, including highly active anti-microbial peptides and inflammatory mediators (Shaykhiev and Bals, 2007). Recently, there is compelling evidence to elaborating the critical functions of epithelial cells-derived TSLP, IL-25 and IL-33 in the initiation, development and regulation of Th2 cytokine-dependent allergic immune responses (Saenz *et al.*, 2008).

A key feature of the inflammatory response in asthma is characterized by intense mucosal inflammation with infiltration and accumulation of activated eosinophils and mononuclear cells in the airways, as well as with epithelial desquamation. Eosinophil-bronchial epithelial cell interactions are thought to be one of central mechanisms for the pathogenesis of asthma, both in terms of the epithelium as a source of

pro-inflammatory mediators and as a target for eosinophil-mediated damage (Sanmugalingham *et al.*, 2000; Walsh, 2001). Epithelial damage in asthma is thought to be primarily mediated by eosinophil-derived basic granule proteins, such as MBP and ECP, which have been shown to be toxic for airway epithelium. Co-culture of IL-5-treated eosinophils and TNF-α-activated bronchial epithelial cell line BEAS-2B resulted in enhanced eosinophil degranulation and adhesion (Takafuji *et al.*, 1996). The interaction of eosinophils and epithelial cells can also result in the induction of inflammatory mediator cysteinyl leukotrienes from eosinophils (Dent *et al.*, 2000). Besides eosinophil-derived mediators for epithelial damage, bronchial epithelium is also an important source of the growth factors and chemoattractants for eosinophils. Epithelium in asthma synthesizes eosinophil growth factors such as GM-CSF, and chemokines such as RANTES and eotaxin, as well as increased amounts of adhesion receptors, particularly ICAM-1 (Sanmugalingham *et al.*, 2000; Walsh, 2001). The interaction between bronchial epithelial cells and eosinophils can up-regulate the survival and activation of human eosinophils and the responsiveness of epithelial cells through the release of cytokines and chemokines and the interaction of adhesion molecules.

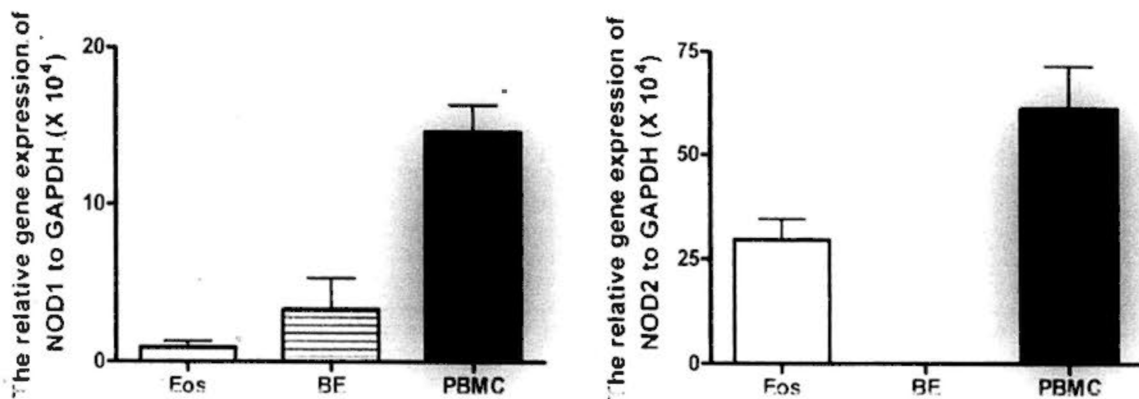
Our group have previously investigated house dust mite allergen *Dermatophagoides pteronyssinus* (Der p) 1 and TLR-mediated activation of eosinophils and bronchial epithelial cells in allergic inflammation (Wong *et al.*, 2006; Wong *et al.*, 2007; Cheung *et al.*, 2008). Although NOD1 and NOD2 polymorphism have been reported to involve in asthma, however, the detailed interaction between eosinophils and bronchial epithelial cells upon NOD1 and NOD2 ligand stimulation in innate immunity and allergic inflammation remain to be elucidated. We therefore have studied the *in vitro* effects of NOD1 and NOD2 ligand in co-culture of human eosinophils and bronchial epithelial cell line BEAS-2B, with a preliminary focus on the aspects of surface adhesion molecule expression, as well as cytokine and chemokine release in the co-culture system.

5.2 Results

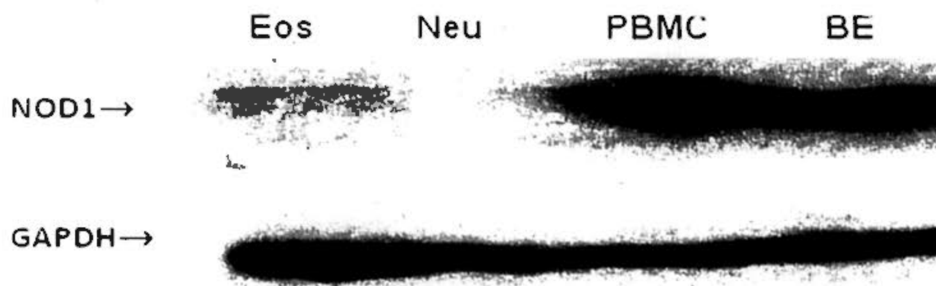
5.2.1 Expression of NOD1 and NOD2 on eosinophils and BEAS-2B cells

The expressions of NOD1 and NOD2 in BEAS-2B cells have been previously described. NOD1 constitutively expressed on BEAS-2B cells (Barton *et al.*, 2007; Slevogt *et al.*, 2007). The expression of NOD2 mRNA and protein was low in unstimulated BEAS-2B cells, but could be elevated by stimulation with the combination of cytokines TNF-α and IFN-γ (Farkas *et al.*, 2008). Then, we further examined NOD1 and NOD2 expression in human primary eosinophils, with BEAS-2B cells and PBMC served as cell controls. Real-time quantitative PCR analysis shows that mRNA of NOD1 and NOD2 were expressed by eosinophils (Figure 5.1A). We further confirmed that NOD1 protein was also constitutively expressed by eosinophils using Western blot (Figure 5.1B). Intracellular staining using flow cytometry showed that NOD2 was constitutively expressed in human eosinophils (Figure 5.1C).

(A)



(B)



(C)

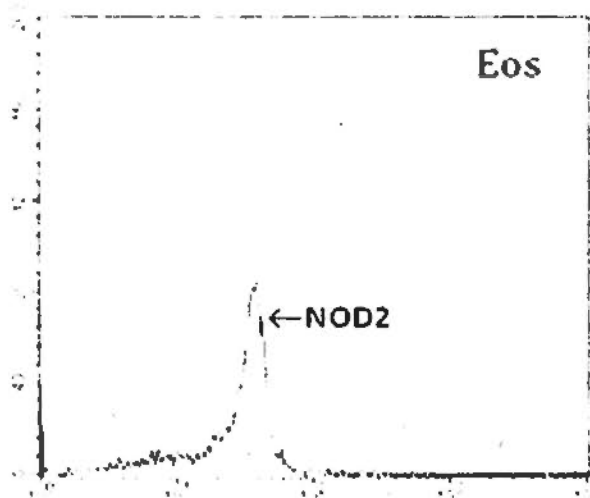


Figure 5.1 Expression of NOD1 and NOD2 in eosinophils, neutrophils, PBMC, and BEAS-2B cells.

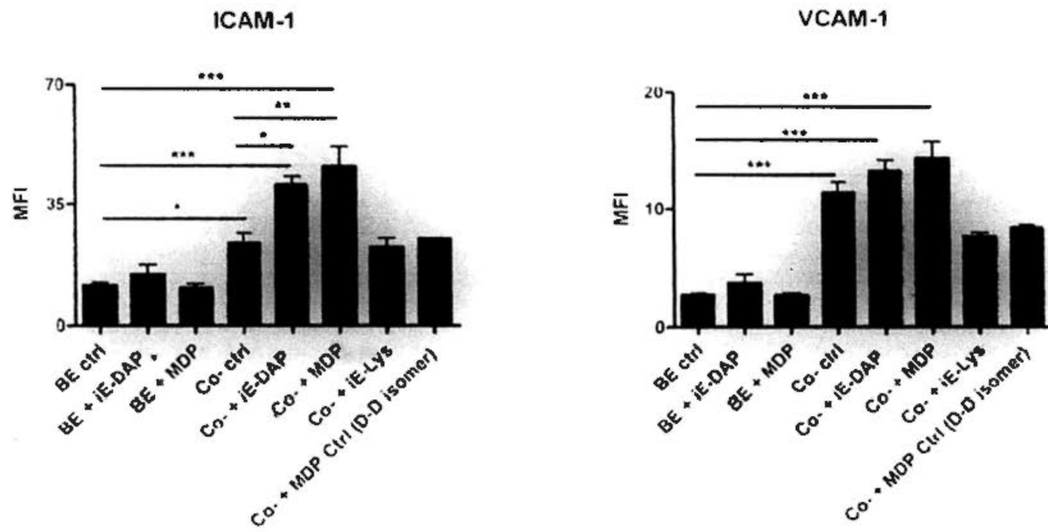
(A) Samples with equal amount of extracted total RNA from different cells were used for qPCR. The relative gene expression was calculated using $2^{\Delta Ct(Ct,NLR-Ct,GAPDH)}$. (B) Samples with equal amount of extracted proteins from different cells were subjected to Western blot for NOD1 expression. Neutrophils, BEAS-2B cells and PBMC were served as controls. (C) Intracellular expression of NOD2 in eosinophils was determined by flow cytometry. All the experiments were performed in triplicates with essentially identical results and representative figure is shown.

Eos: eosinophils; Neu: neutrophils; PBMC: peripheral blood mononuclear cells; BE: BEAS-2B cells.

5.2.2 Effects of NOD1 and NOD2 ligands on the surface expression of adhesion molecules upon the interaction of human eosinophils and BEAS-2B cells

NOD1 ligand iE-DAP (10 μg/ml) stimulation alone had little effects on the surface expression of adhesion molecules on either eosinophils or BEAS-2B cells (Figure 5.2), even with higher dose to 100 μg/ml (data not shown). The similar results of NOD2 ligand MDP (10 μg/ml) stimulation for eosinophils or BEAS-2B cells alone were also observed (Figure 5.2). When co-cultured together without stimulation, the expression of ICAM-1 and VCAM-1 on BEAS-2B cells were significantly enhanced, while no significant changes on the expression of CD18, ICAM-1 and L-selectin on human eosinophils (Figure 5.2). However, upon iE-DAP or MDP stimulation in the co-culture system, the expression of CD18 and ICAM-1 on eosinophils was significantly up-regulated, while L-selectin on eosinophils was markedly down-regulated (Figure 5.2). Moreover, the expression of ICAM-1 and VCAM-1 on BEAS-2B cells was further augmented in the co-culture system with iE-DAP or MDP stimulation (Figure 5.2A). The NOD1 ligand iE-DAP negative control iE-Lys and NOD2 ligand MDP negative control MDP (D-D isomer) showed little effects on the expression of adhesion molecules upon co-culture of eosinophils and BEAS-2B cells.

(A)



(B)

CD18

MFI

30
15
0

***
***
**
**

Eos ctrl
Eos + iE-DAP
Eos + MDP
Co- ctrl
Co- + iE-DAP
Co- + MDP
Co- + iE-Lys
Co- + MDP Ctrl (D-D isomer)

ICAM-1

MFI

20
10
0

**
**
*
*

Eos ctrl
Eos + iE-DAP
Eos + MDP
Co- ctrl
Co- + iE-DAP
Co- + MDP
Co- + iE-Lys
Co- + MDP Ctrl (D-D isomer)

L-selectin

MFI

9
6
3
0

**
**
***
**

Eos ctrl
Eos + iE-DAP
Eos + MDP
Co- ctrl
Co- + iE-DAP
Co- + MDP
Co- + iE-Lys
Co- + MDP Ctrl (D-D isomer)

Figure 5.2 Effect of NOD1 ligand iE-DAP and NOD2 ligand MDP on surface expression of adhesion molecules upon the interaction of human eosinophils and BEAS-2B cells.

Eosinophils (5×10^5 cells) and confluent BEAS-2B (8×10^4 cells) were cultured either together or separately with or without iE-DAP (10 μg/ml), MDP (10 μg/ml), and negative control iE-Lys (10 μg/ml) and MDP Ctrl (D-D isomer) (10 μg/ml) for 16 h. Surface expression of (A) ICAM-1 and VCAM-1 on BEAS-2B cells, and (B) CD18, L-selectin and ICAM-1 on eosinophils was analyzed by flow cytometry and shown as MFI, which was normalized by subtracting appropriate isotypic control and shown as arithmetic mean + SD of three independent experiments.

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

5.2.3 Effects of NOD1 and NOD2 ligands on the cytokine and chemokine release upon the interaction of human eosinophils and BEAS-2B cells

We first screened the cytokine and chemokine release when eosinophils and BEAS-2B cells were cultured either together or separately with or without iE-DAP or MDP treatment using BD CBA Human Inflammation Kit and Human Chemokine Kit. We subsequently confirmed the CBA analysis results using quantitative ELISA assay. NOD1 ligand iE-DAP (10 μg/ml) alone could significantly induce the release of CXCL8 from BEAS-2B cells (Figure 5.3), which was consistent with other report (Bérubé *et al.*, 2009), while NOD2 ligand MDP (10 μg/ml) alone showed little effects on CXCL8 induction from BEAS-2B cells. In addition, iE-DAP (10 μg/ml) or MDP (10 μg/ml) alone exhibited no prominent effects on cytokine and chemokine release from human eosinophils (Figure 5.3). Upon co-culture, the levels of CCL2 and CXCL8 were found to be markedly elevated than those of eosinophils alone or BEAS-2B cells alone (Figure 5.3). Besides, levels of CCL2 and CXCL8 were found to be significantly further enhanced in the co-culture of eosinophils and BEAS-2B cells under the stimulation of iE-DAP (10 μg/ml) or MDP (10 μg/ml). The release of CCL2 and CXCL8 of 24 h culture was higher than those of 16 h. The NOD1 ligand negative control iE-Lys and NOD2 ligand negative control MDP (D-D isomer) showed little effects on the expression of CCL2 and CXCL8 upon co-culture of eosinophils and BEAS-2B cells.

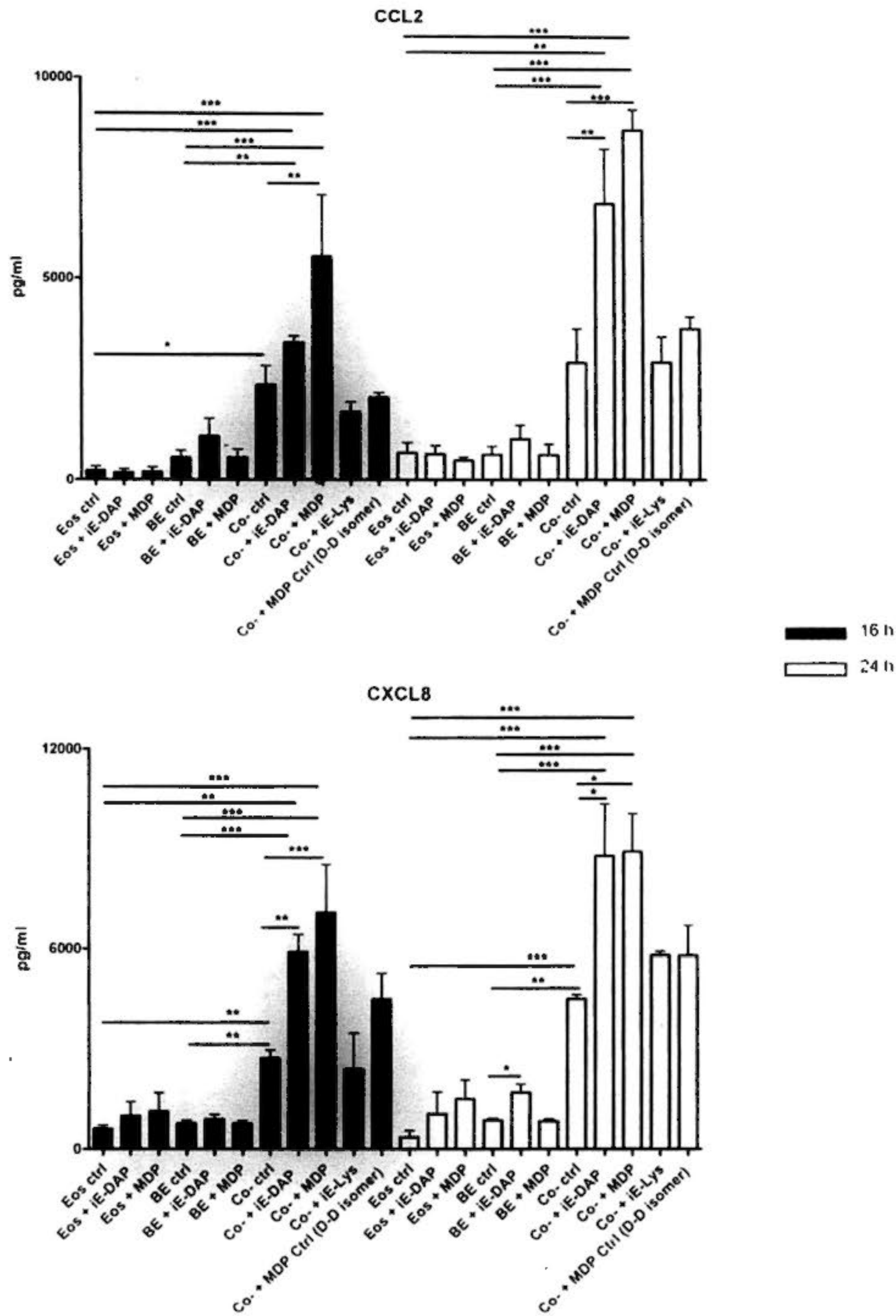

CCL2
pg/ml
10000
5000
0
Eos ctrl
Eos + iE-DAP
Eos + MDP
BE ctrl
BE + iE-DAP
BE + MDP
Co- ctrl
Co- + iE-DAP
Co- + MDP
Co- + iE-Lys
Co- + MDP Ctrl (D-D isomer)
16 h
24 h
CXCL8
12000
6000
0

Figure 5.3 Effect of NOD1 ligand iE-DAP and NOD2 ligand MDP on the release of chemokines CCL2 and CXCL8 upon the interaction of human eosinophils and BEAS-2B cells.

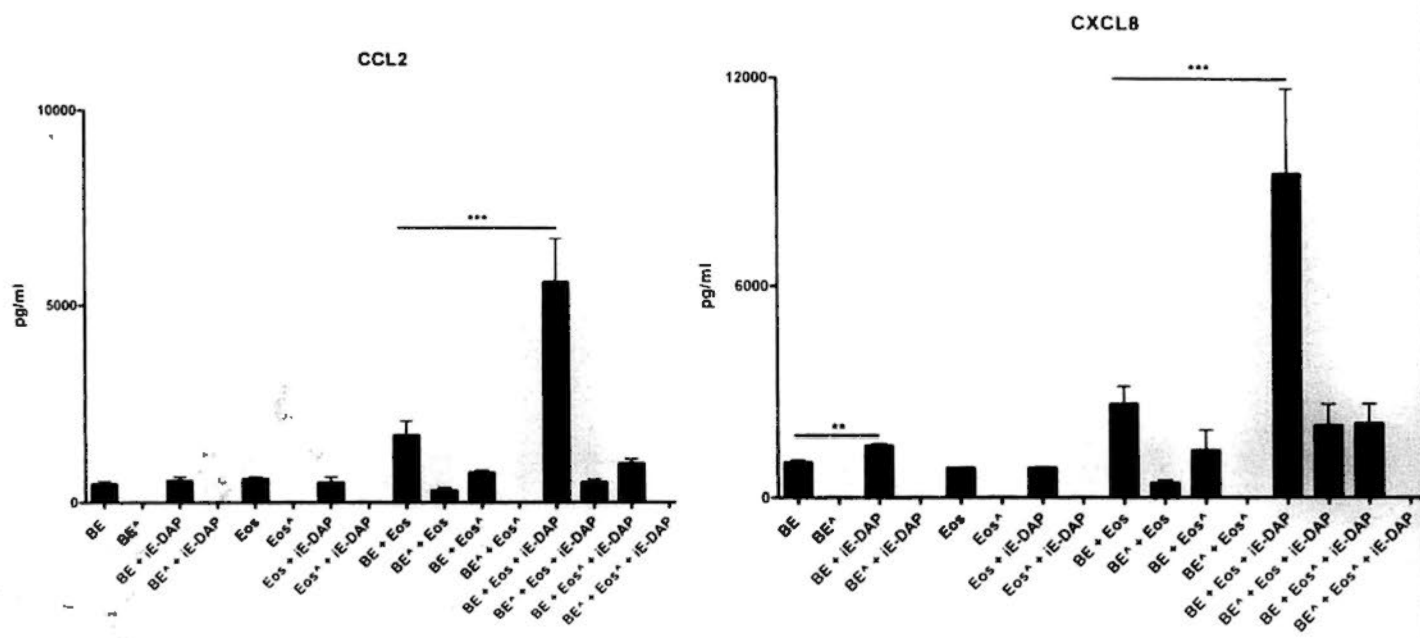
Eosinophils (5×10^5 cells) and confluent BEAS-2B (8×10^4 cells) were cultured either together or separately with or without iE-DAP (10 μg/ml), MDP (10 μg/ml), and negative control iE-Lys (10 μg/ml) and MDP Ctrl (D-D isomer) for 16 h and 24 h. Cell-free culture supernatant was collected, and chemokines CCL2 and CXCL8 released into the supernatant were measured using ELISA. Results are expressed as arithmetic mean + SD of three independent experiments.

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

5.2.4 Source of CCL2 and CXCL8 in co-culture of human eosinophils and BEAS-2B cells upon NOD1 and NOD2 ligand stimulation

Both eosinophils and BEAS-2B cells have the ability to produce CCL2 and CXCL8 (Wong *et al.*, 2006; Wong *et al.*, 2007; Cheung *et al.*, 2008). With a further view to investigate the source(s) of chemokines CCL2 and CXCL8 released in the co-culture system, 1% paraformaldehyde was used to fix eosinophils or BEAS-2B cells to prevent the secretion of cytokines and chemokines, while preserving the cell membrane integrity to maintain the direct intercellular interaction between eosinophils and BEAS-2B cells via the surface adhesion molecules (Lal *et al.*, 1988). We compared CCL2 and CXCL8 release in the co-culture of normal cells with the cells fixed with 1% paraformaldehyde. As shown in Figure 5.4, the co-culture of fixed eosinophils and unfixed BEAS-2B cells, as well as fixed BEAS-2B cells and unfixed eosinophils could almost abrogate the release of chemokines CCL2 and CXCL8 in co-culture system with or without NOD1 ligand iE-DAP or NOD2 ligand MDP stimulation. These results together suggest that both eosinophils and BEAS-2B cells are the sources of CCL2 and CXCL8 in the co-culture system.

(A)



(B)

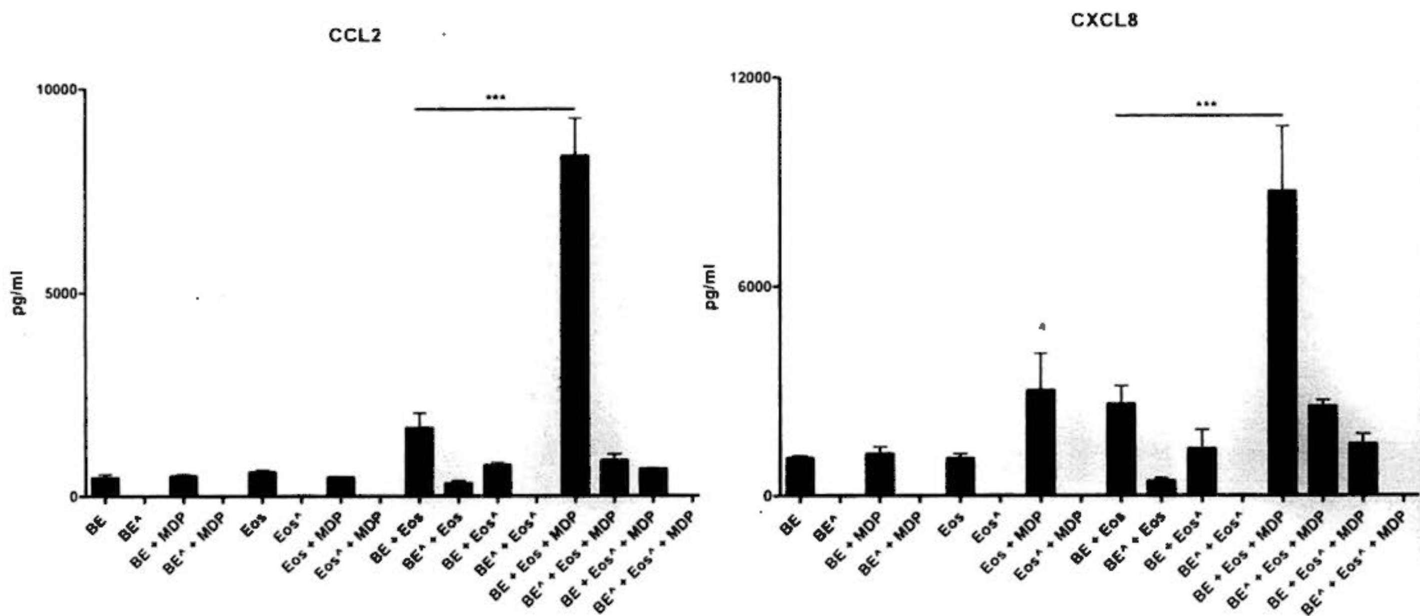


Figure 5.4 Source of CCL2 and CXCL8 in co-culture of human eosinophils and BEAS-2B cells upon NOD1 and NOD2 ligand stimulation.

Eosinophils (5×10^5 cells) and BEAS-2B (8×10^4 cells) were treated with or without 1% paraformaldehyde for 45 min on ice prior to culture together with or without iE-DAP (10 μg/ml) or MDP (10 μg/ml) for 24 h. Cell-free culture supernatant was collected, and CCL2 and CXCL8 released into the supernatant were quantified using ELISA. Results are expressed as arithmetic mean + SD of three independent experiments

Eos: Eosinophils; Eos^: Fixed eosinophils; BE: BEAS-2B cells; BE ^: Fixed BEAS-2B cells.

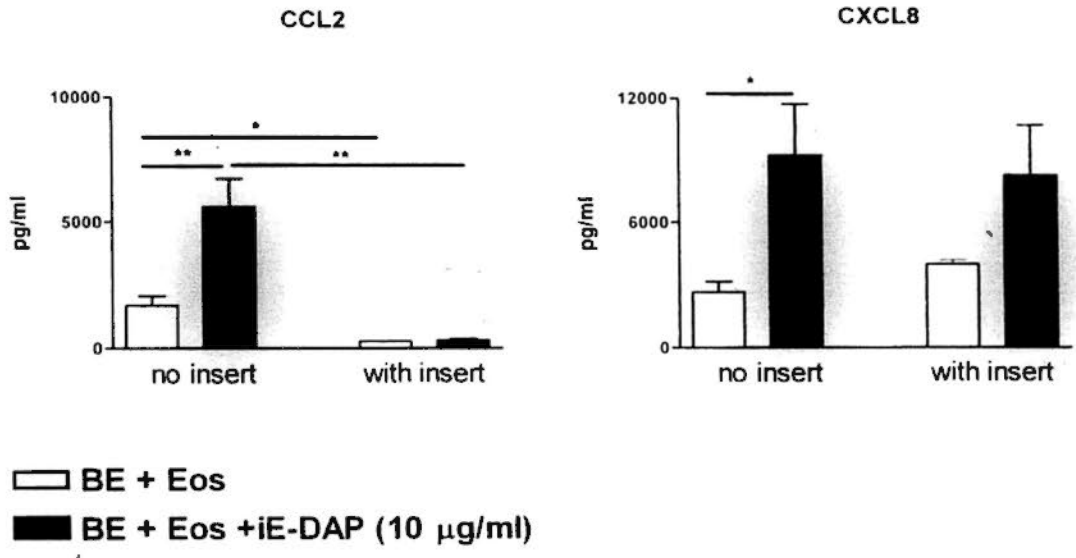
** $p < 0.01$, *** $p < 0.001$ when compared between groups denoted by the horizontal lines.

5.2.5 Effects of transwell insert on the induction of CCL2 and CXCL8 release in co-culture of human eosinophils and BEAS-2B cells upon NOD1 and NOD2 ligand stimulation

To explore whether the direct interaction was essential for the induction of CCL2 and CXCL8 release in the co-culture of eosinophils and BEAS-2B cells upon NOD1 and NOD2 ligands stimulation, transwell insert with pore size of 0.4 μm was used to separate eosinophils and BEAS-2B cells into two compartments in the co-culture system. Intercellular communication through soluble mediators, such as cytokines and chemokines, was allowed in this transwell co-culture system.

Without any stimulation, induction of CCL2 release in co-culture of eosinophils and BEAS-2B cells was totally abolished in the presence of transwell insert, suggesting that CCL2 release in the co-culture system may depend on the direct interaction between eosinophils and BEAS-2B cells (Figure 5.5). Upon iE-DAP or MDP treatment, CCL2 release from the co-culture system was also abrogated using the transwell insert (Figure 5.5). However, the release of CXCL8 in the co-culture of eosinophils and BEAS-2B cells with or without iE-DAP or MDP treatment could not be reversed by the transwell inserts (Figure 5.5), suggesting that soluble mediators may be used for intercellular communication and induction of cytokine and chemokine release.

(A)



(B)

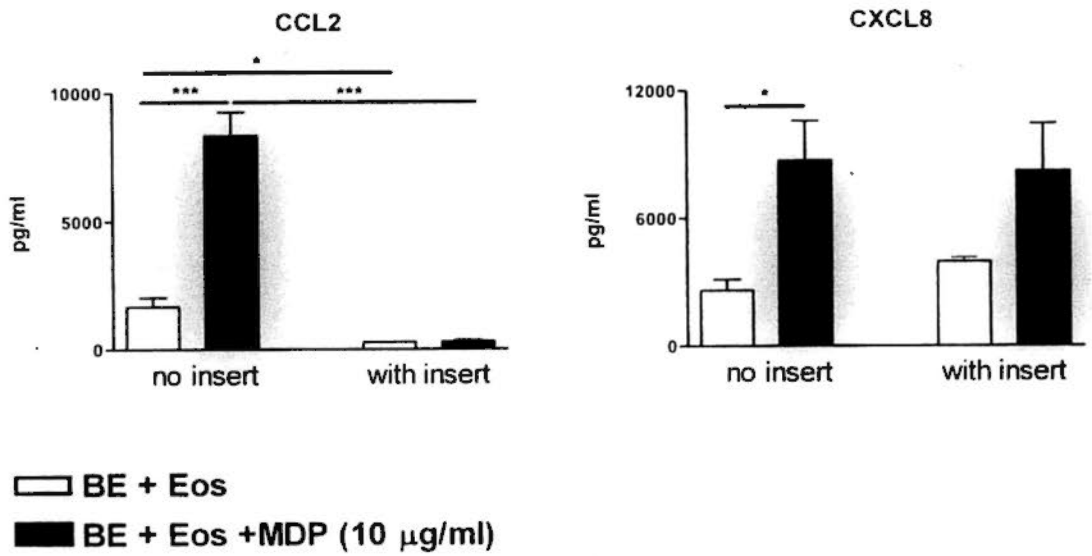


Figure 5.5 Effects of transwell insert on the induction of (A) CCL2 and (B) CXCL8 release in co-culture of eosinophils and BEAS-2B upon NOD1 and NOD2 ligand stimulation.

Eosinophils (5×10^5 cells) and BEAS-2B (8×10^4 cells) were cultured together with iE-DAP (10 μg/ml) or MDP (10 μg/ml) for 24 h in the presence or absence of transwell insert. Cell-free culture supernatant was collected and (A) CCL2 and (B) CXCL8 released into the supernatant were quantified using ELISA. Results are expressed as arithmetic mean + SD of three independent experiments

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

5.3 Discussion

Not only recognizing the conserved domains of bacterial and virus, the important roles of NOD1 and NOD2 as PRR in immune disorders recently have been emphasized. Activation of NOD1 and NOD2 has been shown to involve in Th1 and Th17 cell-mediated diseases, such as Crohn's disease and graft-versus-host disease (Holler *et al.*, 2004; Hugot, 2006). The polymorphisms of NOD1 and NOD2 recently have also been shown to be associated with Th2-mediated atopic diseases, such as allergic asthma (Hysi *et al.*, 2005; Kabesch *et al.*, 2003; Reijmerink *et al.*, 2010; Weidinger *et al.*, 2005). However, the underling mechanisms of NOD1 and NOD2 in atopic diseases have not clearly been characterized. Because of the important role of interaction between eosinophils and bronchial epithelial cells in asthma, we herein investigated the *in vitro* responses upon NOD1 and NOD2 ligand stimulation in co-culture of eosinophils and bronchial epithelial cell line BEAS-2B, which mimic bacterial or virus infection.

NOD1 has been shown to constitutively express in BEAS-2B cells (Barton *et al.*, 2007; Slevogt *et al.*, 2007) and the expression of NOD2 in BEAS-2B cells could be elevated by stimulation with the combination of cytokine TNF-α and IFN-γ (Farkas *et al.*, 2008). We further found that eosinophils also constitutively expressed NOD1 and NOD2. NOD2 ligand MDP exhibit no or weak effects on cytokine and chemokine release in eosinophils or BEAS-2B cells. NOD1 ligand iE-DAP also showed no effects on eosinophils alone but could markedly activate BEAS-2B cells to secrete chemokine CXCL8 and CCL2. Co-culture of eosinophils and BEAS-2B cells could extremely augment the secretion of chemokines CXCL8 and CCL2, which could be further enhanced by iE-DAP or MDP treatment. After fixation of the cells with 1% paraformaldehyde in co-culture system, the results further showed that both eosinophils and BEAS-2B cells were the main source for CXCL8 and CCL2 release in the co-culture system upon iE-DAP or MDP stimulation. Moreover, in the presence of transwell insert, the induction of CCL2 release was almost totally abolished while

the similar amount of CXCL8 was produced upon iE-DAP or MDP treatment in co-culture of eosinophils and BEAS-2B cells, suggesting that distinct mechanisms were involved in CCL2 and CXCL8 release. The direct interaction between eosinophils and BEAS-2B cells is partly responsible for CCL2 release and soluble mediators are implicated in CXCL8 release. However, the cell surface of adhesion molecules for CCL2 induction and soluble mediators for CXCL8 release are still not clear. In further studies, we will use specific antibodies to elucidate the potential targets for these effects.

Eosinophil accumulation in asthmatic airways is a hallmark of allergic diseases and associated with the alteration of adhesion molecules expressed on the surface of eosinophils and their counter ligands (Bue *et al.*, 2009). The immunoglobulin gene superfamily member ICAM-1 is constitutively expressed at relatively low levels on airway epithelial cells or cell lines (Bloemen *et al.*, 1993; Look *et al.*, 1992), while integrin CD18 is constitutively expressed at moderate levels on eosinophils (Cheung *et al.*, 2006), and the role of ICAM-1and CD18 in airway inflammation has been well studied. Our studies found that only upon iE-DAP or MDP stimulation in the co-culture system, the expression of CD18 and ICAM-1 on eosinophils was significantly up-regulated, while L-selectin on eosinophils was markedly down-regulated. Moreover, the expression of ICAM-1 and VCAM-1 on BEAS-2B cells were elevated in co-culture system and further augmented upon iE-DAP or MDP stimulation in the co-culture system. The alteration of CD18 on eosinophils and ICAM-1 on bronchial epithelial cells has been shown to be crucial in allergic inflammation. ICAM-1 on bronchial epithelial cells and BEAS-2B cells could be up-regulated by IFN-γ, TNF-α or IL-1β (Bloemen *et al.*, 1993; Look *et al.*, 1992), which showed increased concentrations in asthmatic BAL fluid (Broide *et al.*, 1992). CD18 expression on eosinophils could be induced by the inflammatory mediators C5a, FMLP, PAF, the chemokines eotaxin and RANTES, as well as phorbol-12-myristate-13-acetate (PMA) (Burke-Gaffney and Hellewell 1998). Further evidence to support the crucial role of ICAM-1 and CD18 in asthma came from *in*

vivo studies. Neutralization antibodies against CD18 or ICAM-1 could inhibit airway eosinophilia in animal models of asthma (Milne and Piper, 1994; Richards *et al.*, 1996; Wegner *et al.*, 1990). There have been results shown that the direct interaction of ICAM-1 and CD18 are implicated in mediating eosinophil adhesion to bronchial epithelial cells (Burke-Gaffney and Hellewell, 1998). RSV-infected type II alveolar epithelial cells (A549) have been shown to support PMA-stimulated eosinophil adhesion via a CD18/ICAM-1-dependent pathway because it could be reduced by blocking epithelial ICAM-1 or eosinophil CD18 (Stark *et al.*, 1996). CD18/ICAM-1 mediated C5a- and PMA-induced eosinophil adhesion to TNF-α/IFN-γ activated-bronchial epithelial cells was CD18/ICAM-1 dependent, but that adhesion to resting bronchial epithelial cells was largely independent of CD18/ICAM-1 (Burke-Gaffney and Hellewell, 1998), which indicated there are other receptors for the adhesion. In addition, TNF-α or IL-1β-treated bronchial epithelia cells significantly enhanced adhesion to PMA-stimulated eosinophils and the adhesion could only be blocked by antibodies to β2 integrins CD18, CD11a, and CD11b, but not antibody to ICAM-1 (Godding *et al.*, 1995), which further indicated other receptors for the β2 integrins. In our studies, VCAM-1 expression could also be induced upon NOD1 and NOD2 ligand stimulation, similar to TNF-α treatment (Atsuta *et al.*, 1997). VACM-1 has been shown to be a receptor for integrin α4β1 and the interaction of both adhesion molecules play an important role in selective eosinophil recuitment from the blood stream into tissue sites (Bochner, 1998). Because of the complicated network and interactions among adhesion molecules and their counter ligands, therefore, we would like to explore the potential molecules involved in the interactions using specific neutralization Abs in the future studies.

NOD1 and NOD2, sharing their structural homology to the apoptosis regulator, Apoptotic protease activating factor 1 (Apaf-1), have been reported to regulate apoptosis. Overexpression of NOD1or NOD2 could promote apoptosis and caspase activation (Geddes *et al.*, 2001; Inohara *et al.*, 1999; Ogura *et al.*, 2001). However, we could not found any

apoptosis effects upon NOD1 or NOD2 ligand stimulation on eosinophils or BEAS-2B cells cultured alone (data not shown). NOD1 and NOD2 proteins could also mediate NF-κB and MAPK activation, which further induce the expression of anti-apoptotic factors, such as A1, c-IAPs, and c-FLIP, and pro-inflammatory factors (Inohara *et al.*, 2003; Inohara *et al.*, 2005; Micheau *et al.*, 2001; Pauleau and Murray, 2003; Wang *et al.*, 1998). NOD1 or NOD2 have no effects on the survival effects of eosinophils and bronchial epithelial cells because the final outcome of cell death was determined by the balance between the NOD-mediated anti-apoptotic power and NOD-mediated activation of pro-apoptotic NF-κB and MAPK pathways.

Although both NOD proteins and TLR belong to PRR and could activate NF-κB and MAPKs, however, NOD proteins act independently of the TLR cascade. RICK is critical in NOD proteins-mediated signaling pathways because NOD1 and NOD2 do not activate NF-κB in RICK-deficient mouse embryo fibroblasts, and the activation is restored upon ectopic expression of RICK in the cells (Chin *et al.*, 2002; Kobayashi *et al.*, 2002). The induction of chemokines CXCL1 and CCL2 upon NOD1 and NOD2 activation was intact in MyD88 deficient mice but abolished in RICK deficient mice (Park *et al.*, 2007). Together, these studies demonstrated that RICK is specifically required for NOD1 and NOD2 signaling, but not for TLR pathways. Although previous studies suggested that RICK is involved in TLR signaling (Kobayashi *et al.*, 2002), however, many preparations of TLR agonists are mixed with impurities with NOD1- and NOD2- agonist activities, which could explain the reduced TLR signaling observed in RICK-deficient macrophages. NOD ligands could synergize with TLR agonists to induce greater amounts of inflammatory cytokines and co-stimulatory molecules (Fritz *et al.*, 2005; Netea *et al.*, 2005; van Heel *et al.*, 2005). The NOD-dependent signaling pathway is far from being fully characterized, and our future studies will therefore focus on the underling signaling transduction in both activated eosinophils and BEAS-2B cells.

Epithelial cells and eosinophils are capable of producing antimicrobial peptides that control bacterial growth. A variety of epithelial cells were reported to produce antimicrobial peptides, including α-defensins, β-defensins and cryptidins, following *in vitro* stimulation by ligands of NOD1 and NOD2 (Boughan *et al.*, 2006; Kobayashi *et al.*, 2005; Sugawara *et al.*, 2006; Uehara *et al.*, 2007). Eosinophil was also shown to produce α-defensins (Driss *et al.*, 2009). Therefore, in the following studies, we also would like to examine whether antimicrobial peptides could be produced in the co-culture system upon NOD1 and NOD2 ligand stimulation.

Not only in innate immune responses, NOD1 and NOD2 have been implicated in the activation of the adaptive immune responses. MDP, totally dependent on the receptor NOD2, has been regarded as a potent adjuvant that drives predominant Th2-responses characterized by the production of isotype IgG1 (Kobayashi *et al.*, 2005). However, the stimulation of DCs with MDP and TLR ligands resulted in Th1-responses via the induction of IL-12 (Kobayashi *et al.*, 2005). Using PGN-NOD2-dependent manner, human DCs upon PGN stimulation were able to secrete IL-23 and IL-1 leading to IL-17 production in memory human CD4+ T cells, while DCs from Crohn's disease patients, with mutations in NOD2 in the LRR domain, are unable to drive Th17 response *in vitro* (van Beelen *et al.*, 2007). Recently, the injection of the antigen OVA together with adjuvant NOD1 agonist FK506 has been shown to induce Th2-biased immunity, similar to Th2 induction by the activation of NOD2 agonist as adjuvant. In contrast, the adjuvant CFA (complete Freund's adjuvant), a mixture of both NOD and TLR ligands, can switch the immune responses to be Th1-biased (Fritz *et al.*, 2007). If synergistically activated by FK156 and TLR4 ligand LPS, the Th17-promoting cytokine IL-23 was observed from DCs and IL-17 release could be detected in splenocytes (Fritz *et al.*, 2007). All the above findings emphasized the important roles of NOD1 and NOD2 in activating and modulating adaptive immune system.

In addition, pattern recognition receptor TLR3 ligand dsRNA could significantly induce the expression of TSLP, a hallmark for initiating and developing allergic diseases as described in Chapter 3 (Kato *et al.*, 2007). The TSLP induction is dependent on NF-κB activation while NOD1 and NOD2 ligand could potentially activate NF-κB (Kato *et al.*, 2007; Lee and Ziegler, 2007). Thus in future studies, we would like to explore whether the stimulation of NOD1 and NOD2, the novel pattern recognition receptors, could possibly induce the expression of TSLP for aggravating allergic responses.

In conclusion, this is the first report on the functions of NOD1 and NOD2 in the interaction between human eosinophils and bronchial epithelial cells, with an initial focus on the aspects of modulation of surface adhesion molecule expression and the stimulation of cytokine and chemokine release. Because of the ubiquitous expression of MDP in both Gram-positive and Gram-negative bacteria and restricted expression of iE-DAP in Gram-positive bacteria, NOD2 could function as a general sensor of most, if not all, bacteria, and NOD1 could detect almost all Gram-negative bacteria. Taken together with previous studies on the potential participation of NOD1 and NOD2 activation in Th2-responses and the polymorphisms of NOD1 and NOD2 with asthma, this study provides further evidence of how NOD1 and NOD2 could be involved in the pathogenesis of asthma. Further investigation of other activation effects and the underlying mechanisms mediated by NOD ligands stimulation may shed light on the development of novel treatment approaches for eosinophil-associated allergic diseases.

Chapter 6
Concluding Remarks and Future Perspectives

6.1 Concluding Remarks

Eosinophils are one of the important effector cells in late reactions of allergic immune responses (Larché *et al.*, 2006). However, a number of recently found stimuli related to atopic diseases still have not been fully clarified for the effects on eosinophils. Our studies here provide new evidence regarding eosinophil activation by TSLP, novel IL-12 family member IL-27, and some microbial products.

TSLP, with similar structure and function to the hematopoietin family cytokines, was originally characterized as a growth factor for the development and proliferation of different subtypes of T cells and B cells (Comeau and Ziegler, 2010). Further studies from both human and animal studies found that TSLP could initiate and maintain allergic inflammation. The effects of TSLP on DCs, mast cells, basophil and NKT cells were also investigated and have implications for Th2-mediated allergic responses. We found that TSLP could directly enhance eosinophil survival, adhesion, and cytokine and chemokine release through ERK, p38MAPK and NF-κB dependent intracellular signaling pathways in eosinophils, which is different from previous studies that TSLP mediated effects through STAT5 or STAT3 dependent pathways in other effector cells. In addition, we found that TLR3 ligand poly I:C has the ability to directly induce TSLP protein secretion from primary epidermal keratinocytes and primary bronchial epithelial cells. Our findings enrich the potential target cells for TSLP, including DCs, mast cells, lymphocytes and granulocytes, as well as their progenitor CD34+ hematopoietic cells,

and give further evidence showing TSLP as attractive target for treatment of allergic diseases (Ziegler and Artis, 2010).

Similar to the findings for TSLP, novel IL-12 family cytokine IL-27 could also activate eosinophils as shown by enhanced survival and adhesion, as well as the production of allergy-related cytokines and chemokines. The intracellular signaling molecules ERK, JNK, p38MAPK and NF-κB were phosphorylated in eosinophils upon IL-27 stimulation and involved in IL-27-mediated cell activation. However, these effects on eosinophils mediated by IL-27 were different from IL-12, which induced eosinophil apoptosis and decreased subsequent tissue eosinophilia (Nutku *et al.*, 2001), but to certain extend similar to the activation effects mediated by IL-23 (Cheung *et al.*, 2008), although the three cytokines belonging to IL-12 family members. In addition, IL-27 exhibited additive effects with TLR4 ligand LPS for the secretion of chemokine CXCL8, that was different from monocytes in which inflammatory activation of IL-27 could be abrogated by LPS (Kalliolias and Ivashkiv, 2008).

The respiratory tract infection has been shown to aggravate allergic asthma (Hashimoto *et al.*, 2008; Johnston, 2007). Bronchial epithelial cells are the first line of defence against exposure to the bacterial and viral infection (Saenz *et al.*, 2008). Eosinophil infiltration and accumulation in the airways and the interaction between eosinophils and bronchial epithelial cells are implicated in the development of asthma (Buc *et al.*, 2009). We found that eosinophils constitutively express cytosolic innate immunity proteins NOD1 and NOD2, belonging to intracellular PRR and recognizing different moieties of bacterial PGN. NOD2 recently was also found to function as a cytoplasmic ssRNA viral PRR (Sabbah *et al.*, 2009). There were little effects of NOD1 ligand iE-DAP on cultured eosinophils alone, and NOD2 ligand MDP on cultured eosinophils or bronchial epithelial cell line BEAS-2B cells alone. However, in the co-culture system of eosinophils and BEAS-2B cells upon iE-DAP and MDP

stimulation, both the two cells significantly changed the expression profiles of adhesion molecules, similar to the expression activated by mediators that were highly expressed in asthma patients (Look *et al.*, 1992; Bloemen *et al.*, 1993), and may facilitate their firm interaction. In addition, large amounts of chemokines were induced in the co-culture system upon iE-DAP and MDP stimulation. Though the study here only showed some pre-screening effects, these results further indicated the inflammatory responses could be induced upon stimulation by iE-DAP and MDP or from the associated bacteria. In the future studies, we will investigate more effects regarding both eosinophils and BEAS-2B cells in the co-culture system upon iE-DAP and MDP stimulation and the underlying mechanisms to explain the potential effects.

Together, all the above observations further emphasized the crucial roles of eosinophils in allergic inflammation, and gave more understanding and insights about the therapeutic approaches to allergic diseases.

6.2 Future Perspectives

Corticosteroids remain the first and available preference of treatment in allergic diseases and most allergic patients respond well to the current therapies (Adcock *et al.*, 2008). However, the treatment is not always completely effective, and associated with side effects and steroid resistance and insensitivity. Because of these limitations, development of new treatments represents a major goal for both the pharmaceutical companies and academic researchers. Whereas normally only accounting for 1 - 3% of circulating leukocytes, the numbers of eosinophils dramatically increase in the peripheral blood and tissues in allergic diseases like allergic asthma, atopic dermatitis and rhinitis. Eosinophilia and eosinophil activation are prominent in the late-phase responses of allergic diseases (Rosenberg *et al.*, 2007; Simon *et al.*, 2004). Noting the potentially important functions of eosinophils in allergic diseases, eosinophils have been

regarded as a novel therapeutic target (Justice *et al.*, 2003; Trivedi and Lloyd, 2007).

IL-5 is a crucial cytokine that regulates the differentiation and development, priming, activation, and survival of eosinophils. Mice deficient in IL-5 have significantly reduced levels of eosinophils (Cho *et al.*, 2004). In OVA-induced murine asthma models, anti-IL-5 antibodies could abolish eosinophilia and AHR (Hamelmann and Gelfand, 2001). However, when treating soluble egg antigen-established airway disease, anti-IL-5 antibody only reduced eosinophilia but had no effect on AHR (Mathur *et al.*, 1999). For human subjects, anti-IL-5 mAb Mebolizumab/SB-240563 (Glaxo SmithKline) prevented the maturation of eosinophils in the bone marrow and significantly reduced the number of eosinophil progenitors in the bronchial mucosa (Menzies-Gow *et al.*, 2003). A single dose of Mebolizumab reduced eosinophils in blood and sputum, but no effects on the late asthmatic responses (Leckie *et al.*, 2000). Additional study suggests that Mebolizumab could ameliorate airway remodelling as shown by the reduction of the deposition of tenascin, lumican and procollagen III, and the percentage of tissue eosinophils expressing TGF-β mRNA (Flood-Page *et al.*, 2003). The administration of another anti-IL-5 mAb, SCH55700 (Schering-Plough Research Institute), to patients with severe persistent asthma showed a long-lasting reduction in blood eosinophils and benefit in improving FEV1 (Forced expiratory volume in one second), but not in any of the other clinical outcomes. Further studies in large-scale clinical study will allow the evaluation of the efficacy of this treatment modality in asthma and other eosinophilic disorders.

CCR3 is the one of principle chemokine receptors binding eotaxin and RANTES for eosinophil accumulation into inflamed tissues. In murine asthma model both anti-CCR3 mAb and low-molecular-weight CCR3 antagonist resulted in a marked reduction of eosinophils in the bronchoalveolar lumen and airway wall tissue, reduction of AHR and prevention of airway remodeling (Wegmann *et al.*, 2007). For human

studies, one of CCR3 antagonists DPC168, has shown therapeutic effects for asthma, allergic rhinitis and atopic dermatitis in Phase I clinical trials (De Lucca *et al.*, 2005), but it was recently found to potently inhibit cytochrome P450 (CYP2D6) and hERG (human ether-related gene) (Pruitt *et al.*, 2007). However, therapies targeting CCR3 may be more beneficial in the treatment of asthma because CCR3 is also expressed on Th2 cells and mast cells, both of which also play important roles in the pathogenesis of asthma. Many potential Abs and antagonists for allergic inflammation are under evaluated and tested in clinical trials.

Allergic inflammation is complex and involves a variety of effector cells and mediators. Besides eosinophils, drug development for allergic diseases also include targeting Th2 cell and mast cell-induced airway inflammatory responses, as well as IgE, allergy-related inflammatory cytokines, chemokines and their receptors as therapeutic resolution (Holgate and Polosa, 2008).

In addition, recent evaluation of signal transduction mechanisms involved in the pathogenesis of allergic inflammation could enrich our understanding on the underlying physiological and pathological processes and help to find novel targets. Different from inflammatory mediators, intracellular signaling molecules can activate specific downstream transcription factors and are less functional redundant. This enables inhibition of cellular activation induced by multiple mediators via the blockage of one common signaling molecule (Gorska and Alam, 2003). The enhanced activation of ERK, JNK, p38 MAPK, NF-κB and AP1 signaling pathway have been proposed a role in allergic asthma (Adcock and Lane, 2003; Duan *et al.*, 2004; Duan and Wong, 2006; Ito *et al.*, 2006; Li *et al.*, 2004; Tsitoura and Rothman 2004;). Selective inhibitors specific for these pathways are under development with the intention of dampening the allergic inflammatory responses (Adcock *et al.*, 2006; Adcock *et al.*, 2008). PI3K regulate cell growth, activation, apoptosis and survival, and may contribute to the

pathogenesis of asthma by affecting airway smooth muscle proliferation and eosinophil recruitment, while ERK pathway is activated upon ligation of TCR in T cells, B cell receptor in B cells, FcεRI in mast cells, and IL-5R or eotaxin in eosinophils, leading to proliferation, differentiation, cytokine and chemokine production, degranulation and chemotaxis (Adcock *et al.*, 2008; Boehme *et al.*, 1999; Finan and Thomas, 2004; Ito *et al.*, 2007; Pazdrak *et al.*, 1998). Intratracheal administration of PI3K inhibitor LY294002 or ERK inhibitor U0126 in OVA-induced murine asthma models could significantly reduce antigen-induced airway eosinophilia, secretion of IL-4, IL-5, IL-13, and eotaxin in bronchoalveolar lavage fluid, goblet cell hyperplasia, IgE and mucus production, and airway hyperresponsiveness (Duan *et al.*, 2004; Duan *et al.*, 2005). Activation of p38 MAPK is a hallmark of allergic diseases and involves in many inflammatory processes. p38 MAPK inhibitor SB203580 was shown to reduce TNF-α production in OVA-challenged rat model of asthma (Escott *et al.*, 2000) and the p38 MAPK antisense oligonucleotide ISIS 101757 could significantly reduce OVA-induced pulmonary eosinophilia, mucus hypersecretion, and AHR in murine asthma model (Duan *et al.*, 2005). JNK activity is increased in corticosteroid-resistant bronchial asthma and JNK inhibitor SP600125 could significantly reduce eosinophil and lymphocyte numbers in BAL fluid and smooth muscle proliferation after repeated allergen exposure in acute and chronic animal models of asthma (Nath *et al.*, 2005; Eynott *et al.*, 2003; Sousa *et al.*, 1999). Transcription factors NF-κB and activator protein (AP)-1 also orchestrate and activate in the airway inflammation of asthma. Numerous inhibitors for NF-κB and AP-1 currently include small molecule inhibitors specific for IKK-β and AP1, and DNA oligonucleotides and DNA-peptide molecules that act as the decoy sequences (Caramori *et al.*, 2008), and all these inhibitors have either shown effective in animal models or clinical trials with promising results. In fact, a large number of commonly used anti-inflammatory drugs, including non-steroidal

anti-inflammatory drugs and glucocorticoids, have been shown *in vitro* to inhibit the activation of these signaling pathways (Vallabhapurapu and Karin, 2009; Gorska and Alam, 2003). Our studies regarding the underlying signaling activation in eosinophils further confirmed the potential roles and effects in allergic therapy.

In conclusion, treatments for allergic diseases are now under development and certain breakthrough has been achieved. Understanding eosinophil activation and the underlying mechanisms based on our results would contribute to elucidate the complicated features of allergic inflammation and be helpful to develop novel targets and strategies for allergic diseases.

Appendix

RayBio® Human Cytokine Antibody Array V Map

	A	B	C	D	E	F	G	H	I	J	K
1	Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	G-CSF	GM-CSF	GRO	GRO-α
2	I-309	IL-1α	IL-1β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
3	IL-12p40p70	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1β
4	MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β1	TNF-α	TNF-β	EGF	IGF-1	Angiogenin
5	Oncostatin M	Thrombopoietin	VEGF	PDGF-BB	Leptin	BDNF	BLC	Ckβ8-1	Eotaxin	Eotaxin-2	Eotaxin-3
6	FGF-4	FGF-6	FGF-7	FGF-9	Flt-3 ligand	Fractalkine	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3α	NAP-2	NT-3
8	NT-4	Osteopontin	PARC	PIGF	TGF-β2	TGF-β3	TIMP-1	TIMP-2	Neg	Pos	Pos

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