Molecular Regulation and Effector Functions of the High Affinity IgE Receptor (FcεRI) in Human Airway Smooth Muscle Cells

By

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THESIS ABSTRACT

The prevalence and economic burden of chronic airway disorders such as asthma is on the rise annually. Allergic asthma is characterized by chronic airway inflammation, airway hyper-responsiveness (AHR), and structural airway remodeling due to increased smooth muscle mass. Most allergic asthma occurs due to the overproduction of immunoglobulin E (IgE) antibodies against common allergens. Classically, IgE has been shown to modulate airway smooth muscle (ASM) contraction/relaxation which is believed to be the underlying cause of airway hyperreactivity. However, the molecular mechanisms underlying IgE effects on ASM cell are not established.

Recently, the high-affinity Fc receptor for IgE (FcεRI) has been identified in human ASM cells *in vitro* and *in vivo* within bronchial biopsies of allergic asthma patients. However, it is unknown whether FcεRI activation on ASM can modulate the immune response within the airways. We hypothesized that the IgE-FcεRI interaction plays a key role in inducing phenotypic and functional changes in ASM cells that eventually contributes to the establishment of airway inflammation, AHR, and remodeling. We sought to investigate the regulation, effector functions, and underlying mechanisms of FcεRI activation in ASM cells. Our work shows that the proinflammatory tumor necrosis factor (TNF) and T helper type 2 (Th2) cytokine interleukin (IL)-4 enhanced the FcεRI abundance and amplified the IgE-induced chemokine (eotaxin-1/CCL11, RANTES/CCL5, IL-8/CXCL8, and IP-10/CXCL10) release in ASM cells via transcriptional mechanisms. Both TNF and IgE induced a novel, Th2-favoring cytokine thymic stromal lymphopoietin (TSLP) through the activation of spleen tyrosine kinase (Syk), and nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1). In

addition, IgE induced *de novo* DNA synthesis and ASM cell proliferation via mitogen-activated protein kinases (MAPKs) and signal-transducer and activator of transcription 3 (STAT3) activation. Collectively, our data suggest that the IgE-induced FcεRI activation leads to the expression of multiple chemokines in ASM which may indirectly recruit inflammatory cells and promote allergic airway inflammation; IgE induces TSLP which can promote the Th2 immune responses within the airways; and IgE may potentially induce airway remodeling by directly inducing ASM cell proliferation. Therefore, targeting the IgE-FcεRI network on ASM may offer a novel therapeutic strategy in allergic asthma.

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If I have forgotten anyone in this journey, I request their forgiveness for my shortsightedness.

DEDICATION

I dedicate this work to my parents and Uncle Shri Rajbir Singh Redhu.

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LIST OF ABBREVIATIONS

([Ca2+]I)	Intracellular calcium concentration
15-HETE	15-hydroxyeicosatetraenoic acid
Ach	acetylcholine
ADAM33	a disintegrin and metalloproteinase domain- containing protein 33
AHR	airway hyperresponsiveness
AID	activation induced cytidine deaminase
AP-1	activator like protein-1
APC	antigen presenting cell
ASM	airway smooth muscle
α-SMA	alpha-smooth muscle actin
ASMC	airway smooth muscle cell
B/TSM	bronchial/tracheal smooth muscle
β2-AR	beta 2- adrenergic receptor
BAF	B cell activating factor
BAL	bronchoalveolar lavage fluid
BCL10	B cell CLL/lymphoma-2
BHR	bronchial hyperresponsiveness
BMMC	bone marrow derived mast cell
BSM	bronchial smooth muscle
BTK	Bruton's tyrosine kinase
C/EBPα	CCAAT/enhancer binding protein-α
Ca2+	intracellular calcium concentration
CADM1	cell adhesion molecule 1
CAM	cell adhesion molecule
cAMP	cyclic adenosine monophosphate
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD40	cluster of differentiation 40
CD40L	CD40 ligand
c-DNA	complementary deoxyribonucleic acid
CH	constant heavy region
COPD	chronic obstructive pulmonary disease
COX-2	cycloxygenase 2
CpG	cytosine phosphate guanine
CRAC-1	calcium release activated channel 1
CSR	class switch recombination
CTLA4	cytotoxic T lymphocyte antigen 4
CXCL	CXC chemokine ligand

DAG	diacyl glycerol
DC	dendritic cell
DMEM	Dulbecco's Modified Eagles Medium
DNA	deoxyribonucleic acid
dnIKKβ	dominant negative inhibitor of kappa B kinase beta
DNP	dinitrophenol
ε-BP	epsilon binding protein
EC	epithelial cell
ECL	electrochemiluminiscence
ECM	extracellular matrix
ECP	eosinophilic cationic protein
EDN	eosinophil derived neurotoxin
EDTA	ethylenediamine tetra acetic acid
EFS	electric field simulation
ELISA	enzyme linked immunosorbant assay
EMSA	electrophoretic mobility shift assay
EMT	epithelial mesenchymal transition
EPO	eosinophil peroxidase
ERK	extracellular signal regulated kinase
Fab	antigen binding fragment
FACS	flow assisted cytometry analysis
FBS	fetal bovine serum
Fc	crystallizable fragment
FcεRI	high affinity IgE receptor
FcεRII/CD23	low affinity IgE receptor
FGF	fibroblast growth factor
FOXP3	forkhead box protein 3
FRET	fluorescence resonance energy transfer
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GINA	global initiative for asthma
GLT	germline transcript
GM-CSF	granulocyte monocyte colony stimulating factor
GPCR	G-protein coupled receptor
GRB2	growth factor receptor-bound protein 2
GRO-α	growth regulated oncogene alpha
GSK-3	glycogen synthase kinase 3
GTPases	guanine triphosphate hydrolase enzyme
GWAS	genome wide association studies
HASM	human airway smooth muscle

HASMC	HASM cell
HAT	histone acetyl transferase
HBEC	human bronchial epithelial cell
HC-IgE	highly cytokinergic IgE
HEK293T	human embryonic kidney 293 T cell
HLAG	human leukocyte antigen G
HRP	horseradish peroxidase
hTERT	human telomerase reverse transcriptase
ICAM-1	intercellular adhesion molecule-1
IDDM	Iscove's modified Dulbecco's medium
IDO	indole 2,3-dioxygenase
IDV	integrated density value
IFN-γ	interferon gamma
Ig	immunoglobulin
IGF	insulin-like growth factor
IL	interleukin
IP-10	interferon-γ-inducible protein 10
IP3	inositol 3-phosphate
ISO	isoproterenol
ITAM	immunoreceptor tyrosine based activation motif
ITS	insulin transferrin selenium
JAK	janus activated kinase
JNK	c-Jun NH2-terminal kinase
KDa	kilo Dalton
LIF	leukemia inhibitory factor
LPR	late phase response
LTB4	leukotriene B4
MAPK	mitogen activated protein kinase
MBP	major basic protein
MC	mast cell
MCP-1	monocyte chemotactic protein 1
MDC	macrophage derived chemokine
MHC	major histocompatibility complex
MLCK	myosin light chain kinase
MMP	matrix metalloproteinase
MOI	multiplicity of infection
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
NFAT	nuclear factor of activated T cells

NF-κB	nuclear factor kappa B
NO	nitric oxide
NOS	nitric oxide synthase
NTAL	non-T cell activation linker
PAF	platelet activating factor
PAGE	polyacrylamide gel electrophoresis
PAR-2	protease activated receptor-2
PBMC	peripheral blood mononuclear cells
PC-IGE	poorly cytokinergic IgE
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PEFR	peak expiratory flow rate
PFU	plaque forming units
PGD2	prostaglandin D2
PHF11	PHD finger protein 11
PI3K	phosphatidylinositol-3 kinase
PKA	protein kinase A
PKB	protein kinase B
PLC-γ	phospholipase C gamma
PMN	polymorphonuclear neutrophils
PSG	penicillin streptomycin glutamate
PVDF	polyvinylidene fluoride
RANTES	regulated upon activation, normal T cell expressed, and secreted
RNA	ribonucleic acid
ROS	reactive oxygen species
RSV	respiratory syncytial virus
RT	reverse transcriptase
RTK	receptor tyrosine kinase
SCF	stem cell factor
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SH2	Src homology 2
shRNA	short hairpin RNA
SLP76	SH2-containing leukocyte protein of 76 kDa
SM22	smooth muscle 22
SNP	single nucleotide polymorphisms
STAT3	signal transducer and activator of transcription 3
STIM-1	stromal interaction molecule 1
SYK	spleen tyrosine kinase

TARC	thymus and activation regulated chemokine
TCR	T cell receptor
TF	transcription factor
TGF-β	tumor growth factor beta
t-GFP	turbo green fluorescent protein
Th2	T helper type 2
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
Treg	regulatory T cell
TRPC3	transient receptor potential channel 3
TSLC-1	tumor suppressor in lung cancer 1
TSLP	thymic stromal lymphopoietin
TSLPR	thymic stromal lymphopoietin receptor
TSM	tracheal smooth muscle
TXA2	thromboxane A2
VAV1	vav1 oncogene
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
Vmax	maximum shortening velocity
WB	Western blot
WHO	World Health Organization

1.0. CHAPTER 1

INTRODUCTION

1.1.0. ASTHMA: A HETEROGENEOUS AIRWAY DISEASE

The prevalence and economic burden of chronic airway diseases such as asthma and chronic obstructive pulmonary disease (COPD) is on the rise annually, particularly in the developed countries. Asthma is one of the most chronic conditions in Western countries, although the global prevalence ranges from 1-18% of the population in different countries. An estimated 8.3% of Canadians over the age of 12 and about 15.6% of children aged 4–11 years have physician-diagnosed asthma (1). With an estimated global burden of 300 million people, asthma is responsible each year for 250,000 premature deaths, as well as 20 million lost working days (2). Asthma is the leading factor behind school absenteeism and is the third leading cause of loss in working days. Moreover, the direct cost of asthma in Canada is above $600 million each year, and the cost of public health care due to asthma is growing every year (3). Major efforts are therefore required to understand the mechanisms of this devastating chronic airway disease.

The Global Initiative for Asthma (GINA) document defines asthma as “a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness and coughing particularly at night or in the early morning. These episodes are usually associated with wide spread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment” (4). Factors that influence the risk of asthma can be divided into those that lead to the development of asthma and those that can trigger symptoms while some may do both. In general, the former include host factors (primarily the genetic) and the latter are usually environmental factors. Therapeutically,

inhaled corticosteroids, leukotriene modifiers, and β2 agonists are the primary controllers. However, at least about 10% of asthma patients have poorly controlled asthma and require further interventions. Recently, a novel biologic agent anti-IgE monoclonal antibody 'Omalizumab/Xolair™' has been introduced with proven efficacy in severe asthma. Another recently approved therapy is 'bronchial thermoplasty', which involves the delivery of radiofrequency energy to the airways to reduce the airway smooth muscle mass. Despite these therapies with promising outcomes in asthma patients, multiple adverse effects warrant further exploration of asthma therapeutics (5).

1.2.0. MECHANISMS OF ASTHMA PATHOPHYSIOLOGY

Asthma manifests in various phenotypes and can be induced by viral infections, exercise, occupational agents, tobacco smoke and pollutants, irritants, weather and allergens (6). Asthma is particularly complex in childhood since various components of the immune system such as antigen presentation, T cell function and antibody production are immature and therefore may facilitate atopic responses (7). "Atopy is a personal and/or familial tendency, usually in childhood or adolescence, to become sensitized and produce IgE antibodies in response to ordinary exposures to allergens, usually proteins. As a consequence, these persons can develop typical symptoms of asthma, rhinoconjunctivitis, or eczema" (8). Asthma resulting from immunological reactions is referred to as allergic asthma and is mostly initiated by IgE antibodies. Eighty percent of childhood asthma and >50% of adult asthma is reported to be allergic (8, 9). The mechanisms initiating the 'non-allergic' asthma are poorly defined, though similar inflammatory responses occur in both forms of asthma. The most important link between atopy and asthma is the development of persistent inflammation in the airway wall leading to

major structural changes collectively termed as 'airway remodeling' which is responsible for many of the asthma symptoms. When atopic asthmatics are challenged with a specific allergen (to which they are sensitized), they have a biphasic response **(Fig. 1.2.1).** The first, early or immediate phase occurs within minutes while the late phase response appears after about 6-8 hours of allergen challenge.

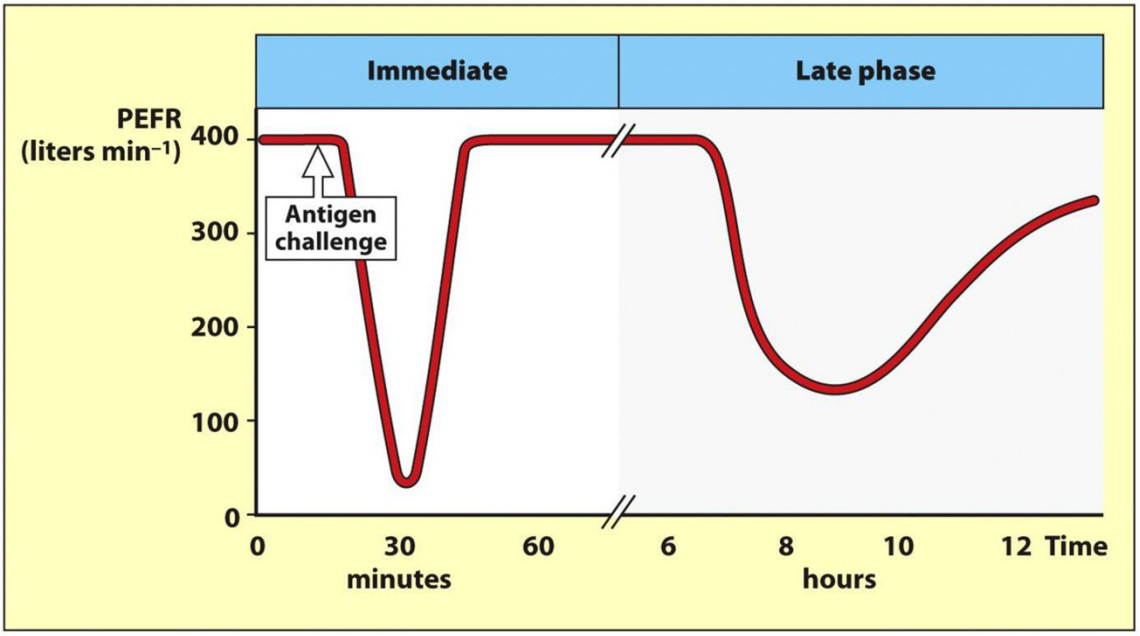

Fig. 1.2.1: Allergic reactions can be divided into an immediate response and a late-phase response. An asthmatic response in the lungs with narrowing of the airways caused by the constriction of bronchial smooth muscle can be measured as a fall in the peak expiratory flow rate (PEFR). The immediate response peaks within minutes after antigen inhalation and then subsides. Approximately 8 hours after antigen challenge, there is a late phase response that also results in a fall in the PEFR. The immediate response is caused by the direct effects on blood vessels and smooth muscle of rapidly metabolized mediators such as histamine and lipid mediators released by mast cells. The late phase response is caused by the effects of an influx of inflammatory leukocytes attracted by chemokines and other mediators released by mast cells during and after the immediate response (10).

1.2.1. Immunoglobulin E (IgE) mediated mechanisms: Immediate hypersensitivity

IgE is the principal mediator in allergic reactions such as seasonal rhinitis, urticaria,

allergic asthma, and anaphylaxis (11). IgE is overproduced in allergic individuals in response to pollens (trees, grasses and weeds), dust mites, pet dander, occupational substances and, sometimes foods. Both IgE and mast cells are heavily concentrated in mucosal tissues (12). Upon binding of allergens to IgE bound to its high affinity Fc receptor (FcεRI), the mast cell is activated and immediately releases the substances from their pre-stored granules, histamine being the most important among others (13, 14). This sets the clinical manifestations of the so-called 'early phase', which includes increased mucus secretion, vasodilation, stimulation of the nerve ends and bronchospasm. In addition to the release of preformed mediators, activated mast cells also begin to synthesize other inflammatory mediators and cytokines such as leukotrienes, and interleukin (IL)-4 and IL-5. This process occurs rapidly after the antigen encounter of immune system and is therefore called as 'immediate hypersensitivity' or 'type I hypersensitivity'(13). Some of the released mediators also promote the local recruitment and activation of leukocytes, orchestrating the development of 'late-phase reactions' (LPR) **(Fig. 1.2.1)**. LPR often develops after 2–6 h and peaks 6–9 h after allergen exposure. LPR in lungs is characterized by airway narrowing and mucus hypersecretion. It also reflects the local recruitment and activation of T helper type 2 (Th2) cells, eosinophils, basophils and other leukocytes, and persistent mediator production by resident cells (such as mast cells) (13).

1.2.2. Th1/Th2 hypothesis

Asthma pathogenesis is credited largely to an abnormal T cell response, particularly to the imbalance in Th1/Th2 immune response. In 1986, Mosmann and Coffman suggested that the CD4+ T cells can be categorized into T helper type 1 (Th1) and Th2 subsets based on their secretion of cytokines upon stimulation (15). Th1 cells were described as those which make

interferon-γ (IFN-γ) as their hallmark cytokine, whereas the Th2 were classified based on their production of IL-4, IL-5 and IL-13. Subsequent to this landmark dichotomy, considerable efforts were made to discover the cytokine signaling pathways and transcription factors that aid in differentiation of the Th1/Th2 lineage. Eventually, two major transcription factors, T box expressed in T cells (T-bet) and GATA-3 were recognized as the master regulators of the Th1 and Th2 differentiation programs, respectively (16, 17). In the differentiation of Th1 cells, IL-12 (produced by macrophages, dendritic cells) is known to signal via signal transducer and activator of transcription (STAT)-4 pathway, which increases the t-bet expression and eventually the IFN-γ production (16). In case of Th2 differentiation, IL-4 acts via STAT6 signaling pathway leading to the activation of GATA-3 transcription factor, necessary for Th2 cytokines production (17).

Early detection of increased number of Th2 cells in asthmatic airways categorized asthma as a Th2 cell-driven disease (18, 19). Further studies in humans and animal models provided evidence that Th2 cells, through the production of IL-4, -5, and -13 cytokines, can instigate and maintain key pathophysiological features of asthma (20, 21). While IL-4 is pivotal in allergic sensitization and IgE production, and IL-5 is crucial for eosinophil survival, IL-13 has numerous effects, including a central role in the development of bronchial or airway hyperresponsiveness (BHR or AHR) and tissue remodeling (22). Notably, Th1 cells may be expected to have an inhibitory role in asthma as they inhibit the development of Th2 cells (23). Moreover, the deletion of t-bet transcription factor in mice leads to the development of spontaneous AHR and IL-13-dependent eosinophilia (24). However, recent cluster analysis of asthma phenotypes shows that the eosinophilic and non-eosinophilic asthma subtypes exist, suggesting that pathways other than just Th2 may be involved in some patients (19, 25). Some of the recently discovered Th cell phenotypes with potential role in allergic asthma include Th9, Th17, Th22, and Treg (regulatory

T cells) (19).

1.2.3. Other factors- Genetics/ Environment

Genetic susceptibility is one of the most prominent factors in determining the development of asthma. Asthma is known to run strongly in families and has a heritability of up to 60% (26-28). As such the genetics of asthma is extremely complex and does not follow simple Mendelian inheritance. Several candidate genes are linked to the development of allergic diseases, and are studied mainly by assessing the single nucleotide polymorphisms (SNPs) in controls versus disease cases. In addition to the candidate gene approaches (dealing with a single gene), genome-wide association studies (GWAS) have gained popularity in discovering novel genes and pathways involved in disease pathogenesis. Approximately 20 genome-wide linkage studies in different populations have investigated the chromosomal regions that are linked to asthma and atopy, or relevant phenotypes such as elevated serum IgE levels, wheezing, and bronchial hyperresponsiveness (29). Multiple chromosome regions have been identified the genes of strong biological association with allergic asthma. This includes the cytokine clusters on chromosome 5q containing the genes for IL-3, IL-5, granulocyte/ macrophage colony stimulating factor (GM-CSF); FCER1B on chromosome 11q; IFNG and STAT6 on 12q; and IL-4R (the IL-4Rα chain, also part of the IL-13R) on chromosome 16p. Linkage studies assisted with positional cloning approaches have resulted in the identification of some novel asthma susceptibility genes, including CYIP2, DPP10, HLAG, PHF11, GPRA, and ADAM33 (29). In particular, GPRA (G protein coupled receptor for asthma) and ADAM33 (a disintegrin and metalloproteinase domain-containing protein 33) have generated substantial interest since their validated expression in smooth muscle cells suggests a convicing role in the pathophysiology of asthma and allergic

airway disease (30).

Besides genetic component, environmental factors are considered critical in affecting the development and outcome of allergic asthma. Several hypotheses have been put forward to explain the role environment plays in allergic disease pathogenesis. One of the most popular and reliable among these is the "hygiene hypothesis" which asserts that the microbiologic factors may be inherently involved in suppression of the asthmatic phenotype, with childhood exposure to microbiologic stimuli bestowing a protective effect against the development of atopy and subsequent asthma (31, 32). The hypothesis suggests that infection in early life stimulates a Th1 immunologic response, rather than the Th2 response associated with allergy and asthma. The initial exposure to microorganisms occurs in the birth canal during normal vaginal delivery. Hygiene hypothesis has got credence from a recent Dutch birth cohort study which suggests an increased occurrence of asthma in children by 8 yearof age s delivered by caesarean section (lack of microbial exposure which occurs in vaginal delivery) (33). Other studies suggest that a rural or farm upbringing has a protective effect on later development of allergy (34). In summary, both environment and genetic factors coalesce together to contribute to the initiation and/or progression of allergic disease.

1.3.0. CELLULAR COMPONENTS OF ALLERGIC ASTHMA

Many cellular elements play an active role in initiation or progression of IgE-mediated allergic response in asthma **(Fig. 1.3.0).** Some of the key cell types are discussed here briefly.

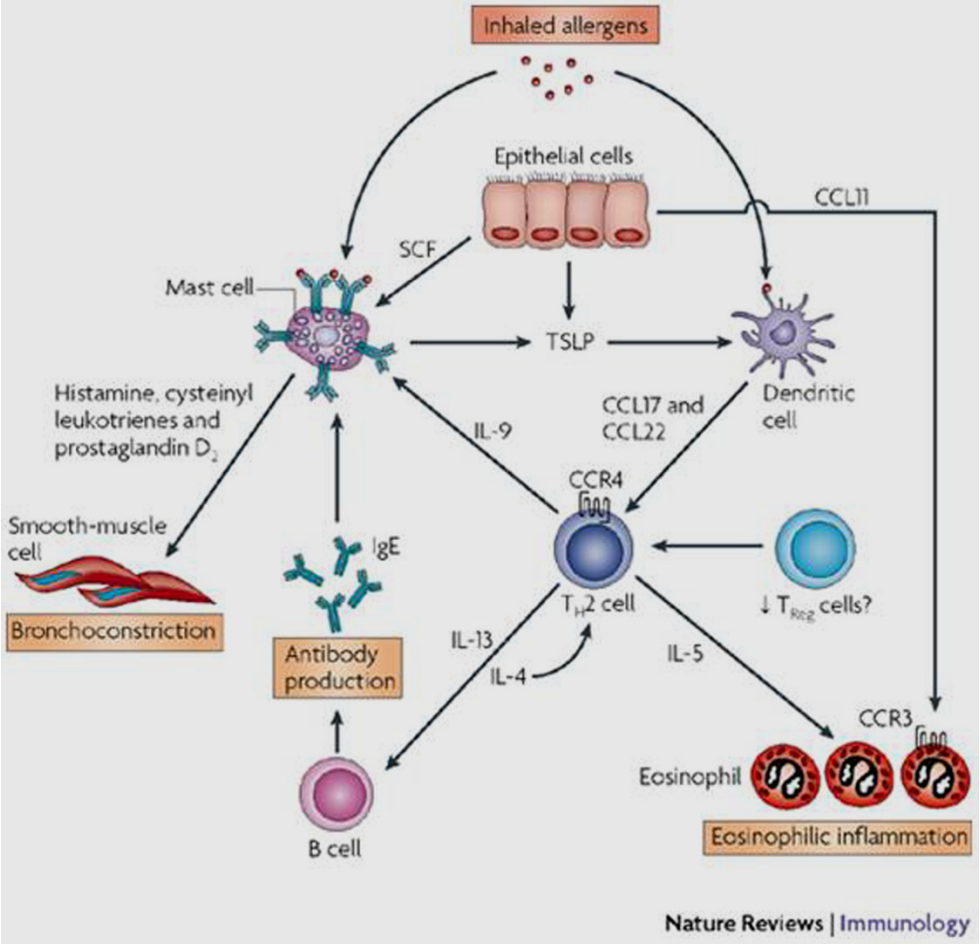

Figure 1.3.0: Inflammatory and immune cells involved in asthma. Inhaled allergens activate sensitized mast cells by crosslinking surface-bound IgE molecules to release several bronchoconstrictor mediators, including cysteinyl leukotrienes and prostaglandin D2. Epithelial cells release stem-cell factor (SCF), which is important for maintaining mucosal mast cells at the airway surface. Allergens are processed by myeloid dendritic cells, which are conditioned by thymic stromal lymphopoietin (TSLP) secreted by epithelial cells and mast cells to release the chemokines CC‑chemokine ligand 17 (CCL17) and CCL22, which act on CC‑chemokine receptor 4 (CCR4) to attract T helper 2 (Th2) cells. Th2 cells have a central role in orchestrating the inflammatory response in allergy through the release of interleukin‑4 (IL‑4) and IL‑13 (which stimulate B cells to synthesize IgE), IL‑5 (which is necessary for eosinophilic inflammation) and IL‑9 (which stimulates mast-cell proliferation). Epithelial cells release CCL11, which recruits eosinophils via CCR3. Patients with asthma may have a defect in regulatory T (TReg) cells, which may favour further TH2-cell proliferation.(35).

1.3.1. Lymphocytes

Both T and B Lymphocytes are parts of an adaptive immune response, the original player

in instigating the IgE response. B cells play an important part in allergic asthma through the production and release of allergen-specific IgE which bind to the FcεRI on mast cells and basophils, and to the low affinity IgE receptor FcεRII/CD23 expressed on B cells, macrophages, and eosinophils. The Th2 cytokines IL-4 and IL-13 and T cell derived CD40 signals induce the B cells to undergo class switch recombination (CSR), a mechanism wherein IgE is produced from IgM+ or IgG+ B cells (11).

The CD4+ T helper cells are considered as a critical regulator of allergic immune response by an imbalance in their prototype Th1/Th2 phenotype. The Th1 cells produce IFNγ and are found in higher numbers in normal airways; while Th2 cells have been detected predominantly in asthmatic airways. By releasing IL-4 and IL-13, which drive IgE production from B cells, IL-5 which induces eosinophil differentiation in bone marrow, and IL-9 which attracts and drives the differentiation of mast cells, Th2 cells have emerged at the forefront of allergic process in asthma which is an intensive area of investigation (19, 35) **(Fig. 1.3.0)**. The transcription factor GATA3 which drives Th2 differentiation, is increased in the airways of asthmatic patients (36), and knockdown of this factor prevented the development of AHR and inflammation in a murine model of allergic asthma (37).

Other Th subtypes namely Th17 have been shown to induce neutrophilic, steroid-resistant inflammation in one asthma model (38), enhanced Th2 inflammation in another (39), and surprisingly the reduction of eosinophilic inflammation and AHR in another model (40). Although a concrete role for Th17 cells in human asthma remain to be investigated, they appear to contribute to neutrophilic, steroid-resistant severe asthma (41). Another CD4+ T cell subset called Th9 was observed to secrete IL-9 that depends on TGF-β for their development (42). The IL-9 was detected in biopsies from asthma patients and colocalized with CD4+ T cells. Although

IL-9 transgenic mice exhibit baseline AHR and airway eosinophilia without allergen exposure, IL-9 deficient mice produced mixed results, suggesting a redundant function of IL-9 (43, 44). Therefore, the *in vivo* role of IL-9 as anti- or pro-inflammatory remains yet to be determined (19).

In addition to proinflammatory Th subsets, a regulatory phenotype (Treg) has emerged as pivotal in regulating multiple immune disorders, including allergic asthma. Naturally occurring forkhead box P3 (FOXP3)+ CD4+ CD25+ Treg cell transfer ameliorates the development of airway inflammation and AHR and prevents the allergen-induced dendritic cell activation in the airways (45). The inhibitory actions of Treg cells are mediated through the production of anti-inflammatory cytokines IL-10 and TGF-β, expression of inhibitory molecules such as cytotoxic T lymphocyte antigen 4 (CTLA4), and the downregulation of MHC class II and the co-stimulatory molecules CD80 and CD86 by antigen-presenting cells (19, 45).

1.3.2. Mast cells

Human mast cells (MC) are derived from CD34+/CD117+/CD13+ hematopoietic bone marrow progenitors that migrate through blood to tissues to differentiate. Although the MC progenitors are abundant in small intestine, they are scanty in normal lung. However, they have been detected abundantly in the bronchial epithelium and airway smooth muscle, and are associated with airway inflammation in mouse models (46), and in human asthma (47). Similar to rodent MCs (classified as mucosal-type, and connective tissue-type), human MC also exhibit heterogeneity and are classified according to their serine protease content as tryptase only MC (MC_T) located in the mucosa of the lung and small intestine, chymase only (MC_C), or both tryptase and chymase-positive (MC_{TC}) and are found in skin, gastrointestinal submucosa and

vasculature (48-50).

Mast cells are considered to be one of the first cells to respond to an allergen and are principal player in instigating the type-I or IgE-mediated immediate type hypersensitivity. Mast cells are activated when a polyvalent allergen/agent binds to the IgE bound to FcεRI. The abundance of FcεRI on the surface of mast cells is enhanced by increased serum IgE levels and the presence of IL-4, thereby amplifying the activation (51). The early phase reaction (EPR) is characterized by the release of prestored (in granules) and newly synthesized mediators namely histamine, tryptase, prostaglandin D_2 (PGD_2), leukotriene C_4 (LTC_4), platelet activating factor (PAF), CCL2, IL-13, vascular endothelial growth factor (VEGF)-A, and TNF; which have immediate effects on epithelial, airway smooth muscle, and endothelial cells and nerves **(Fig. 1.3.0)**. These mediators lead to increased epithelial permeability and mucus production, smooth muscle contractions, vasodilation, and neurogenic signals; altogether translating into bronchoconstriction, vasodilatation, and plasma exudation leading to the symptoms of asthma (wheezing and dyspnea) (13, 52). Early response mediators such as TNF, LTB4, IL-8/CXCL8, and CCL2 help to orchestrate the late phase response by recruiting neutrophils, eosinophils, and basophils (50). The presence of FcεRI on mast cells is critical to IgE-mediated allergic reactions in vivo and no other mechanism has been known to compensate for its absence (53).

1.3.3. Basophils

Basophils are derived from CD34+ progenitors, differentiate and mature completely in the bone marrow with the help of IL-3. They have many features similar to mast cells such as expression of FcεRI, secretion of Th2 cytokines, and granule exocytosis and release of histamine after activation. Unlike MC however, basophils circulate in the periphery and have half-life of

few days, have little proliferative potential, and have fewer but larger granules. One of the potent features of basophils is their expression of IL-4, IL-13, and CD154 (CD40L), which is thought to be the initiation of 'pump priming' for subsequent Th2 cell differentiation (51). Although predominantly a circulating granulocyte, basophils express integrins and chemokine receptors and infiltrate the inflamed tissues such as airways. Besides their appreciated role in IgE-FcεRI-mediated allergic reactions, basophils were recently shown in murine models (in context of immunization with protease allergens, ovalbumin, and helminth infection) to be capable of antigen presentation for induction of Th2 responses, with expression of MHC-II molecules and production of 'early IL-4' (54).

1.3.4. Eosinophils

Eosinophils differentiate from CD34+ hematopoietic progenitor cells under the influence of IL-5 **(Fig. 1.3.0)**. Eosinophils develop in the bone marrow and are released into the circulation under the effect of distal IL-5 (e.g. at the sites of allergic inflammation, helminth infection), allergen challenge, and experimental administration of eosinophil chemoattractant CCL11/eotaxin-1, acting through the CCR3 receptor. Eosinophils enter the circulation in response to IL-5 where their half-life is only about 8-18 hours. Eosinophils also reside in tissues, particularly at the mucosal sites of gastrointestinal tract and sites of Th2 inflammation including allergic asthma (51).

Eosinophils are known to express FcεRI in hypereosinophilic syndrome, parasitic infections and allergic asthma patients (53). The cross-linking of FcεRI and IgE on eosinophils in hypereosinophilic disease is known to cause cell degranulation (55, 56). Eosinophil activation leads to the release of proinflammatory mediators including newly synthesized eicosanoids,

cytokines, and granule-stored cationic proteins such as major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), and eosinophil derived neurotoxin (EDN). These granules are highly toxic to respiratory epithelial cells and parasites (51). Moreover, most asthma phenotypes have a hallmark increase in eosinophils in tissue, blood, and bone marrow; and the elevated numbers correlate with disease severity (57).

1.3.5. Neutrophils

Neutrophils are also called as polymorphonuclear leukocytes (PMN) which play an essential role in the immune system, acting as the first line of defence against bacterial and fungal infections. Although the role of mast cells, basophils, and eosinophils is well established in asthma, the neutrophils were one of the least appreciated cells until recently. It was summarized recently that only about 50% of the asthma cases were associated with eosinophilic inflammation, while the most other cases were accompanied by increased airway neutrophilia and IL-8CXCL8 expression (58). The number of neutrophils in induced sputum and bronchial biopsy specimens in control subjects and mild to moderate asthma patients was comparable, while it was very high in severe asthma (59, 60). Bronchoalveolar lavage (BAL) of severe asthmatics also showed enhanced neutrophils compared to control or mild asthmatics (61). Similar to other granulocytes, neutrophils produce a wide range of inflammatory products including cytokines (IL-1β, IL-6, IL-8, TNF, and TGF-β), lipid mediators (LTB_4, LTA_4, PAF, and TXA_2), proteases (elastase, collagenase, and matrix mettaloproteinase MMP-9), microbial products (lactoferrin, myeloperoxidase-MPO, and lysozyme), reactive oxygen intermediates (superoxide, H_2O_2, OH^-), and nitric oxide (62-64).

Neutrophils express all three IgE receptors namely high affinity (FcεRI), and low affinity

(FcεRII/CD23) and Galactin 3 (Mac-2/εBP) (65-67). Neutrophils from atopic asthmatic subjects displayed enhanced expression of FcεRI compared with atopic non-asthmatics or healthy subjects, particularly notable that this expression is regulated in vivo in pollen season, suggesting a very important link between neutrophils and IgE-dependent asthma. Cumulative data from others (61) and our group suggests that PMNs may be involved in immunomodulation such as allergen presentation, production of proinflammatory cytokines, chemokines and release of reactive oxygen species (ROS), which altogether may contribute to allergic diseases by airway inflammation and hyperresponsiveness (63, 64).

1.3.6. Antigen presenting cells (APCs)

Monocytes, dendritic cells, and epidermal Langerhans cells are antigen presenting cells (APC), which play a significant role in the pathogenesis of allergic asthma. All of these cells have been shown to express FcεRI to a variable level and under different physiological conditions (68). In contrast to the well characterized role of tetrameric (αβγ2) FcεRI on mast cells and basophils in immediate type hypersensitivity, the trimeric (αγ2) FcεRI aggregation on APCs is involved in 'antigen focusing'. Studies using birch and grass pollen allergen showed that the allergens recognized by antigen-specific IgE on APCs were presented to T cells in an FcεRI-dependent manner (69, 70). Interestingly, this process was 100 to 1000-fold more efficient when monocyte-enriched PBMCs presented allergen (targeted to FcεRI on these cells via allergen-specific IgE) to T cells. After polyvalent allergen ligation, the FcεRI-bound IgE is internalized in acidic proteolytic compartments, degraded, and delivered into organelles containing MHC-II, HLA-DM, and lysosomal proteins (69). After internalization, APCs migrate to the lymphatic tissues where they prime T cells into effector and memory cells; whereas both APC and Th2 cells

can modulate the differentiation of B cells into cells producing allergen-specific IgE (11). In addition to the antigen presentation, FcεRI activation on APC can initiate signal transduction via NF-κB activation (71), which can initiate or perpetuate the local inflammation by secretion of a wide variety of proinflammatory mediators such as IL-1β, IL-6, IL-8, TNF, IL-12, TARC, MDC, and MCP-1 (69). Interestingly, FcεRI activation on APC is also shown to induce the release of some regulatory mediators such as IL-10, TGF-β, and indoleamine 2,3-dioxygenase (IDO), which have been shown to suppress the T cell responses (72). Therefore, besides remarkable insights into the role of FcεRI expression on APCs, further studies are required to establish its function on APCs in allergic response.

1.4.0. AIRWAY STRUCTURAL CELLS IN ASTHMA

Although the role of lymphocytes and inflammatory cells is undeniable, tissue-forming structural cells have an inherent role in secretion of multiple inflammatory mediators and in maintaining chronic allergic inflammation. Furthermore, the most prominent feature in chronic allergic asthma- airway remodeling is due to underlying structural alterations such as epithelial denudation, subepithelial fibrosis, goblet cell metaplasia, increased smooth muscle mass, angiogenesis, and altered extracellular matrix (ECM) components (73). Some of the key structural cellular elements are discussed below.

1.4.1. Epithelial cells

Airway epithelial cells (EC) are the first structural components that encounter inhaled allergens, dust particles, chemicals, microbes and other environmental agents. Besides being a source of conventional array of cytokines, chemokines, growth factors, and other markers such as 15-hydroxyeicosatetraenoic acid (15-HETE), Fibronectin, and endothelin; recent evidence

suggest that epithelial cells may produce various inflammatory mediators such as GM-CSF, TSLP, IL-25, and IL-33 that are involved in interactions between epithelium and DCs, eventually favoring a Th2 immune response. Epithelial cell-derived TSLP can facilitate an OX40 ligand and OX40-dependent interaction between DC and T cells to promote a Th2 bias (74). The epithelial cells in asthma appear to be fragile and shed easily due to weak intercellular connections. This aspect in asthma may also be due to a failure in repair process due to chronic allergic inflammation in airways. While a progressive loss of lung has been postulated to occur in severe asthma exacerbations, the remodeling process may occur very early in asthma and may even predate clinical symptoms in some cases. Recent airway biopsy studies in children suggest that pathological changes such as epithelial shedding, basement membrane thickening, and angiogenesis occur very early in the asthmatic airways (31, 75-78). Although epithelial progenitor cells with capacity for renewal following injury exist, the repair pathways are likely dysfunctional in asthma (79).

Airway EC (80, 81) and intestinal epithelial cells (82, 83) have been shown to express both FcεRI, and FcεRII/CD23. While the function of FcεRII/CD23 was shown to be the trans-epithelial transport of IgE and allergens in intestinal compartments (82, 84), its activation in airway EC induced endothelin (well known to be pro-fibrotic) expression but not 15-HETE or any other inflammatory markers (81). The trimeric ($\alpha\gamma 2$) FcεRI expression in airway epithelium was confined to asthmatic subjects only and not normal subjects, nonasthmatic allergic subjects, or patients with chronic bronchitis, suggesting an asthma-specificity of its expression. The activation of FcεRI by an alpha chain-specific antibody led to the release of eicosanoids 15-HETE in asthmatic epithelial cells (80). Altogether, the function of IgE on epithelium so far seems to be in promoting the local inflammation and allergen/antigen transportation across

epithelial barrier to the sub-mucosal compartment.

1.4.2. Fibroblasts

Fibroblasts are another important structural entity in airways that contribute to the pathogenesis of allergic asthma. Subepithelial fibrosis is observed in most asthma patients, which may be due to the activity of fibroblasts, myofibroblasts, or infiltrating fibrocytes. Allergen challenge in asthmatic subjects leads to enhanced accumulation of myofibroblasts in the airway mucosa, the source of which remains a topic of debate. Fibroblastic infiltration of the lung is conceived as a result of (i) either the recruitment of circulating bone marrow-derived progenitors termed fibrocytes to the airways, or (ii) due to the proliferation and differentiation of resident fibroblasts, or (iii) plausibly due to the epithelial cells which may undergo phenotypic changes to effector fibroblasts through a process called epithelial-mesenchymal transition (EMT) (31). Recently, asthmatic primary airway EC were shown to exhibit increased susceptibility to TGF-β-induced EMT compared to the EC derived from normal subjects (85).

The direct effects of IgE and/or allergens on Fibroblasts have not been determined, although indirect evidence of IgE-responsiveness is available. Knight and colleagues showed that anti-IgE and IL-1β stimulation of human lung tissue enhanced the leukemia inhibitory factor (LIF) and its receptor (a pathway known to modulate neural and contractile responses), particularly in fibroblasts (86). However, the use of isolated lung tissue may in fact imply the fibroblast activation by mast cells instead of IgE (87).

1.4.3. Smooth muscle cells

Asthma was originally defined in 1860 by Sir Henry Hyde Salter with remarkable insight

as “Paroxysmal dyspnoea of a peculiar character with intervals of healthy respiration between attacks”, and recognized that these episodes were caused by abnormal smooth muscle contraction (88, 89). The role of ASM in healthy lung is still a matter of debate since it has been named as ‘appendix of the lung’ by some (90), meaning it is one of the rudimentary elements of no use; and ‘architect of the lung’ by others (91), implying its importance as a structural entity. However, ASM appears to be a critical contributor to asthma pathophysiology. The contraction of ASM causes acute narrowing of the airway and airflow obstruction in asthma, the smooth muscle mass is increased in airways of patients with asthma, and ASM from asthmatic patients demonstrate enhanced secretary function (92, 93). Contemporary understanding of smooth muscle contribution to asthma features suggests that ASM could be the most attractive target for therapeutics (94). Indeed, the newly introduced severe asthma therapy ‘bronchial thermoplasty’ is aimed at ablating the ASM mass with radiofrequency heat applied directly to airways with the help of a bronchoscope (92, 95) **(Fig. 1.4.3)**.

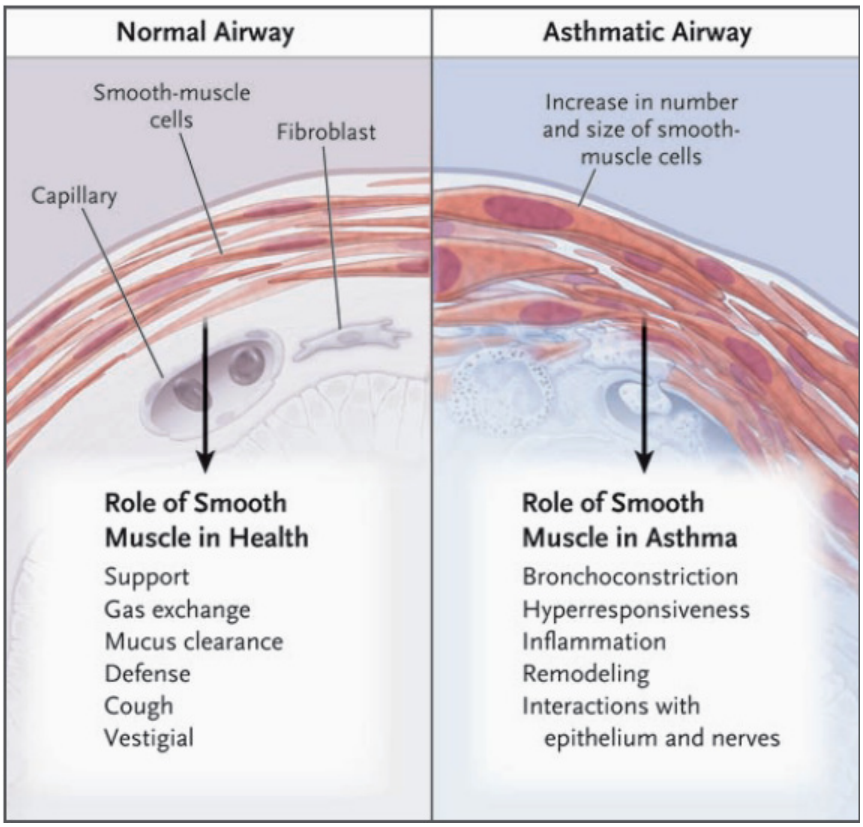

Figure 1.4.3.: Features of the Normal Airway and the Asthmatic Airway. In normal airways, smooth muscle might provide structural support, help regulate gas exchange, and contribute to mucus clearance, defense mechanisms, and cough or it might be vestigial (resulting from lung development) and not play an important role. In asthma, airway smooth muscle mediates acute bronchoconstriction and participates in airway hyperresponsiveness. Accumulating evidence implicates smooth muscle in the pathogenesis of airway inflammation and remodeling and points to interactions with bronchial epithelium and nerves. The benefit of current asthma treatments, including bronchodilators, inhaled corticosteroids, and anti-IgE antibody, as well as bronchial thermoplasty, may be due in part to their actions on airway smooth muscle. Potential therapies (now theoretical) that target airway smooth-muscle contraction or abundance might include the induction of apoptosis of airway myocytes, alteration of transcription to shut down expression of the contractile apparatus, paralysis of airway smooth muscle, and enhancement of force-fluctuation–induced relengthening of airway smooth muscle (92).

Airway smooth muscle has been shown recently to respond to inhaled environmental factors directly through various mechanisms, including dust, allergens, microbes, and pollutants. Notably, ASM express both FcεRII/CD23 (96) and FcεRI (97, 98) (see our work), and their activation has functional outcomes on ASM activation.

1.5.0. AIRWAY SMOOTH MUSCLE (ASM) CELL: AN IMPORTANT CELL IN HEALTH AND DISEASE

The function of ASM can be defined in context of two developmental stages in health. In the prenatal stage, ASM behaves as a *phasic* smooth muscle which contracts rhythmically, and stretch-induced signals produced by this ASM tone are implicated in muscle myogenesis and lung growth. Postnatally, ASM behaves as a *tonic* smooth muscle. ASM cells are packed together as bundles or fascicles. The ASM exhibits active tone that confers radial stiffness on the airway wall, while the discontinuity of adjoint circumferential bands would confer longitudinal extensibility, thus facilitating the length changes that occur during spontaneous airway narrowing. The postnatal tone of ASM bundles serves to stiffen the airways preventing collapse during compressive narrowing (99). The role of ASM in adults upon phasic-to-tonic contractility switch is debatable. Some believe that it gives the airway rigidity and therefore, protects the airways from collapsing during tidal breathing (100), whereas others have suggested that the ASM contraction is involved in the tradeoff between dead space and airway resistance during expiration (99, 101). However, normal ASM contraction in disease (asthma) can easily cause dyspnoea, the shortness of breath (102). The role of ASM in asthma pathogenesis is discussed in next sections.

1.5.1. Pathogenesis of asthma and the role of ASM cells: airway hyperresponsiveness

Bands of submucosal smooth muscle run through the conducting pulmonary airways, wrapping circumferentially around the lumen. “Like a boa constrictor squeezing its prey, these smooth muscle bundles narrow the airway lumen when they contract” (93). ASM cells are

thought to be the principal effector component of airway narrowing. A modest degree of bronchoconstriction occurs in normal airways, however it is often abnormally exaggerated in asthmatic airways, resulting in excessive airway narrowing due to nonspecific irritants or pharmacological agonists, a phenomenon known as bronchial or airway hyperresponsiveness (BHR or AHR). Notably, it is the ASM contraction which has long been a target of asthma therapeutics in the form of inhaled beta-2 (β2)-adrenergic receptor (β_2-AR) agonists (which provide quick relief of airflow obstruction) (93). As such, agonists of ASM contraction include histamine, acetylcholine (Ach), bradykinin, neurokinin A, and leukotriene (LT)D_4, which act on the Gq family of seven transmembrane G-protein coupled receptors (GPCR) to activate phospholipase C (PLC) which eventually induces inositol 1,4,5-triphosphate (IP3)-dependent intracellular calcium ($[Ca^{2+}]i$) flux. On the contrary, the agonists of ASM relaxation or bronchodilation are β_2AR-agonists such as isoproterenol (ISO) which activate the Gs subunit of GPCR to generate cyclic AMP (cAMP) which activates protein kinase A (PKA) to sequester $[Ca^{2+}]i$ and result in ASM relaxation (103). Furthermore, AHR is included as one of the cardinal features in the definition of asthma (2), and is considered to be driven primarily by altered ASM function (104). Cumulative evidence from past two decades suggests that ASM in asthmatic airways is intrinsically different from healthy ASM. For instance, ASM from asthmatic subjects was thought to generate more force and hence contract to a greater extent (105), and that the asthmatic ASM have increased maximum shortening velocity (Vmax) and capacity (106). The ASM from asthmatics had higher mRNA expression of myosin light chain kinase (MLCK) than from normal subjects (106). MLCK phosphorylates myosin light chain (MLC), eventually resulting in contraction. Interestingly, the promoter of MLCK gene has several binding sites for the transcription factor CCAAT/enhancer binding protein-α (C/EBPα) (107), the expression of

which was shown to be decreased in asthmatic ASM (108). It was therefore proposed (109) that the deficit in C/EBPα expression may account for the enhanced MLCK expression in asthmatic ASM and hence the increased Vmax, eventually contributing to AHR (106). A recent study further demonstrated that the asthmatic ASM exhibits enhanced expression of the fast myosin heavy chain isoform transgelin, and MLCK, with additional experiments in rat model suggesting that this may be associated with increased velocity of shortening, thus contributing to AHR (110). Besides AHR, contemporary evidence implicates the role of ASM in other features of asthma such as modulation of inflammatory response, and airway remodeling, discussed in next sections (92) **(Fig. 1.4.3)**.

1.5.2. ASM as an 'inflammatory-like cell'

1.5.2.1. Mediator release. Much of the knowledge we have about ASM phenotype and function began to uncover in 1980s and 1990s with the realization of their isolation and culture from lung tissue- trachea and bronchi, by either explants or enzymatic digestion method (111, 112). First evidence of the role of smooth muscle in regulation of inflammation came in 1980s when vascular smooth muscle was shown to release an IL-1-like T cell-activation factor (113). Since then, smooth muscle of both vascular and airway origin have been shown to secrete a plethora of inflammatory mediators. ASM in particular, has been named by some as 'biological factories', owing to its unbelievable synthetic potential (114). One of the first convincing evidence showing ASM's contribution to allergic inflammatory response came from the study showing a strong mRNA and protein signal of eosinophil-attracting chemokine CCL11/eotaxin-1 in ASM of asthmatic airways compared with that of normal airways which had only a minor immunoreactivity (115). Interestingly, freshly isolated ASM cells have 'contractile' phenotype

which is switched to a 'synthetic-proliferative' phenotype upon culture in serum-rich conditions. The latter is unresponsive to contractile agonists and has reduced expression of contractile proteins such as smooth muscle myosin heavy chain (smMHC), alpha smooth muscle actin (α-SMA), and smMLCK. However, these switched cells proliferate faster in response to mitogens and produce multiple cytokines and extracellular matrix (ECM) proteins (116). Currently, ASM is known to produce significant amounts of proinflammatory (TNF, IL-6), Th-1 (IFN, IL-1, IL-2, IL-12, IL-33), Th-2 (IL-4, IL-5, IL-13), Th-9 (IL-9), and Th-17 (IL-17) cytokines; chemokines (CCL5/RANTES, CCL11/eotaxin-1, CCL17/TARC, CXCL8/IL-8, CXCL10/IP-10, CX3CL1/Fractalkine, MCPs), growth factors (PDGF, VEGF, FGF, IGF, Thrombin, IGF-1, TGF-β, SCF, GM-CSF), matrix proteins (fibronectin, perlecan, decorin, elastin, laminins, thrombospondin, chondroitin sulphate, collagen I-V, verisican), and other inflammatory molecules (LIF, NO, PGE_2, COX-2, PAF, bradykinin, adenosine), summarized in (114, 116-118). Many of these mediators activate the ASM in an autocrine/paracrine fashion by inducing the release of chemokines that eventually recruits and activates multiple inflammatory cells. In summary, these inflammatory mediators can modulate the synthetic, contractile, and proliferative responses of ASM, eventually contributing to inflammation, AHR, and airway remodeling in vivo.

1.5.2.2. ASM response to proinflammatory cytokines. ASM also expresses the receptors of multiple inflammatory mediators, suggesting an autocrine/paracrine network of inflammation around ASM. Activation of some of these receptors (such as TNFR, IL-1R, IL-4Rα, IL-13R) is known to play a critical role in modulating both acute and chronic airway inflammation, and AHR (119). Proinflammatory cytokines IL-1β and TNF are probably the most extensively

studied modulators of ASM function. Predominant role of TNF in asthma pathogenesis is evident from studies showing that the airways of asthmatics exhibit enhanced prevalence of TNF (besides IL-4, -5, and -6) (120), and TNF inhalation in normal individuals leads to the development of AHR (121). Cumulative evidence implicates that TNF induces airway neutrophilia, eosinophilia, mucus hypersecretion, T cell activation, and expression of cell adhesion molecules along with multiple other inflammatory mediators (119, 122). ASM expresses both the receptors (TNFR-1 and -2) for TNF, although the former is thought to be the predominant in mediating TNF effects on ASM synthetic response (123). IL-1β expression is also increased in asthmatic airways, and has been shown to activate various cell types such as mast cells, T and B lymphocytes, epithelial cells, and ASM cells (35). In human ASM cells, IL-1β treatment enhances the productions of several inflammatory mediators such as CCL11/eotaxin-1, RANTES, GM-CSF, growth-related oncogene-α, PGE_2, nerve growth factor, and MCP-1, -2, and -3 (119). Furthermore, IL-1β synergizes with TNF to modulate various inflammatory pathways in ASM cells, such as induction of β2-AR hyporesponsiveness, the phenomenon which is ablated by selective COX-2 inhibition (124). Moreover, glucocorticoids turn both (IL-1β and TNF) into mitogenic to ASM via suppression of COX-2-dependent protein kinase A activity (125). IL-4 and IL-13 are other important cytokines that have classical ability to switch the immune response towards a Th2 arm, and have been shown in higher amounts in asthmatic airways. In human ASM cells, both IL-4 and IL-13 signal through a common type-II IL-4 receptor, consisting of IL-4Rα and the IL-13Rα1. Both IL-4 and IL-13 can augment the chemokine release from ASM, particularly the IL-1β- and TNF-induced IL-6, IL-8, RANTES, MCP-1, and -2 (126).

1.5.2.3. Cell adhesion molecule (CAM) expression in ASM. Besides cytokine/chemokine receptors, ASM cells express many CAMs, implying that these cells may have the ability to interact directly with immune cells, or even have an inherent immune 'inflammatory' nature of their own. ASM cells express the integrins, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and CD44 which renders T cell binding to ASM cells, eventually leading to DNA synthesis in ASM (127). In addition, ASM express MHC-II, and the co-stimulatory molecules CD80, CD86, and CD40, although their expression does not enable ASM to present antigens to ASM cells (128-130). Nonetheless, both T cells and ASM can activate each other via these integrins and co-stimulatory molecules. Also, ASM cells were shown to express OX40 ligand (OX40L), the activation of which induced IL-6 release in ASM (131). Besides T cells, neutrophils (132), eosinophils (133), and mast cells (47) have been shown to adhere to ASM cells. Recent reports suggest the mast cell-ASM interaction via expression of a heterophilic adhesion molecule, tumor suppressor in lung cancer-1 (TSLC-1) (134). The CAM-mediated T cell adherence to ASM cells enhanced the acetylcholine (Ach)-induced bronchoconstriction and impaired the isoprenaline-induced bronchodilation (130). Recent reports from an experimental asthma model further show that the adoptive transfer of antigen-specific CD4+ T cells to naïve mice leads to T cell-ASM co-localization and subsequent enhanced proliferation and attenuated apoptosis of ASM cells, suggesting the functional outcome of this interaction in airway remodeling (135).

1.5.2.4. ASM-Mast cell interaction. Mast cells are known to infiltrate the ASM tissue in asthma (47). Mast cells interaction with ASM is thought to be critical in modulating airway immunopathology in asthma (136). Therefore, this interaction is of particular interest to many.

Interestingly, ASM can be induced to secrete potential mediators of mast cell chemotaxis, proliferation, and survival, such as fractalkine, CADM1, and SCF (31). Mast cells expressing IL-4 were detected recently in histological sections of ASM bundles from asthmatics but not from normal subjects, suggesting a novel role of IL-4 through mast cell-ASM interaction (137).

1.5.3. The role of ASM cell in airway remodeling

Increased ASM mass is considered one of the hallmark features of airway remodeling (73, 93). Although the precise mechanisms remain yet to be established, this increase in ASM mass has been postulated to arise because of increase in cell number (hyperplasia) and in cell size (hypertrophy), dysfunctional ASM apoptosis, ASM migration, and recruitment of 'smooth muscle-like cells' or 'myofibroblasts' to the subepithelium (138). Huber and Koessler (1922) first described the increased ASM mass in asthmatic patients (139), while other reports showed an overall abundance of ASM in lungs of patients who died of asthma compared with the patients who died suddenly without any history of chronic bronchitis or emphysema (140, 141). In fact, AHR and increased smooth muscle mass have been shown to coexist in patients with fatal asthma (140-142). In support of ASM hyperplasia, Woodruff and colleagues have provided convincing evidence by showing almost two-fold higher number of ASM cells (but not ASM mass) in subjects with mild-to-moderate asthma, with up to about 80% increase in α-SMA expression in these patients whereas there was no change in expression of contractile proteins (143). Moreover, ASM proliferation in severe asthma was shown to be a dynamic process *in vivo* recently by staining with proliferating cell nuclear antigen (PCNA) and Ki67 markers (144). Increased amount and altered ECM composition, observed in asthma (145), can also modulate the phenotype and responsiveness of ASM (146). Indeed, it has been shown that fibronectin and

collagen I enhance ASM cell proliferation in response to PDGF or thrombin, while laminin promotes contractile phenotype (147). As such, due to intrinsic phenotype plasticity, airway myocytes exhibit multifunctional behavior and play an active role in airway inflammation, fibrosis, AHR, and airway remodeling in vivo (116). Observations showing increased ASM mass in asthma (143, 144) and studies wherein airway remodeling was present in animals models prior to allergen challenge (148), and in young children prior to eosinophilic inflammation and clinical asthma (149, 150); suggest altogether that ASM remodeling may in fact be a cause, instead of consequence of asthma pathophysiology. Interestingly, ASM cells have been shown to possess an intrinsic ability to exhibit excessive proliferation in asthma (151), which is also known to be insensitive to glucocorticoids (108), owing to the lack of C/EBPα expression. Furthermore, bronchoalveolar lavage (BAL) fluids from asthmatics were also shown to induce ASM proliferation (152). In vitro, ASM proliferation has been studied widely in response to growth factors, cytokines and other inflammatory mediators including PDGF, FGF, EGF, TNF, histamine, leukotrienes, tryptase, endothelin-1, thrombin, and elastase (138).

1.5.4. Signaling pathways regulating ASM synthetic and mitogenic function

Functional outcome of cytokine/chemokine receptor activation on ASM involves the activation of multiple signaling pathways, which has been an active area of investigation in recent past. These pathways predominantly include mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription (STAT), and nuclear factor kappa B (NF-κB) pathways. MAPK are a family of ser/thr kinases which transduce extracellular signals to the nucleus, and are of three main types: the extracellular signal-regulated kinases (Erk), c-Jun NH_2-terminal kinases (JNK), and p38 MAPKs. Activation of these kinases results in the

phosphorylation of various intracellular substrates including transcription factors, which turn on multiple inflammatory genes such as cytokines and chemokines expression in ASM (153). Transcription factors such as NF-κB, AP-1, STATs are activated upon stimulation with cytokines (e.g. TNF, IL-1β, IL-4), and regulate the gene expression of various inflammatory mediators by activating their respective transcriptional promoters. Furthermore, mitogens affecting ASM proliferation are divided into two broad categories: (i) those that activate the receptor tyrosine kinases (RTK), (ii) those that exert their effects through GPCR. Cumulative evidence shows that phosphatidylinositol-3-kinase (PI3K) and Erk MAPK are principal signaling pathways activated upon both RTK and GPCR stimulation in ASM (117, 138).

1.5.5. Immune mechanisms of smooth muscle sensitization

Prausnitz and Kustner were the first to recognize that the allergen sensitivity can be transferred to nonallergic individuals through the transfer of serum from an atopic subject (154). Subsequent studies showed that the exposure to serum from asthmatic subjects caused an increase in mediator release from isolated cells (155), and initiated allergen-induced bronchoconstriction in isolated human airways (156). However, the detailed studies to understand the contractile properties of ASM in asthma began with a report from Antonissen and colleagues (1979), in which a canine (dog) model of asthma was used to sensitize them with dinitrophenol and ovalbumin (DNP-OVA) injections intraperitoneally along with aluminium hydroxide (alum) as adjuvant, leading to the production of anti-DNP IgE antibodies. Tracheal smooth muscle (TSM) strips were then isolated from these animals and studied *in vitro*. Interestingly, TSM from sensitized animals exhibited significantly greater shortening velocity and an increased isotonic shortening at any given load after maximal electrical field stimulation

(EFS) (157). This study led to the idea of passive sensitization of isolated bronchial tissue with serum from atopic individuals (containing high levels of IgE), which was used by many investigators to study the release of bronchoconstricting mediators and the *in vitro* responsiveness to histamine and other mediators, summarized in (158). Interestingly, similar to the canine study, passive sensitization of isolated human airway tissue also enhanced the smooth muscle shortening velocity and capacity in response to EFS, and augmented the myogenic responses (159, 160). Since smooth muscle contraction depends on increase in $[Ca^{2+}]i$ and subsequent activation of MLCK, it was postulated that the sensitization modulates this pathway, leading to smooth muscle hyperreactivity (161) **(Fig. 1.5.5)**. In fact, the atopic sensitization of canine and human airways resulted in enhanced abundance and activity of MLCK (162, 163). In addition, allergen sensitization of guinea pig airways was found to enhance the content and activity of Rho kinase, which inhibits MLC phosphorylation and therefore alters the balance towards MLCK activity and contraction (164). The role of ($[Ca^{2+}]i$) in smooth muscle contraction was confirmed by the use of drugs interacting with calcium channels which were shown to reduce the already established airway hyperreactivity (165).

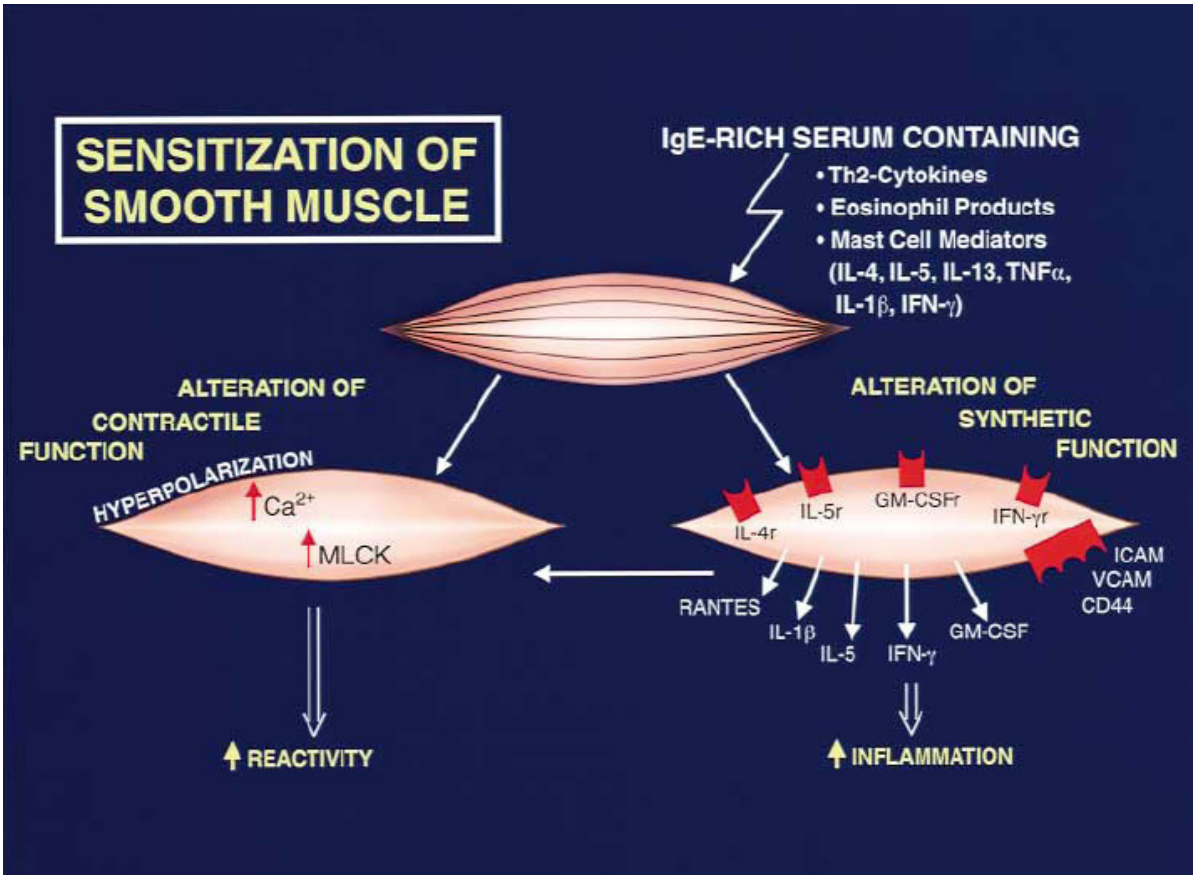

Figure 1.5.5.: Sensitization-induced changes in smooth muscle function. The incubation of smooth muscle with IgE-rich serum or the exposure to certain inflammatory cytokines causes changes in smooth muscle contractile and synthetic function. Alteration in contractile function in reflected by an increase in smooth muscle shortening velocity and capacity as well as increased reactivity to bronchospasmogenic stimuli. These changes are believed to occur through an increase of $[Ca^{2+}]i$ concentrations, enhanced MLCK activity, or changes in the cell membrane potential. Moreover, sensitization might alter smooth muscle synthetic function through increased production of inflammatory cytokines, chemotaxins, and expression of cytokine receptors and adhesion molecules, all of which can contribute to the changes in smooth muscle reactivity and initiate or maintain airway inflammation (161).

From atopic serum sensitization studies, it was suggested that serum IgE levels play an important role in smooth muscle hyperreactivity (166-168). Indeed, the bronchial hyperresponsiveness was shown to be associated with serum IgE levels (166), and transferable by IgE-rich serum from asthmatic to non-asthmatic individuals (169). In fact, serum IgE levels correlated with human airway reactivity *in vitro* (170). Incubation of IgE-rich serum from atopic

individuals was shown to cause hyperreactivity in isolated airway preparations (171). Moreover, IgE was proposed to cause smooth muscle contractile function through binding to the smooth muscle membrane and cause subsequent hyperpolarization (172) **(Fig. 1.5.5)**. Finally, the pre-incubation of an IgE neutralizing antibody (anti-IgE, 17-9), which reduces free IgE to undetectable levels, prevented the IgE-dependent passive sensitization of human airways and subsequent allergen-induced contractile responses *in vitro* (173). Cumulatively, the passive sensitization studies of airway tissue suggested that the resulting smooth muscle contraction is an indirect effect of IgE-mediated activation of other cell types such as mast cells. However, ASM of both rabbit and human origin were shown recently by others and our group to express FcεRI which is activated by IgE with multiple functional outcomes (96-98, 174). ***This led us to hypothesize that the synthetic and contractile responses observed upon passive sensitization with IgE-rich serum may indeed be due to the direct effects of IgE on ASM.***

1.6.0. IgE AND IgE RECEPTORS

Ishizaka and Ishizaka (1967) described a new antibody isotype called immunoglobulin E (IgE) (175). Since its discovery, IgE has been shown to be critical in allergic reactions and immune response to parasitic infections (53, 176).

1.6.1. Structure of IgE

IgE is one of the five immunoglobulin isotypes and has similar molecular structure to IgM, IgD, IgG, and IgA, consisting of two identical light (L) chains and two identical heavy (H) chains with variable (V) and constant (C) regions made up of Ig domains. Each Ig domain contains about 110 amino acids and comprises a β-sheet sandwich with three and a C-type topology with four β-strands (177). IgE is characterized by epsilon (ε) H chains, which similar to

IgM, contains one variable heavy chain (VH) and four heavy chain constant domains (Cε1-4), in contrast to IgG, IgD, and IgA which contains only three heavy chains **(Fig. 1.6.1)**. The additional domain Cε2 in IgE replaces the hinge region in IgG, and is a critical determinant of its distinctive physical properties and IgE isotype-specific functions. Due to this disparity, C_H2 and C_H3 domains of IgD, IgG, and IgA are homologous to C_H3 and C_H4 domains of IgE and IgM (11, 12). Interestingly, the Cε2 region was initially thought to act as a 'spacer' between the antigen-binding Fab arms and Cε3-Cε4 region, but recent studies using florescence resonance energy transfer (FRET) elucidated that the distance between the N- and C-termini of the molecule (6.9 Å) was less than half of the expected extended Y-shaped structure (17.5 Å) (12, 178).

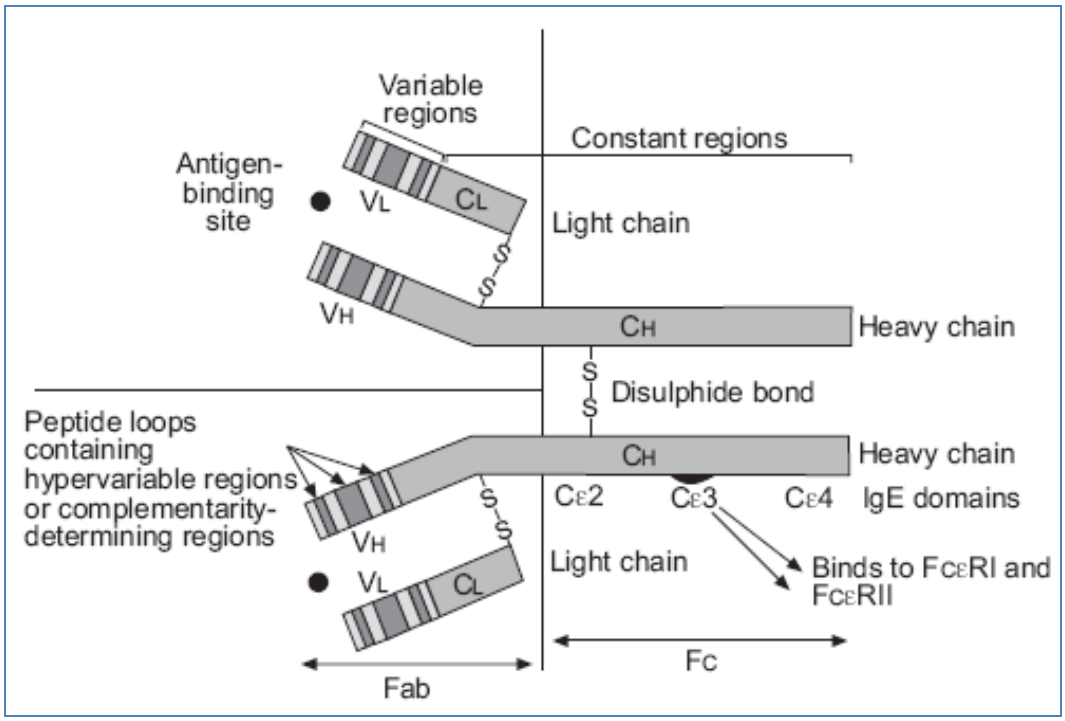

Figure 1.6.1.: Schematic diagram of IgE. The variable domain (V) binds antigen. The constant region (C) domains determine secondary biological function (e.g. cell-surface binding). FcεRI and FcεRII are high-, and low-affinity IgE receptors, respectively. Fab: antigen-binding fragment; Fc: crystallizable fragment; VH: heavy-chain variable domain; VL: light-chain variable domain; CH: heavy-chain constant domain; CL: light-chain constant domain (179).

The V regions of both H-, and L-chains pair up to form two identical antigen-binding sites. These along with C_H1 domain pair form the antigen binding fragment (Fab) region of the antibody. The remaining Ig domains constitute the constant fragment (Fc) region of the antibody, which binds to the cellular receptors (FcR). IgE binds to its receptors at the Cε3 region. Similar to other immunoglobulin isotypes, IgE is heavily glycosylated and the glycosylation patterns are critical determinants of the interaction of IgE with its receptors (180).

1.6.2. Abundance and regulation of IgE synthesis

1.6.2.1. IgE characteristics

The half-life of IgE in serum is only about 2.5- 3 days, compared with 20 days for IgG. However, most of the IgE is sequestered in tissues (181), and it is believed that the immune surveillance by IgG occurs in circulation, by IgE primarily in tissues, and by IgA in mucosal surfaces. IgE has been shown to be synthesized early in uterine life and is detectable in cord-blood and human placenta (182, 183). IgE is measured in kU per litre units, 1kU being equal to 2.4ng/ml (WHO standard 75/502). Normal levels of IgE in newborns are considered to be about 0.8- 1.2ng/ml, which tend to gradually increase up to about 25-100ng/ml in adolescence (184). In adulthood, mean serum IgE levels are considered to be ~150ng/ml in serum which still makes IgE the least abundant antibody class, compared with ~10mg/ml for IgG in non-atopic individuals. Serum IgE concentrations reflect the number of circulating IgE-committed B cells. In certain parasitic diseases or hyper-IgE syndrome, serum IgE levels may increase to up to three orders of magnitude than normals without any signs of allergic disease. In allergic individuals, IgE levels in circulation may reach over ten times the normal, and allergen specific IgE which may be over 1000 times higher than the detection limit in normals, tend to associate with disease symptoms.

1.6.2.2. IgE synthesis and isotype switching

In subjects with asthma or hay fever, IgE may be detected only in secretions from target organs, suggesting the occurrence of local IgE synthesis (11). Interestingly, high serum IgE levels are considered to reflect high synthetic rates. However, it is also important to realize that high levels of IgE lead to lower catabolism rates; the half-life of IgE increased from 1.8 to 5.8 days when moving from normal IgE levels of 0.07 to about 35µg/ml in patients with hyper-IgE

syndrome or atopy. Moreover, the half-life of IgE is increased to about 8-14 days when it is bound to its high affinity receptor FcεRI (185).

In spite of the short half-life of IgE itself, the IgE response is believed to be long lasting, even in the absence of allergen stimulation. This fact may be explained by long-lived IgE-producing plasma cells, molecular mimicry by cross-reacting antigens, or by long-time deposition of allergen in follicular DCs. Mature B cells begin by expressing IgM but may switch to IgE or other isotypes upon antigen activation. There are three crucial steps in mounting an IgE immune response: (i) differentiation of DC type 2 (DC2) that promotes (ii) the formation of allergen/antigen-specific IL-4- and IL-13-producing Th2 cells which consequently (iii) induce the differentiation of B lymphocytes to IgE switch and transformation of IgE-producing plasma cells (184). In the first step, DC is thought to acquire type-2 phenotype in the presence of IL-4 and absence of IL-12. Recent studies implicate the potential of a novel cytokine TSLP to induce strong OX40L expression besides costimulatory molecules CD80/86 and MHC-II, while inhibiting the IL-1, IL-6, IL-12, and IFN-α/β expression. This provides the TSLP-stimulated DCs the precise phenotype to instruct a naïve CD4+ T cell to become a Th2 cell (186). The final step, IgE class-switch recombination (CSR) occurs through two pathways: the classical and the alternative pathways (187, 188). The classical or T cell-dependent pathway involves the presence of cytokines IL-4 and IL-13 during the interaction of CD40-CD40L in B cells that leads to the induction of Cε germline transcription and activation induced cytidine deaminase (AID) expression. IL-4 and IL-13 induce the activation of STAT6 which can synergize with NF-κB to induce the Cε GLTs (188). The alternate pathways require just the presence of IL-4 and can occur without the help of T cells. For instance, glucocorticoids can induce IgE synthesis in the presence of IL-4 by enhancing CD40L expression on B cells (189); in presence of IL-4, B cell activating

factor (BAF) and proliferation-inducing ligand expressed on monocytes/DCs can bind to their receptors on B cells to induce IgE synthesis; and Epstein-Barr virus can nonspecifically induce IgE switch plausibly through CD40 molecular mimicry by virus-encoded latent protein 1 (187, 190). Negative regulation of IgE class switching can be induced by cytokines such as IFN-γ, IL-21 which inhibit the development of Th2 cells and Cε GLTs. Certain B-cell surface receptors such as CD45, cytotoxic T-lymphocyte antigen 4 (CTLA-4), and CD23 can also inhibit the IgE CSR through altering the signal transduction pathways (187, 188).

Allergen-specific IgE has been detected in nasal mucosa from allergic patients suffering from allergic rhinitis, nasal polyposis, and allergic fungal rhinosinusitis. In fact, germinal centre like reactions and CSR can occur in target organs such as respiratory tract mucosa where IgE-expressing B cells and plasma cells were detected in atopic asthma patients (12, 191).

1.6.3. IgE receptors

IgE has three known receptors; the high affinity FcεRI, and the low affinity receptors FcεRII/CD23 and Galactin-3/Mac-2/ε-BP, reviewed below.

1.6.3.1. High affinity IgE receptor (FcεRI)

FcεRI belongs to the immunoglobulin (Ig) superfamily of proteins which is abundantly expressed in mast cell and basophils (~200,000 molecules/cell) and is expressed at much lower levels in other cells such as monocytes, Langerhans cells, platelets, and eosinophils (11).

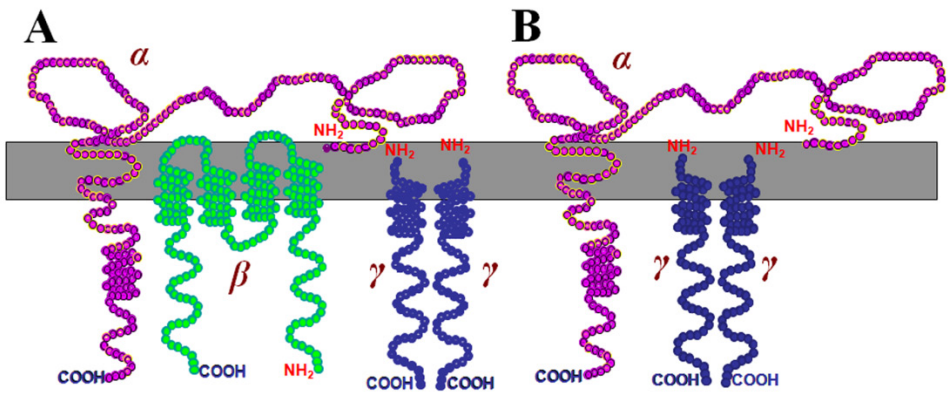

Figure 1.6.3.1: Structure of the high-affinity IgE receptor (FcεRI). (**A**): FcεRI was first described as a tetrameric complex (αβγ2). In rodents, FcεRI β-chain is required for cell surface expression of FcεRI so that FcεRI is only expressed as αβγ2 tetramers on mast cells and basophils. **(B)**: in human monocytic lineage, e.g., dendritic cells, monocytes, and Langerhans cells, FcεRI is expressed exclusively as αγ2 complex (97).

FcεRI was first demonstrated in rodents, in which it is exclusively present on mast cells, basophils, and non-B non-T cells. Murine FcεRI exists as an obligate tetramer made up of one α chain, one β chain and two identical disulfide-linked γ chains. However, the human FcεRI can exist in both tetramer and trimer (αγ2) isoforms. The tetramer (αβγ2) is maintained as a complex in the plasma membrane through hydrophobic and electrostatic noncovalent interactions between both covalently and noncovalently bound lipid molecules (53, 192, 193).

The FcεRI-α chain is a type I integral membrane protein that contains the Fc binding sites for IgE in its extracellular domain. It consists of two extracellular Ig-related domains, one transmembrane domain with a conserved aspartic-acid residue, and a short cytoplasmic tail **(Fig. 1.6.3.1)**. The α-chain has seven N-glycosylation sites which are required to mediate proper interaction between the α-chain and folding machinery in the endoplasmic reticulum (ER). The α-chain core protein is about 27KDa heavy but its heavy glycosylation makes it to appear as a broad, extremely heterogeneous band centered at ~45KDa (53). The α-chain also contains an ER

retention signal which has a di-lysine motif located in its cytoplasmic domain, and keeps it in an immature form in the ER (194). The intracellular tail of α chain does not have any known function in signaling (53).

The β chain has four transmembrane domains which separate the amino (N)- and carboxy (C)-terminal cytoplasmic tails (195). In mast cells, FcεRI-β chain is also shared by the low affinity IgG receptor FcγRIII (196). The γ chain is a member of the γ/ζ/η family of antigen receptor subunits and has a transmembrane region and a cytoplasmic tail. The FcεRI-γ chain is commonly referred to as FcRγ and is shared among FcγRIII, FcγRI, FcεRI, FcαR, and T cell receptor (TCR) depending upon the cellular context (53). Both FcεRI-β and -γ chains have their characteristic immunoreceptor tyrosine-based activation motifs (ITAM), which get phosphorylated upon antigen cross-linking of FcεRI-bound IgE molecules. The β chain has been shown to possess an amplifier function for FcεRI signaling and the cell-surface expression of FcεRI itself (197). The γ chains, on the other hand, serve as indispensable FcεRI signaling units. In both rodents and humans, FcεRI has been described as a modular unit; the α chain as binding module, and the β and γ chains serve as signaling modules in either αβγ2 or αγ2 complex (53) **(Fig. 1.6.3.1)**.

Human FcεRI can be expressed as either tetramer or trimer. The tetramer (αβγ2) form is expressed on the classical effector cells of allergic reactions, for instance mast cells and basophils, although recent evidence implicates this form of receptor expression on allergic asthmatic neutrophils, and smooth muscle cells (see our work). The trimeric (αγ2) form of the receptor is expressed on APCs such as Langerhans cells, peripheral blood DCs, and monocytes (**Table 1.6.3.1**). Although the mRNA for FcεRI- α, -β, and -γ chains has been detected on eosinophils, and platelets (198, 199), further studies are required to confirm the expression of

tetrameric structure on these cells.

Table 1.6.3.1.: An overview of the FcεRI subtypes expressed by different human cells and their associated functions.

Cell type	FcεRI type	Cell function
Mast cells, Basophils	Tetrameric	Cell activation, degranulation
Monocytes	Trimeric	Antigen presentation
Blood DCs	Trimeric	Antigen presentation
Langerhans cells	Trimeric	Modulation of cell differentiation
Eosinophils	Trimeric/tetrameric	Defence against parasitic infections
Platelets and megakaryocytes	Trimeric/tetrameric	Inflammation
Neutrophils	Trimeric/Tetrameric	Allergic disease
Epithelial cells	Trimeric/tetrameric	Inflammation
ASM cells	Tetrameric*	Cell activation, Allergic inflammation*

Source: Adapted from (199) with updates from (64, 83, 98, 200, 201). *see our work.

1.6.3.2. Low affinity IgE receptor (FcεRII/CD23)

FcεRII/CD23 is a ~45KDa type II transmembrane glycoprotein of C-type lectin superfamily. The membrane bound form of CD23 consists of three globular lectin domain 'heads' separated from the membrane by a triple α-helical coiled-coil 'stalk' region (12). CD23 is widely expressed in T, B lymphocytes, neutrophils, monocytes, follicular DCs, and intestinal epithelial cells (202). Proteolysis of the stalk region of CD23 by proteases such as ADAM10 leads to a range of soluble fragments (sCD23) of 37KDa, 33KDa, 25KDa, and 16KDa size, all of which can bind to IgE (203). The N-terminal sequence of CD23 exists in two splice variants: CD23a which is expressed by antigen-activated B cells and eosinophils, and CD23b is IL-4-inducible on inflammatory cells, monocytes, macrophages, B cells, and epithelial cells (204).

CD23 activation has both inhibitory (monomeric sCD23) and stimulatory effects (trimeric sCD23) on IgE synthesis in activated B cell, depending upon its oligomerization state. CD23 can regulate the IgE synthesis in vivo, as evident from the studies showing that the CD23-deficient animals had higher antigen-specific IgE levels and the CD23 transgenic animals had reduced IgE levels (205). In fact, sCD23 has many cytokine-like activities, such as maintaining the growth of activated mature B lymphocytes, promoting the differentiation of germinal centre centroblasts towards plasma cell pool (in association with IL-1α), and inhibition of germinal centre B cell apoptosis (206). In addition, sCD23 promotes the differentiation of myeloid precursors, thymocytes, and bone marrow CD4+ T cells; and in conjunction with IL-1, sCD23 drives cytokine release from monocytes (202). Although CD23 binds to IgE in a carbohydrate-independent manner, it can bind to CD21 in a carbohydrate-dependent manner, and also to several integrins such as $\alpha_M\beta_2$ integrin, $\alpha_X\beta_2$ integrin, $\alpha_V\beta_3$ integrin, and $\alpha_V\beta_5$ integrin (12).

In recent years, CD23 has been shown to play a central role in food allergic reactions. The transepithelial antigen transport was enhanced in allergic rats through a mechanism involving IgE and FcεRI/CD23 (207). CD23 has also been shown in multiple studies to facilitate IgE and IgE-derived immune complex (IC) transport across polarized human intestinal epithelial monolayers (82, 208, 209). A recent study further extends these observations and strengthens the role of CD23 in transepithelial antigen and IgE-IC transport in respiratory epithelial cells; the process was positively regulated by IL-4 (210). Collectively, antigen transport across epithelial barriers appears to be a hallmark function of CD23.

1.6.3.3. Low affinity Galectin-3/Mac-2/ε-BP

Galectin-3 also known as Mac-2 or ε-binding protein (ε-BP) is a secretory protein of

~30KDa belonging to the family of β-galactoside-binding lectins. It consists of a carbohydrate-recognition domain linked to a non-lectin region of proline- and glycine-rich tandem repeats. The name ε-BP came from the observation that it can bind to IgE as well as FcεRI. Indeed, differentially glycosylated IgE isoforms display distinct binding capacities for galectin-3. Besides, galectin-3 can also bind to other cell surface and extracellular matrix proteins (211-213). Galectin-3 is expressed on the cell surface of eosinophils, neutrophils, mast cells, macrophages, DCs, T cells, and B cells (213, 214). Interestingly, Th2 cytokines IL-4 and IL-13 enhance the alternatively activated macrophage expression of galectin-3, while LPS or IFN-γ can inhibit its release (215); eosinophils from allergic donors exhibit increased levels of galectin-3 (216).

Galectin-3 can activate various cell types either by directly binding at cell surface or through endocytosis leading to the modulation of intracellular signaling pathways. It is known to play a large role in inflammatory response including adhesion, migration, and activation of neutrophils, chemoattraction of monocytes and macrophages, migration of DCs, and regulation of apoptosis in immune cells (213, 214). Notably, extracellular galectin-3 can potently activate mast cells via cross-linking of FcεRI in an IgE-dependent or IgE–independent manner. In fact, galectin-3 can induce inflammatory mediator release from IgE-sensitized and non-sensitized mast cells and macrophages (211, 217).

1.6.4. IgE binding with its Fc receptors

IgE binding to its high affinity FcεRI receptor is bimolecular with a stoichiometry of 1:1 (218). The IgE- FcεRI complex has an association constant of Ka of $10^{10}M^{-1}$ which may be due to a slow dissociation rate with a half-life of about 20h for IgE and its Fc receptor. The half-life

of the complex on mast cells is further enhanced to ~14 days (11, 219). Interestingly, the crystal structure of IgE bound to FcεRI shows that the IgE molecule is acutely bent to a larger degree than anticipated, with the Cε2 domains folded back and making maximum contact with Cε3 and slightly touching the Cε4 domains (12, 220) **(Fig. 1.6.4)**.

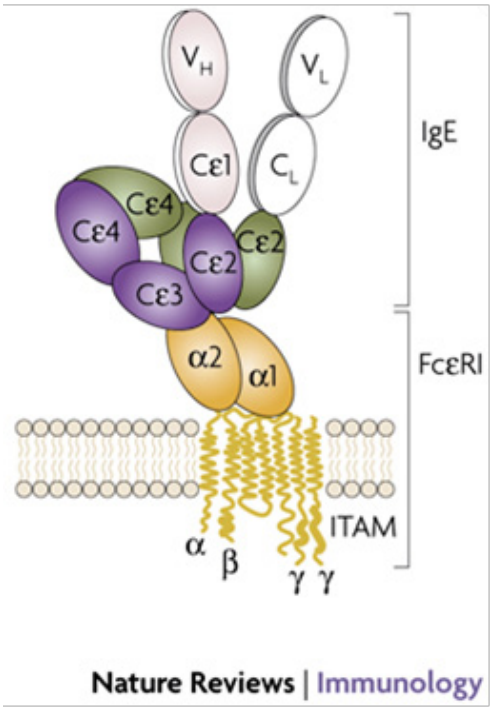

Figure 1.6.4.: Schematic representation of the entire IgE molecule bound to the extracellular domains of the FcεRI α-chain, according to the structural information from the Fcε3-4 complex and the bent IgE-Fc structure. The β- and γ-chains of FcεRI, with their immunoreceptor tyrosine-based activation motifs (ITAMs), are also shown (12).

The complex of IgE with a monomer of low affinity FcεRII/CD23 has a binding affinity of 2-7 x $10^{6\text{-}7}$ M^{-1} (221). Interestingly, the three molecules of CD23 head (trimer) leads to a cumulative avidity of Ka of ~$10^{8\text{-}9}$ M^{-1}, almost equivalent to the high affinity of FcεRI. Although the precise binding site of CD23 with IgE is not determined, it is assumed to lie in the Cε3 domains distinct from the FcεRI site, in accordance with a stoichiometry of 2:1 (two CD23 molecules are engaged by one IgE molecule) (12, 222).

1.6.5. Signal transduction through FcεRI

FcεRI-induced signaling pathways are best characterized in mast cells and basophils. The antigens cross-linking of IgE bound to FcεRI leads to a multitude of signaling pathways culminating in mediator release. In a simplified model of mast cell signaling **(Fig. 1.6.5)**, aggregation of FcεRI leads to the ITAMs phosphorylation on β- and γ- chains by Src family tyrosine kinase Lyn. ITAM phosphorylation then serves as a docking site for another Src homology domain 2 (SH2) containing kinase, spleen tyrosine kinase (Syk) which eventually leads to a conformational change of Syk leading to increase in its enzymatic activity (223). Syk can autophosphorylate at tyrosine residues which then leads to downstream propagation of signals (224). Syk then leads to tyrosine phosphorylation of the adaptors LAT1 and LAT2 (NTAL), activation of PI3K pathway and the protein tyrosine kinase Btk. Phosphorylated LAT serves as a scaffold for multiple signaling complexes including cytosolic adaptors such as Gads, Grb2, SLP76, Shc; guanosine triphosphate exchangers Sos, Vav1 (225); and the enzymes PLC-γ1 and PLC-γ2. With the help of Btk and PI3K, PLC-γ then hydrolyzes PI(4,5)P2 in the plasma membrane to form diacylglycerol (DAG) and IP_3 (226) **(Fig. 1.6.5)**.

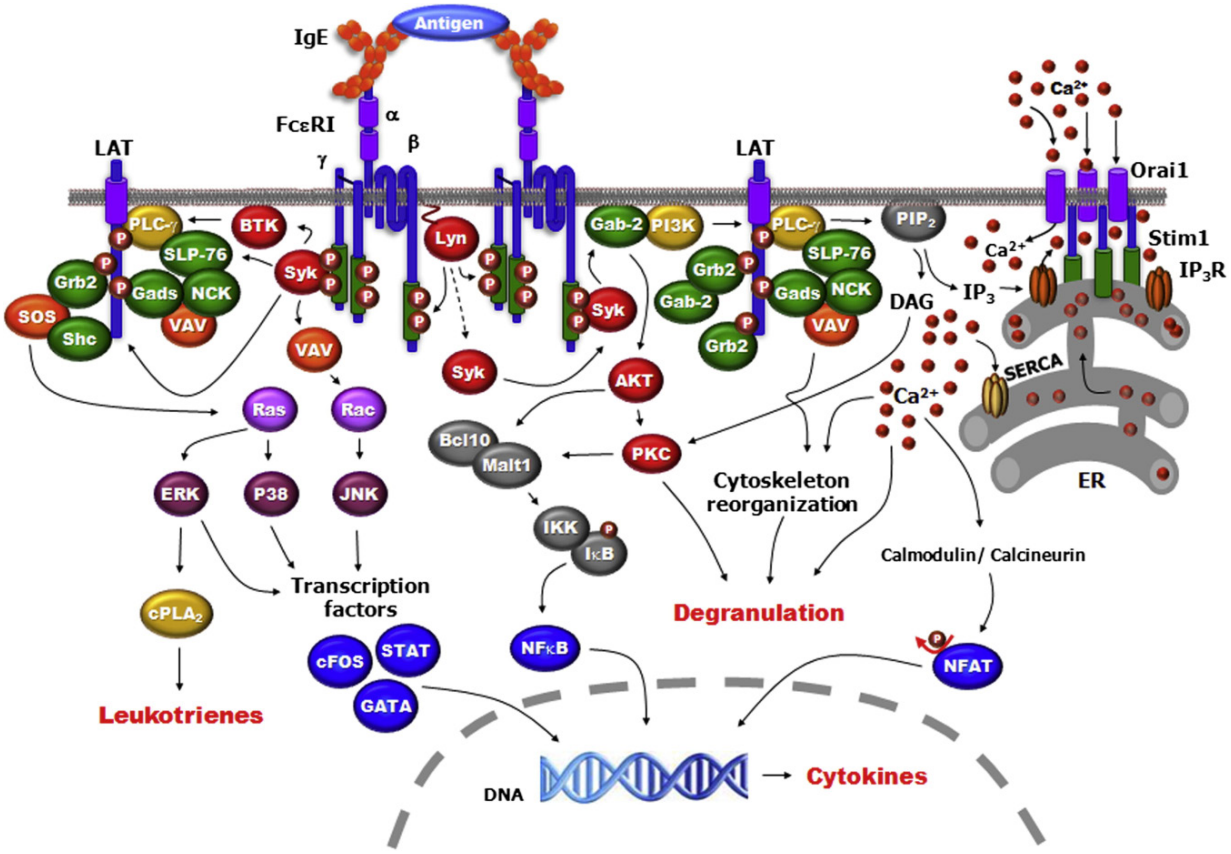

Figure 1.6.5.: A simplified model depicting major molecules and events in mast cell activation (227).

The IP_3 activates the IP_3 receptor on endoplasmic reticulum leading to the calcium release. The calcium sensor Stim-1 then interacts with Orai-1 which leads to the opening of the CRAC channels, eventually allowing the increase in ($[Ca^{2+}]i$) (228). DAG leads to the activation of PKC and also interacts with calmodulin to activate calcineurin leading to the transcription factor NFAT translocation into the nucleus to regulate the transcription of several cytokine genes. The activation of small GTPases such as Ras, Rac, and Rho leads to the stimulation of the Erk, JNK, and p38 MAPK pathways, which serve as signaling mediators between the cytosol and the

nucleus to regulate a variety of transcription factors. FcεRI cross-linking also leads to the phosphorylation and degradation of IκB which allows the release and nuclear translocation of the NF-κB pathway. Some other signaling molecules involved in this pathway include PKCβ, CARMA1, Bcl10, and MALT1. The activation of transcription factors NFAT and NFκB, and MAPK pathways leads to the synthesis of several cytokines. PKC and MAPKs also act on the cytosolic PLA2 which releases arachidonic acid that is metabolized by the cycloxygenase or the lipoxygenase pathways to synthesize the inflammatory mediators, prostaglandins, and leukotrienes (227) **(Fig. 1.6.5)**.

1.6.6. Effects of IgE sensitization (without allergen/antigen cross-linking)

Conventional dogma dictates that the FcεRI-mediated mast cell or basophil activation occurs in two steps. First step occurs when antigen-specific monomeric IgE binds to the FcεRI, the step itself called as 'IgE sensitization' as it sensitizes the mast cell to a specific allergen so that it can be activated immediately upon subsequent allergen exposure (53). Next, antigen/allergen (or anti-IgE in experimental settings) binding to this specific IgE cross-links the FcεRI (FcεRI 'cross-linking' or aggregation), consequently inducing various cell activation events such as degranulation, de novo synthesis and secretion of inflammatory mediators, and promotion of survival and migration (229) **(Fig. 1.6.5)**.

Recent studies, however, have established a new paradigm in which monomeric IgE binding alone (i.e. sensitization), in the absence of allergen/antigen or anti-IgE antibodies, can activate cells with multiple functional outcomes. In fact, these effects are not limited to mast cells or basophils alone. Two groups concluded from their independent studies that monomeric IgE can inhibit mast cell apoptosis (i.e. enhances survival) induced by growth factor removal

(230, 231). Cumulative evidence now suggests that IgE sensitization can induce multiple effects in murine and human origin mast cells such as upregulation and stabilization of FcεRI (230), increased histamine content (232), histamine and leukotriene release, cytokine and chemokine expression (IL-1β, IL-6, IL-8/CXCL8, MCP-1, -3, MIP-1β, GM-CSF, GRO-α, and -γ) (233-235), nitric oxide (NO)-generating synthases (NOS)-2, and -3 (236), membrane ruffling (237), and fibronectin adhesion and migration (238, 239). Although all of these effects are induced by antigen/anti-IgE-mediated FcεRI cross-linking as well, the 'sensitization' mode requires IgE concentrations of 2-3 logs higher than those required for 'cross-linking' mode (240). Interestingly, monomeric IgE was also shown to promote the survival of monocytes, and asthmatic neutrophils (241, 242).

Pro-survival and synthetic effects of IgE on mast cells are induced by activation of multiple signaling pathways such as Lyn, Fyn, and Syk activation, activation of PLC-γ, PI3K, phosphorylation of Erk, p38, JNK MAPKs, PKB, increase in intracellular Ca^{2+}, and nuclear translocation of NFAT (237, 239, 240). Evidence implicates that monomeric IgE induces these effects with certain degree of receptor aggregation (233). Studies, through using size-exclusion chromatography, high-speed centrifugation, and competitive binding between polymeric and monomeric haptens, have denied the occurrence of these effects due to 'IgE aggregates' formation prior to binding to FcεRI, thus mimicking the allergen/antigen cross-linking (237). Moreover, monomeric IgE was classified into two subtypes depending upon their 'level' of mast cell activation. The HC-IgE or 'highly cytokinergic' IgE, such as SPE-7 clone, not only prolonged mast cell survival but also enhanced degranulation, proliferation, migration (243), adhesion to fibronectin, leukotriene release, cytokine/chemokine (such as IL-6, TNF) release, activation of multiple signaling pathways, and FcεRI aggregation and internalization. On the

other hand, PC-IgE or 'poorly cytokinergic' IgE (H1DNP-ε-206 clone) affects only mast cell survival to a lesser extent (233, 239, 244-246). Most of the above described studies have dealt with the effect of monoclonal monomeric IgE, while the monomeric IgE in serum of atopic patients is polyclonal. Interestingly, a recent study suggests that the polyclonal monomeric IgE from the serum of atopic dermatitis patients induced higher levels of IL-8/CXCL8 release in human peripheral blood-derived mast cells compared with IgE from serum of healthy subjects, extending the role of monomeric IgE in modulation of mast cell function (247).

1.6.7. Regulation of FcεRI surface expression

Since the binding of IgE to FcεRI is the first step in allergic cascade, the amount of FcεRI present on cell surface determines the effector functions of inflammatory cells and thus the intensity of allergic reaction (248). FcεRI expression on cell surface is tightly regulated. Earliest observation in this context dates back to 1978 (249) when serum IgE levels were found to be closely associated with the amount of IgE bound to basophils. Later, monomeric IgE was shown to enhance the FcεRI expression on the surface of mast cells and basophils (250, 251). IgE-deficient mice expressed low levels of FcεRI on mast cells which could be markedly upregulated with IgE incubation in vitro or by IgE injection in vivo (252). Other studies later showed similar effects of IgE on surface expression of FcεRI on mast cells, basophils, monocytes, and DCs. Moreover, this IgE-induced upregulation leads to enhanced effector function of cells such as IgE-dependent mast cell mediator release and antigen presentation in DCs (252-256). Mechanistically, IgE-mediated upregulation of FcεRI is described to occur from stabilization of the already IgE-occupied receptors on the cell surface. This stabilization protects FcεRI from degradation while maintaining the basal synthesis, eventually leading to an increase in

accumulation of FcεRI at the cell surface. This accumulation occurs firstly from the use of a preformed receptor pool derived from recycled and recently synthesized receptors, and later, the receptor accumulation depends on the basal FcεRI protein synthesis. Interestingly, there is no evidence of IgE-induced transcription or translation of FcεRI (248).

The regulation of surface FcεRI expression is not restricted to IgE alone. Mast cells from the mice deficient in IgE still expressed basal levels of FcεRI (252), suggesting that there are other mechanisms which can regulate FcεRI expression. Cytokines, in particular Th2 signature cytokine, IL-4 induces the transcription of FcεRI-α chain in human eosinophils from AD patients (257), human mast cells (258), and human DCs (259). Indeed, a study of the distal promoter of human FCERIA gene confirmed that IL-4 increases the intracellular expression of FcεRI-α chain (260). Moreover, IL-9 induced the FcεRI expression in murine Th cells (261), and IL-9, IL-4, and GM-CSF upregulated the FcεRI-α chain expression in human asthmatic neutrophils (64). The presence of IL-3 during early differentiation stages, before granule formation, induced FcεRI expression in murine bone-marrow derived mast cells and human basophil precursors (262). In recent years, the neuropeptide substance P downregulated the surface expression of FcεRI and transcripts of α, β, and γ chains in human mast cells (263), TGF-β showed both inhibitory (in high dose) and stimulatory effects (in low dose) on FcεRI expression in cord blood CD34+ stem cell-derived CD1a+ dendritic cells DCs (264). Collectively, surface expression of FcεRI is highly regulated and the local inflammatory milieu in allergic diseases may play a critical role in this process.

Besides IgE and cytokine milieu, the assembly of FcεRI modular unit itself has several regulatory checkpoints. The mRNA transcripts of FcεRI subunits α-, β-, and γ-chains are not equally abundant and have expression levels in decreasing order of γ-, α-, and β-chains,

suggesting that these subunits are not coordinately synthesized (53). For instance, the co-expression of FcεRI-γ chain is a requirement for FcεRI-α chain to reach the surface and eventually for the FcεRI complex to function. In the absence of γ-chains, the α-chain is retained in its immature form in the ER; the former is also known to mask an ER-retention signal in the α-chain leading to its export and maturation (248, 253, 265-267). In addition, the classical full-length β-chain acts as an amplifier of human FcεRI cell surface expression by promoting maturation and trafficking of FcεRI-α chain and stabilizing the surface-expressed FcεRI complex (197). An alternative splice variant of FcεRI-β chain named truncated domain (FcR-βT) blocked the FcεRI-α subunit expression, impaired its association with γ-chains and promoted its degradation through ubiquitin-proteasome dependent pathways (268). Interestingly, βT isoform competed with the wild-type β-subunit during FcεRI assembly in ER, and prevented proper folding of the FcεRI-α chain, eventually decreasing FcεRI cell surface expression (269). Moreover, human basophils co-express both βT and wild-type β-chains in variable proportions, suggesting that the ratio of βT:β-chain could regulate FcεRI expression and thus influence the susceptibility to allergic disorders (268).

1.6.8. FcεR expression in ASM cells

Despite tremendous evidence from 1980s-1990s on atopic/IgE-rich serum-induced ASM responses (161), only recently these cells have been shown to express both low affinity FcεRII/CD23 and high affinity FcεRI (97). In attempts to investigate the mechanisms underlying altered ASM responsiveness in atopic-sensitized state, Hakonarson and colleagues showed that both human and rabbit ASM tissues constitutively express FcεRII/CD23 and Fc receptors for IgG, FcγRIII (CD16), FcγRII (CD32), and FcγRI (CD64). The FcεRII/CD23 expression was

upregulated in presence of atopic (IgE rich) serum or IgE-immune complexes. Interestingly, FcεRII/CD23 activation by IgE immune complexes or atopic serum induced 'pro-asthmatic' like changes in ASM responsiveness, such as increased contractility and attenuated relaxation (96, 174). Moreover, Rhinovirus (RV) and atopic sensitization displayed cooperative effects in enhancing the FcεRII/CD23 expression and subsequent induction of 'pro-asthmatic' like changes in ASM, potentially by ICAM-1-coupled LFA-1 signaling in ASM itself (270). The same group later showed that the IgE sensitization of ASM cells leads to autocrine release of IL-13 and IL-5, which may underlie the altered ASM responsiveness including heightened agonist-induced constrictor responsiveness and impaired β2-agonist-mediated relaxation (271-273). Other groups have also showed FcεRII/CD23 expression in ASM constitutively (98). Belleau and colleagues (2005) also showed constitutive FcεRII/CD23 expression in human ASM cells which was further upregulated by IL-4, GM-CSF, or both. Moreover, the cytokine-upregulated FcεRII/CD23 was also accompanied by changes in cell morphology such as depolymerization of actin fibers, cell spreading, and membrane ruffling, and an overall increase in cell volume and protein content, suggesting hypertrophy of ASM (274). So far, there is no report showing the expression of galectin-3/Mac-2/ε-BP in ASM cells, although a weak signal in immunostaining was observed in vascular smooth muscle regions of human atherosclerotic lesions (275).

Expression of tetrameric (αβγ2) FcεRI was demonstrated in bronchial/tracheal ASM cells in normal and asthmatic patients. FcεRI expression was detected in cultured ASM cells in vitro by using Western blotting (WB), flow cytometry, immunocytochemistry, immunofluorescence, and real-time RT-PCR; and in vivo by immunohistochemistry showing the FcεRI-α chain immunoreactivity in ASM of patients with mild atopic asthma. FcεRI cross-linking by IgE and anti-IgE antibodies led to Th2 (IL-4, -5, and -13) cytokines and CCL11/eotaxin-1 chemokine

release; and ([Ca^{2+}]i) mobilization, suggesting a likely IgE-FcεRI-ASM-mediated link to airway inflammation and AHR (98). Although one group denied the existence of both FcεRII/CD23 and FcεRI expression in ASM cells (276), at least two other groups have confirmed the FcεRI expression in ASM. One study showed an IL-13-inducible FcεRI-α chain mRNA expression in ASM cells in a microarray-screen (277). Roth and Tamm (2010) recently showed the expression of both FcεRII/CD23 and FcεRI on ASM cells, and clearly depicted an IgE-induced ASM synthetic function which was inhibited by a therapeutic monoclonal anti-IgE antibody (278-280). Collectively, IgE appears to modulate ASM responsiveness through the activation of FcεRI receptor and deserve further investigation to appreciate the role of this pathway in allergic airway disease.

1.7.0. THYMIC STROMAL LYMPHOPOIETIN (TSLP): A NOVEL PLAYER IN TH2 INFLAMMATION

TSLP is a novel hematopoietic, interleukin 7 (IL-7)-like pro-allergic cytokine, originally isolated from a murine thymic stromal cell line (281) and characterized as a lymphocyte growth factor (282). The human TSLP gene is located on chromosome 5q22.1 next to the atopic cytokine cluster on 5q31 (283). Both human and mouse TSLP exert their biologic activities by binding to a high-affinity TSLPR complex that is a heterodimer of TSLPR chain which is closely related to the common receptor γ chain (γc) and interleukin 7 receptor-α (IL-7Rα) **(Fig. 1.7.0)** (284-286). Although the sequence homology for both TSLP and its receptor between mouse and human is only about 40%, their biological functions appear to be similar (287).

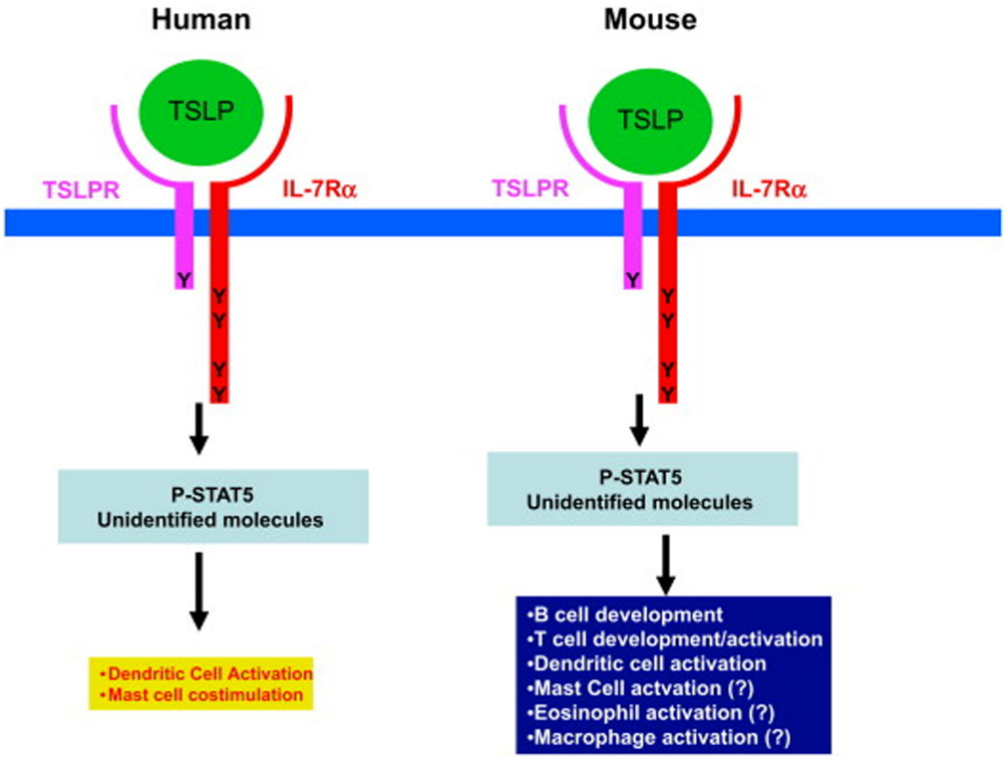

Figure 1.7.0.: Structure of the TSLP receptor complex. Heterodimer of TSLPR and IL-7Rα. TSLP stimulation induces activation and phosphorylation of STAT5 (P-STAT5), as well as activation of other as yet unidentified pathways. TSLP function: in human beings, TSLP directly activates DCs by upregulating MHC class I and II molecules and costimulatory molecules, promotes cell survival, and induces secretion of chemokine. TSLP also directly costimulates mast cells to produce proinflammatory Th2 cytokines in the presence of TNF-α and IL-1. In mice, TSLP has additional functions, including promoting B-cell and T-cell development and promoting activation of T cells, mast cells, eosinophils, and macrophages (284).

Initially discovered as thymic epithelial cell-derived cytokine, TSLP is now known to be produced by epithelial cells in the lungs, gut and skin, fibroblasts, airway smooth muscle, endothelial cells, mast cells, macrophages, granulocytes (288-293), synovial fibroblasts, intervertebral disc cells (294), and more recently by dendritic cells (DCs) (295). Of great interest, airway structural cells including ASM appear to be a rich source of this potent pro-allergic cytokine (296). The expression of TSLP is regulated by several factors including both exogenous stimuli including trauma, mechanical injury, infection with microbes, Toll-like receptor (TLR)

ligation, and host-derived pro-inflammatory and Th2 cytokines (297, 298). TSLPR activation involves components of different signaling pathways such as protein kinases, MAPKs, JAKs, and transcription factors (STAT3, STAT5) in different cell types. Atopic diseases can be classified into a triad of asthma, allergic rhinitis, and atopic dermatitis (AD) (299). Although these share a common pathogenesis pattern characterized by common allergic effectors such as Th2 cytokines, IgE, mast cells, and eosinophils, the mechanisms underlying a preferential 'Th2' response in atopic patients still remain obscure (300). Interestingly, high levels of TSLP have been detected in all of these atopic conditions (293, 301, 302). Emerging interest has also depicted a significant role of TSLP in inducing protective Th2-dependent immunity against helminth infections. In *Trichuris muris* helminth infection model, the disruption of TSLP/TSLPR interaction (by using TSLP blocking antibody or deletion of TSLPR) in normally resistant wild-type mice leads to lower expression of pathogen-specific Th2 cytokine responses which result in a failure to control the infection. This response occurs by mechanisms involving enhanced expression of IL-12p40 subunit, IFN-γ, IL-17A, and the development of severe infection-induced inflammation (300, 303).

1.7.1. TSLP in atopic diseases

The first indication of a role of TSLP in allergic inflammatory response came from AD patients, in whom epidermis of lesional skin has higher TSLP expression than that of epidermis from uninvolved skin or skin from people with nonallergic dermatitis (302). Mouse model studies further showed that inducible TSLP expression in epidermis leads to spontaneous AD-like disease which displayed all the cardinal features of human AD disease (304). TSLP was found to be necessary and sufficient to drive Th2 cytokine-mediated airway inflammation in

murine models of asthma. Lung-specific expression of a TSLP transgene induced allergic inflammation characterized by a massive infiltration of inflammatory cells, goblet cell hyperplasia, and subepithelial fibrosis in airways, with increased serum IgE levels (305). On the contrary, TSLPR deficiency in mice failed to develop asthma in response to inhaled antigen (ovalbumin plus alum), probably due to the inability of CD4+ T cells to respond to TSLP, as reconstitution with TSLPR-positive T cells restores the aspects of the inflammatory disease (306).

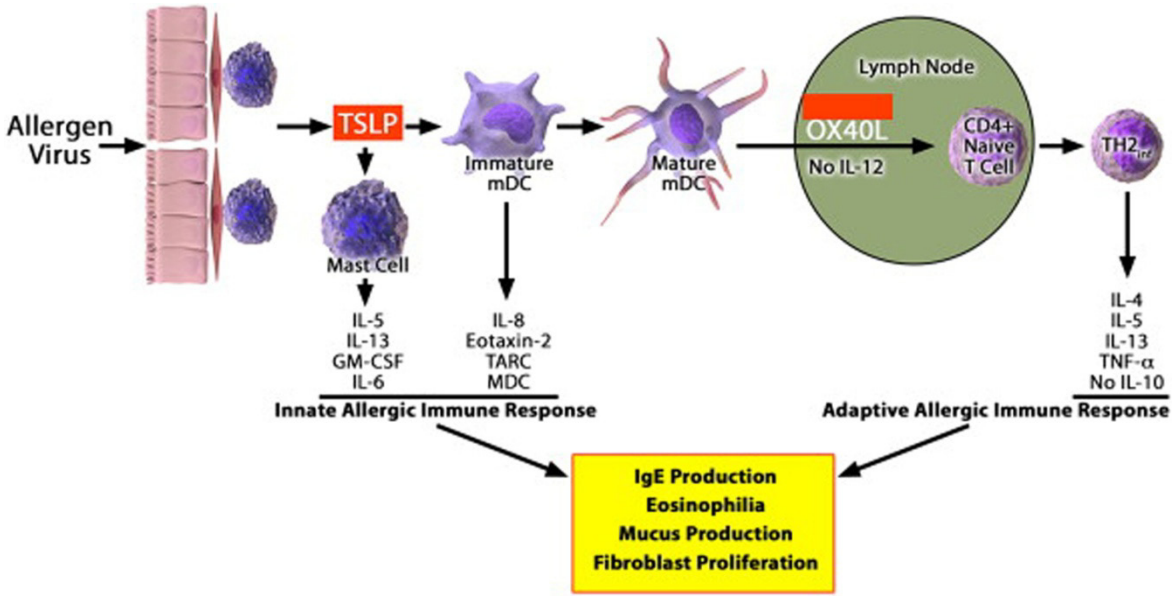

Figure 1.7.1.: Pathophysiology of TSLP in allergic inflammation. Insults from allergens or viruses trigger mucosal epithelial cells or skin cells (keratinocytes, fibroblasts, and mast cells) to produce TSLP. TSLP initiates the innate phase of allergic immune responses by activating immature DCs to produce the chemokines IL-8/CXCL8, eotaxin-2, and the Th2 attracting chemokine TARC and MDC and by costimulating mast cells to produce IL-5 and IL-13, as well as GM-CSF and IL-6. TSLP-activated mDCs mature and migrate into the draining lymph nodes to initiate the adaptive phase of allergic immune responses. TSLP-activated DCs express OX40L, which triggers the differentiation of allergen-specific naïve CD4+ T cells to inflammatory Th2 cells that produce IL-4, IL-5, IL-13, and TNF but not IL-10. Inflammatory Th2 cells then migrate back to the site of inflammation because of the local production of TARC and MDC. The Th2 cytokines IL-4, IL-5, IL-13, and TNF-α, produced by the inflammatory Th2 cells, initiate allergic inflammation by triggering IgE production, eosinophilia, and mucus production (284)

Mechanistically, TSLP-stimulated myeloid DCs (mDCs) can prime naïve CD4+ T cells in an antigen-specific manner, resulting in T cells showing characteristic features of Th2-differentiated inflammatory cells (i.e. production of IL-4, IL-5, IL-13 and TNF), with the exception of IL-10 production (302). Interestingly, in the absence of IL-12, TSLP induces the expression of OX40L, the ligand for cell survival factor OX40 on DCs; and OX40-OX40L interactions were found to be critical for the ability of the mDCs to drive Th2 cell differentiation (307). Besides activating mDCs and inducing proallergic CD4+ or CD8+ responses (302, 308), TSLP can also synergize with IL-1 and TNF to induce Th-2 cytokine and chemokine (IL-5, IL-13, IL-6, GM-CSF, CXCL8, and CCL1) expression in mast cells (289). A brief outline describing the key mechanisms of TSLP pathophysiology in allergic inflammation is provided (284) **(Fig. 1.7.1)**.

1.7.2. TSLP in chronic obstructive pulmonary disease (COPD)

Although many studies have reported a preponderance of Th-1 cytokines in COPD, numerous studies also show a Th2-biased response in the airways, especially in subjects with chronic bronchitis (309). This Th2 response appears to be modulated by CD8+ T cells. Interestingly, human TSLP wasss showwwnnn to prime DCs to induce naïve CD8+ T cell differentiation into IL-5-producing cells (308). With emerging evidence for TSLP in inflammatory diseases, an enhanced TSLP expression was observed in ASM bundles of COPD patients (292). Ying et al (291) showed an elevated mRNA expression of TSLP, and chemokines TARC/CCL17, MDC/CCL22, and IP-10/CXCL10 in bronchial mucosa, with corresponding elevation of these proteins in the BAL fluid of COPD patients. Collectively, it is indeed

surprising to find elevated TSLP expression in COPD, which is conventionally thought to be a 'Th-1-dominated' inflammatory disease, and therefore merits investigation that can demarcate the exact role of TSLP in this airway pathology.

1.7.3. TSLP expression in ASM cells

ASM cells express TSLP constitutively *in vitro* and in ASM bundles of COPD patients *in vivo*. Proinflammatory cytokines TNF, and IL-1β enhanced the TSLP mRNA and protein release, which was abolished upon the use of pharmacological inhibitors of MAPK (p38 and ERK1/2) in ASM cells (292). Various other groups have also confirmed the expression of TSLP in ASM cells; which is modulated by proinflammatory and Th2 cytokines, β2-agonists (296), and cigarette smoke extract (310). However, the detailed molecular mechanisms regulating TSLP expression in ASM remain to be elucidated. A significant part of this project deals with the triggers and molecular signaling mechanisms of TSLP expression and TSLP-induced activation of ASM.

1.8.0. SUMMARY OF LITERATURE

- Asthma affects about 1-18% of the population worldwide. An estimated 8.3% of Canadians over the age of 12 and about 15.6% of children aged 4–11 years have physician-diagnosed asthma.
- GINA defines asthma as a chronic condition in which inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness and coughing particularly at night or in the early morning. These episodes are usually associated

with wide spread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment.

- Therapeutically, inhaled corticosteroids, leukotriene modifiers, and β2 agonists are the primary therapies. Recently approved therapies include the use of a novel anti-IgE antibody Omalizumab/Xolair^{TM}, and bronchial thermoplasty.
- Asthma resulting from immunological reactions is referred to as allergic asthma and is mostly initiated by IgE antibodies (atopy). Eighty percent of childhood asthma and >50% of adult asthma is reported to be allergic.
- An imbalance in Th1/Th2 profile of immune responses, thus a preponderance of Th2 (IL-4, IL-5, and IL-13 cytokines, increased IgE synthesis) character has been considered a critical determinant of asthma, besides genetic and environmental factors such as the elements of 'hygiene hypothesis'.
- IgE exerts its biological effects through binding to the Fc receptors of high affinity (FcεRI), and the low affinity (FcεRII/CD23) and galectin-3/Mac-2/ε-BP receptors.
- In a classical IgE-dependent reaction, mast cells or basophils are first primed by allergen-specific IgE on their surface (sensitization phase), then get activated when a polyvalent allergen/agent binds to the IgE bound to FcεRI (challenge or effector phase) leading to the degranulation and inflammatory mediator release.
- FcεRI can be expressed as either tetrameric ($\alpha\beta\gamma_2$ complexes, as in mast cells, basophils, and rodents) or trimeric ($\alpha\gamma_2$ complexes, in APCs, eosinophils, platelets, and epithelial cells in human). FcεRI-mediated response is amplified by the agents that can enhance FcεRI abundance on cell surface. These factors include serum IgE levels, cytokines IL-4, IL-9, and

GM-CSF. Moreover, FcεRI-β- and -γ-chains assembly is critical in finally enabling FcεRI complex expression on cell surface.

- When atopic asthmatics are challenged with a specific allergen (to which they are sensitized), they have a biphasic response. The first or early phase occurs within minutes to 2 hours and causes increased mucus secretion, vasodilation, stimulation of the nerve ends and bronchospasm; while the late phase response appears after about 8 hours of allergen challenge and manifests as airway narrowing and mucus hypersecretion. The latter also reflects the local recruitment and activation of Th2 cells, eosinophils, basophils and other leukocytes, and persistent mediator production by resident cells.
- Contemporary evidence suggests that IgE binding alone (i.e. sensitization itself) can activate various murine and human cells such as mast cells and basophils; induce plethora of signaling pathways, and impart survival benefits to mast cells, asthmatic neutrophils, and monocytes.
- Aside from the 'classical' inflammatory immune cells, the resident airway structural components have gained considerable interest as determinants of asthma pathophysiology.
- The role of airway smooth muscle (ASM) in healthy lung is a matter of debate while it is agreed widely that ASM manifests as the 'bad guy' in airway pathology in asthma. The contraction of ASM causes acute narrowing of the airway and airflow obstruction in asthma, the smooth muscle mass is increased in airways of patients with asthma, and ASM from asthmatic patients demonstrate enhanced secretary function.
- Nonetheless, ASM displays intrinsic phenotype plasticity (contractile and synthetic-proliferative) and respond to mitogens (like PDGF, FGF, IGF) and proinflammatory cytokines (e.g. IL-1, TNF) to synthesize multiple pro-asthmatic mediators including

cytokines (e.g. IL-4, IL-5, IL-13, IL-6), chemokines (such as CCL11/eotaxin-1, CXCL8/IL-8, CXCL10/IP-10, CCL5/RANTES), and ECM proteins.

- Early studies suggest that ASM sensitization with serum from atopic subjects (rich in IgE) induce greater shortening velocity, increased isotonic shortening, and increased abundance and activity of MLCK. Later, serum IgE levels were shown to be associated with ASM hyperreactivity, abnormal contractile function, airway reactivity in vitro, and AHR in vivo.
- ASM cells express FcεRI, FcεRII/CD23, and Fc receptors for IgG such as FcγRIII (CD16), FcγRII (CD32), and FcγRI (CD64).
- TSLP is a novel hematopoietic, IL-7-like pro-allergic cytokine. High levels of TSLP have been detected in asthma, allergic rhinitis, and atopic dermatitis patients. Mouse model studies further show that lung-specific TSLP expression leads to an 'asthma-like' phenotype, and mice lacking TSLPR failed to develop these features in response to inhaled antigen, likely due to the inability of CD4+ T cells to respond to TSLP.
- Recently, ASM cells were shown to express TSLP constitutively in vitro and in COPD in vivo.

2.0. CHAPTER 2

2.1.0. STUDY RATIONALE

Asthma is a chronic inflammatory disease which affects an estimated 15% of children and more than 8% of adults in Canadian population. Clinically, asthma manifests as chronic airway inflammation, airway obstruction, enhanced bronchial responsiveness, and airway remodeling. Besides inflammatory cells, ASM cells have emerged as key determinants of asthma owing to their ability to contract aggressively in response to inflammatory cell products, synthesize pro-asthmatic mediators, and grow rapidly to culminate into airway tissue remodeling, a hallmark feature of allergic asthma (93, 161). Despite numerous studies clearly showing the relationship of asthma to specific allergens, only recently total serum IgE level were documented to be associated with airways hyperreactivity and atopic asthma (311). Recent studies on ASM have proposed that these cells may contribute to airway inflammation, AHR, and remodeling in asthma by mechanisms depending on IgE-Fc receptor interaction, involving the low affinity FcεRII/CD23 activation (96, 174, 272), and more recently the high affinity FcεRI. It was shown that the ASM cells in vitro and in vivo are capable of expressing a tetrameric FcεRI (αβγ2) and its activation led to marked transient increases in intracellular Ca2+ concentration, Th-2 cytokines (IL-4, IL-5 and IL-13) and CC chemokine (eotaxin-1/CCL11) release in ASM cells (97, 98).

Although substantial research in recent years has been performed on FcεRI in immune cells (53, 227, 248), the factors that influence FcεRI expression and the contribution of this receptor in ASM cell functions have not been investigated. It is not clear whether FcεRI-induced signaling events in ASM are comparable to those in mast cells. Importantly, it is largely unknown and debatable whether ASM can participate in airway immune response via IgE/FcεRI

pathway. Given the emerging role of TSLP as a master switch of Th2-dominated allergic inflammation (312), it is a novel and enticing possibility to evaluate the effect of IgE on ASM TSLP expression. Moreover, it is unclear whether IgE sensitization via FcεRI alone confers survival or proliferation advantage to ASM cells, as has been demonstrated in past in monocytes (241), mast cells (230, 231) and, asthmatic neutrophils (242) which may influence the cell growth and/or proliferation.

2.2.0. GLOBAL HYPOTHESIS

The IgE-dependent activation of ASM cells via FcεRI is a critical determinant of ASM cell synthetic, contractile, and proliferative/survival functions, which may eventually contribute to airway inflammation and remodeling.

2.3.0. SPECIFIC HYPOTHESES

FcεRI expression in ASM cells is modulated by local airway milieu; IgE-mediated FcεRI activation on ASM induces selective gene expression of CC and CXC chemokines, and pro-allergic TSLP; and IgE induces ASM cell proliferation.

2.4.0. OVERARCHING GOAL AND SPECIFIC AIMS

The overarching goal of this study is to elucidate the molecular regulation and function of FcεRI, a central molecular component in allergic asthma, expressed by human ASM.

Specifically, this study investigates the regulatory role of proinflammatory (TNF, IL-1β) and Th2 (IL-4) cytokines in IgE-binding FcεRI-α chain expression and subsequent IgE-induced ASM activation *(Chapter 4)*; the molecular mechanisms of TNF-induced TSLP expression in

ASM *(Chapter 5)*; to examine the effect of IgE sensitization on TSLP expression in ASM and relevant signaling mechanisms *(Chapter 6)*; and finally to understand the effect of IgE on ASM proliferative response and IgE-induced signaling pathways *(Chapter 7)*.

3.0. CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1.0. ETHICS STATEMENT

All the experimental procedures were approved by the Human Research Ethics Board of the University of Manitoba, Winnipeg, Manitoba, Canada. Written informed consent for ASM harvesting was obtained from all patients.

3.2.0. REAGENTS

3.2.1. Chemicals

Recombinant human TNF (or TNF-α), IL-1β, IL-4, recombinant human TSLP, and platelet-derived growth factor-BB (PDGF-BB) were purchased from R&D Systems (Minneapolis, MN, USA). Fetal bovine serum (FBS), and sodium pyruvate were purchased from HyClone (Logan, UT, USA). 100X L-glutamine, Dulbecco's modified Eagle's medium (DMEM), Ham's F-12, trypsin-EDTA, and antibiotics (penicillin, streptomycin) were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). Unless stated otherwise, all other reagents were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

3.2.2. Antibodies

Recombinant human IgE was obtained from Diatec (BioPorto Diagnostics A/S, Denmark). Murine anti-human FcεRIα-chain mAb, 15/1 (313), was kindly donated by Dr. Franz Kricek (NOVARTIS Institute for Biomedical Research GmbH & Co KG, Vienna, Austria). Sheep polyclonal anti-human TSLP Ab was purchased from R&D Systems (Minneapolis, MN). Rabbit anti-human p38 MAPK mAb, rabbit anti-human ERK1/2 mAb, affinity-purified rabbit

anti-phospho-p38 MAPK (T180/Y182), affinity-purified mouse anti-phospho-ERK1/2 (T202/Y204), and rabbit anti-phospho and total-specific SAPK/JNK (T183/Y185) Abs, rabbit mAb phospho-Akt (Ser 473) and total-Akt antibody were purchased from Cell Signaling Technology, Inc (Danvers, MA). Mouse mAb anti-phospho-tyrosine STAT3 (Y705) was from BD Biosciences (Mississauga, ON), and affinity purified rabbit anti-total STAT3, and rabbit polyclonal anti-Syk (C-20) antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse IgG1 isotype control (clone MOPC21) was from Sigma (Oakville, ON).

3.2.3. Inhibitors

The p38 MAPK inhibitor, SB-203580 [4-(4-fluorophenyl)-2-(4-methyl-sulfinylphenyl)-5-(4'-pyridyl)-1H-imidazole], the JNK inhibitor, SP-600125 [anthra(1,9-cd)pyrazol-6(2H)-one; 1,9-pyrazoloanthrone], and the p42/p44 ERK inhibitor, U-0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene], were purchased from Calbiochem (Mississauga, ON, Canada). The glycogen synthase kinase 3 beta (GSK-3β) inhibitor 3-[[6-(3-aminophenyl)-1H-pyrrolo[2,3-d]pyrimidin-4-yl]oxy]-phenol (TWS119) was purchased from OTAVA Ltd., Toronto, ON, Canada. Cell-permeable peptide inhibitors and control peptides of IκB kinase, NF-κB, and Akt inhibitor VII, TAT-*Akt-in* were purchased from CALBIOCHEM® (EMD), San Diego, CA, USA. Description of these peptides is provided:

P1- IκB kinase inhibitor peptide (Ac-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala- Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Asp-Asp-Arg-His-Asp-Ser-Gly-Leu-Asp-Ser-Met-Lys-Asp-Glu-NH_2; cat# 401477);

P2- IκB kinase inactive control peptide (Ac-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala- Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Asp-Asp-Arg-His-Asp-Ala-Gly-Leu-Asp-Ala-Met-Lys-Asp-Glu-NH_2;

cat# 401478). P1 contains the IκB recognition sequence for IκB kinases (IKK) and a cell-permeable peptide sequence, derived from the hydrophobic region of the signal peptide fibroblast growth factor. P2 is a control peptide containing a mutated IκB sequence, linked to the same carrier peptide (314). Compared to P2, the P1 specifically inhibits LPS-induced IκB degradation by IKK in RAW 264.7 cells (< 50 μg/ml) and prevents NF-κB activation.

P3- NF-κB SN50 inhibitor peptide (H-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala- Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Val-Gln-Arg-Lys-Arg-Gln-Lys-Leu-Met-Pro-OH; cat# 481480);

P4- NF-κB SN50M inactive peptide control (H-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala- Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Val-Gln-Arg-Asn-Gly-Gln-Lys-Leu-Met-Pro-OH; cat# 481486). P3 contains the nuclear localization sequence (NLS) of the transcription factor NF-κB p50 linked to the hydrophobic region (h-region) of the signal peptide of Kaposi fibroblast growth factor (K-FGF). The N-terminal K-FGF h-region confers cell-permeability, while the NLS (360-369) inhibits translocation of the NF-κB active complex into the nucleus. In murine endothelial LE-II cells, LPS-induced NF-κB nuclear translocation is maximally inhibited at 18 μM (315).

3.2.4. Promoter constructs

Plasmids encoding luciferase reporter driven by respective wild-type chemokine promoters were kindly gifted (pGL3-EO2 eotaxin-1/CCL11 pr by Dr. Jutta Horejs-Hoeck, Institute for Chemistry and Biochemistry, Salzburg, Austria; pUHC13-3-IL-8pr-wild type by Dr. Michael Kracht, Medical School Hannover, Hannover, Germany; pGL3-RANTES-Luc wild-type by Dr. Akira Andoh, Shiga University of Medical Science, Seta-Tukinowa, Otsu 520-2192, Japan; pGL3-IP-10 (-533) by Dr. Daniel A Muruve, University of Calgary, Calgary, Alberta, Canada; pGL3-basic vector (Promega, Madison, WI) encoding wild-type human TSLP promoter,

other constructs containing mutations in the human TSLP promoter, and plasmids containing dominant-negative mutant of IKKβ (referred to as dnIKKβ), and NF-κB/p65 overexpressing plasmids were kindly provided by Dr. Steven F. Ziegler, Benaroya Research Institute, Seattle, WA, USA) (290). The mutations in the binding sites for human NF-κB (GggaAACTCCA changed to GttcAACTCCA), human activator protein 1 (AP-1) (TGTcaTTG changed to TGTtgTTG) were verified by DNA sequencing analysis (290).

3.3.0. PREPARATION OF HUMAN AIRWAY SMOOTH MUSCLE CELLS

Three sources of human airway cells, namely bronchial smooth muscle (BSM), tracheal smooth muscle (TSM), and hTERT-immortalized bronchial smooth muscle cells were used. For ease of understanding, all of these cells will be collectively referred in this thesis as airway smooth muscle cells (ASM or ASMC or HASM/HASMC for human ASM). ASM cells were prepared as follows. Human bronchial smooth muscle cells were obtained from macroscopically healthy segments of 2-4th generation lobar or main bronchus of patients undergoing surgery for lung adenocarcinoma (Dr. H Unruh, Section of Thoracic Surgery, University of Manitoba, Canada). These cells were used as such and were classified as 'primary ASM/BSM cells' (316). The expression of human telomerase reverse transcriptase (hTERT) is known to extend the life span of endothelial cells, fibroblasts, and smooth muscle cells (317-319). To extend the life span of cultured ASM/BSM cells, primary and low-passage cultures were infected with a retrovirus vector encoding the hTERT gene. The hTERT expression was confirmed in immortalized cells by RT-PCR using telomerase-specific primers. Immortalized cells were passaged up to 60 times with no evidence of senescence (319, 320).

Primary human tracheal smooth muscle (HTSM) cells were obtained from macroscopically healthy segments of the trachea during post-mortem in Respiratory Hospital at Health Sciences Centre, Winnipeg, MB, Canada, and were isolated and cultured similar to primary HBSM cells (98, 152, 319). At confluence, ASM cells demonstrated spindle-shaped morphology and a hill-and-valley pattern, characteristic of smooth muscle in culture. Moreover, ASM cells at confluence retain smooth muscle-specific actin, SM22, and calponin protein expression and mobilize intracellular Ca2+ in response to acetylcholine, a physiologically relevant contractile agonist (152). In all the experiments, primary ASM cells were used at passages 2-5, and hTERT cells at passages 10-17.

3.3.1. Cell Stimulation

For all experiments, low passage ASM cells grown on uncoated plastic dishes in complete DMEM (DMEM supplemented with 100µg/ml streptomycin, 100U/ml penicillin, and 10% fetal bovine serum) were used. Unless otherwise mentioned, cells were grown to a subconfluent (~70%) condition and serum starved to synchronize and growth-arrest at G_0/G_1 phase for 2 days (48h) in Ham's F12 supplemented with 100µg/ml streptomycin, 100U/ml penicillin, and 1X ITS (5µg/ml insulin, 5µg/ml transferrin, and 5ng/ml selenium) before each experiment. Cells were then stimulated in fresh FBS-free Ham's F12 medium containing human recombinant IL-1β, TNF-α, IL-4, IgE or vehicle (medium alone) for time periods specific to experiments.

3.4.0. RNA ISOLATION AND RT-PCR

Forty eight hour serum-deprived confluent ASM cell cultures were harvested, and total cellular RNA was extracted using TRIzol® method (Invitrogen Canada Inc., Burlington, ON). Briefly, 5-10 x 10^6 cells were homogenized/lysed in 1 ml TRIzol® reagent on ice; organic-aqueous phase separation was performed by adding 0.2 ml chloroform per 1 ml TRIzol® and shaking vigorously at room temperature (RT) and centrifugation at 12000 xg for 15 minutes at 4°C. The aqueous phase was separated into a fresh RNase-free tube and 0.5 ml isopropyl alcohol per 1 ml TRIzol® was added before incubation at RT for 10 minutes and centrifugation at 12000 xg for 10 minutes. Supernatant was discarded; the RNA pellet was washed with 1 ml of 75% alcohol per 1 ml of TRIzol®, vortexed once, and centrifuged at 7500 xg for 5 minutes at 4°C. Supernatant was discarded; the RNA pellet was air-dried and resuspended in 30-50 μl of RNase-free water. Total RNA was quantified by taking absorbance at 260nm and 280nm, or directly with NanoDrop (Thermo Scientific, Wilmington, DE, USA) instrument. Reverse transcription was performed by using 2 μg of total RNA in a first-strand cDNA synthesis reaction with High Capacity cDNA Reverse transcriptase kit as recommended by the supplier (Applied Biosystems, Foster City, CA, USA). Oligonucleotide primers were synthesized on the basis of the entire coding region of human genes FcεRI-α, FcεRI-γ, TSLP, or GAPDH as described in aim-specific methods. The RT-PCR was conducted in a thermal cycler 'Mastercycler' (Eppendorf Canada, Mississauga, ON). Each cycle included denaturation (94°C, 1 min), annealing (primer-specific T°C, 1 min), and extension (72°C, 1 min 30 s). The initial denaturation period was 5 min, and the final extension was 10 min. The size of the amplified fragment was analyzed by DNA gel electrophoresis in 1.5% agarose and visualized by ethidium bromide staining under ultraviolet illumination. The specificity of the amplified bands was confirmed by nucleic acid sequencing (data not shown). GAPDH was amplified as internal control.

3.4.1. RT-PCR Primers and specific PCR conditions

Oligonucleotide primers were synthesized on the basis of the entire coding region of human FcεRI-α (GenBank accession no. NM 002001.2) as follows: Forward primer 5'-CTCCATTACAAATGCCACAGTTG-3' and Reverse primer 5'-CACGCGGAGCTTTTATTACAGTA-3'; and for human FcεRI-γ (GenBank accession no. NM004106) were: Forward primer, 5′-CCA GCA GTG GTC TTG CTC TTA C-3′ and reverse primer, 5′-GCA TGC AGG CAT ATG TGA TGC C-3′. Primers for human housekeeping gene, glyceraldhyde-3-phosphate dehydrogenase (GAPDH) are forward primer 5'-AGCAATGCCTCCTGCACCACCAAC-3' and reverse primer 5'-CCGGAGGGGCCATCCACAGTCT-3'. Human TSLP primer were synthesized (GenBank accession no.: NM_033035) as follows: forward primer, 5'-TATGAGTGGGACCAAAAGTACCG-3'; and reverse primer, 5'-GGGATTGAAGGTTAGGCTCTGG-3'. The PCR for (FcεRI-α, 35 cycles; FcεRI-γ, 30 cycles; GAPDH, 25 cycles) was conducted in a thermal cycler 'Mastercycler' (Eppendorf Canada, Mississauga, ON). Each cycle included denaturation (94°C, 1 min), annealing (FcεRI-α, 59°C, 1 min; FcεRI-γ, 64°C, 1 min and; GAPDH, 55°C, 1 min), and extension (72°C, 1 min 30 s). The initial denaturation period was 5 min, and the final extension was 10 min. The size of the amplified FcεRI-α, -γ and GAPDH fragment was 116 bp, 338 bp and 137 bp, respectively (321). The PCR (for TSLP, 35 cycles; GAPDH, 25 cycles) was carried out in a thermal cycler (Mastercycler, Eppendorf). Each cycle had denaturation (94°C, 1 min), annealing (TSLP, 62°C, 1 min; GAPDH, 55°C, 1 min), and extension phase (72°C, 1 min 30 s). The initial denaturation period was kept 5 min, and the final extension was for 10 min. The size of TSLP and GAPDH

amplified fragments was 97 bp and 137 bp, respectively (292, 322). GAPDH was amplified as internal control.

3.4.2. Real-Time RT-PCR Analysis

The FcεRI-α and GAPDH standards were prepared using PCR-amplified cDNA from a human basophilic cell line (KU812, ATCC® # CRL-2099™). PCR products were isolated from 2% w/v agarose gel using QIAEX II Agarose Gel Extraction kit (Qiagen Inc., Mississauga, ON, Canada). The primers used were same as we used for RT-PCR, as mentioned above. The amount of extracted cDNA was quantified by spectrophotometry and expressed as copy number. A serial dilution was used to generate each standard curve. Real-time quantitative PCR was carried out by ABI 7500 Real-Time PCR System and analyzed by 7500 System SDS software version 1.3.1 (Applied Biosystems, Foster City, CA, USA), following manufacturer's instructions. Product specificity was determined by melting curve analysis and by visualization of PCR products on agarose gels. Calculation of the relative amount of each cDNA species was performed according to standard protocols of absolute quantification. Briefly, the amplification of FcεRI-α gene in stimulated cells was calculated first as the copy number ratio of FcεRI-α to GAPDH, and then expressed as normalized values of fold increase over the value obtained with unstimulated (control) cells. The relative quantification protocol (Applied Biosystems, Foster City, CA, USA) was followed for TSLP mRNA expression analysis as per the instructions by the manufacturer.

3.5.0. IMMUNOPRECIPITATION AND WESTERN BLOT

B/TSM FcεRIα-chain protein expression was analyzed by immunoprecipitation and

Western blotting with minor modifications from the protocol described earlier (64). Briefly, B/TSM cells were lysed for 30 min 4°C in NP-40 lysis buffer supplemented with a cocktail of protease inhibitors (2mM sodium orthovanadate, 1mM phenyl-methylsulfonylfluoride, 10 μg/ml leupeptin, 0.15 units/ml approtinin, 1 μg/ml pepstatin A) (Sigma-Aldrich) and centrifuged for 20 min to remove nuclei. Cell lysates from B/TSM or basophilic cell line KU812 (positive control) were pre-cleared with protein G sepharose-coated beads (Amersham-Pharmacia) for 2h at 4°C in a rotating mixer, followed by incubation with protein G sepharose-coated beads conjugated with 2 μg/ml of murine anti-human FcεRIα mAb 15/1 or isotype mouse IgG1 monoclonal antibody (MOPC 21) for 16h at 4°C. Immuno-complexes were then pelleted by centrifugation and washed six times with the wash buffer (PBS /1% NP40). For immunoblotting, samples were separated on SDS polyacrylamide gel (11-13%) and electro-transferred onto PVDF membrane (Millipore, Mississauga, ON). The membrane was blocked at RT for 2 hrs with 5% Blotto, (Santa Cruz Biotechnology, CA, USA), incubated with rabbit anti-human FcεRIα-chain polyclonal Ab (Upstate Biotechnology, Inc., Lake Placid, NY) (1 μg/ml) at room temperature for 2 h, followed by secondary antibody HRP-goat anti-rabbit IgG (H+L) prepared in TBST (1:5000). The blots were developed by enhanced chemiluminescence as recommended by the supplier (Amersham Pharmacia, ON). A band non-specific to FcεRI in the same gel was used as loading control. On the other hand, 15 μg of B/TSM and KU812 cell lysates were directly loaded onto 14% SDS polyacrylamide gel (11-13%) and electro-transferred onto PVDF membrane. The membrane was blocked at RT for 2 hrs with 5% Blotto, incubated with goat anti-human FcεRI-γ polyclonal Ab (K-16 clone) (Santa Cruz, CA) prepared in TBST (1:500) at RT for 2h, followed by secondary antibody HRP-rabbit anti-goat IgG whole molecule (Sigma-Aldrich) prepared in TBST (1:5000), and the blots were developed as described above. After stripping, the blots were probed for

GAPDH and used as a loading control. The corresponding values of pre-stained protein molecular weight marker were scaled to the FcεRI-α and -γ protein bands. The intensity of FcεRI-γ bands was determined by using AlphaEase FC software version 3.1.2 relative to control loading levels. IgE-induced ASM signaling pathways were studied by performing Western blotting for phosphorylated MAPK and STAT3, as described (323, 324). Briefly, sub-confluent HASM cells were growth-arrested by serum deprivation for 48 h as described above. HASM cells were then stimulated in fresh FBS-free medium with IgE (10 μg/ml) or medium alone. At indicated time points, cells were washed once with chilled PBS, and total proteins were extracted with lysis buffer. Harvested lysates were centrifuged for 10 min at 4°C to remove cellular debris. The supernatants were collected and stored at –70°C. Protein lysate (10 μg) was loaded onto 10% SDS-PAGE, followed by transfer to PVDF membranes (Amersham Pharmacia, Baie D'Urfe, Quebec, Canada). The blots were then blocked with 5% nonfat dry milk in TBS/0.1% Tween 20 (TBST) for 1 h at RT, and then incubated overnight at 4°C with Abs specific for phosphorylated STAT3, ERK1/2, p38, and JNK. After washing with TBST, the blots were subjected to incubation with goat anti-mouse or goat anti-rabbit HRP-conjugated secondary Abs and bands were revealed with ECL reagents (Amersham Pharmacea). After stripping, total anti-STAT3, -ERK, -p38, and -JNK were used as loading controls. Densitometric analysis was performed on gels and integrated density value (IDV) was presented as the fold-increase in phosphorylated over total levels compared to time zero.

3.6.0. CYTOKINE AND CHEMOKINE ELISA FROM ASM CELL SUPERNATANTS

In order to study the functional significance of cytokine stimulated FcεRIα-chain upregulation, primary human B/TSM cells were grown until 65% confluency in 12-well culture

plates and 48 h serum-deprived cells were then stimulated for 48 h with recombinant human IL-1β, TNF-α, IL-4 (10 ng/ml each) or vehicle (medium alone). The supernatants were removed and cells were washed twice with serum-free Ham's F12 media. Thereafter, the cells in each treatment group were either left unstimulated (medium alone) or stimulated with mIgG1 (1 μg/ml) or recombinant human IgE (1 μg/ml) for 24 h under the similar culture conditions. In some experiments, cell-permeable peptide inhibitors for IκB kinase (P1), NF-κB (P3) and related control peptides (P2, P4) were used at 10μg/ml concentration for 1 hour at 37°C prior to stimulation with TNF-α (10ng/ml) (292, 322). ASM cell culture supernatants were collected at specified times, centrifuged at 1200rpm for 7 min at 4^{o}C to remove cellular debris, and stored at -80^{o}C until further use. Immunoreactive TSLP, eotaxin-1/CCL11, IL-8/CXCL8, IP-10/CXCL10 (10 kDa interferon-gamma-induced protein), RANTES/CCL5 (Regulated upon Activation, Normal T-cell Expressed, and Secreted), and TARC/CCL17 (Thymus and activation-regulated chemokine) released into ASM culture supernatants were quantified using ELISA with matched Abs according to basic laboratory protocol provided by the manufacturer (R&D Systems, Minneapolis, MN, USA). Cytokine and chemokine proteins were quantified in reference to serial dilutions of recombinant standards falling within the linear part of the standard curve for each specific chemokine sample measured. The sensitivity limits of these chemokine assays are 3.5 pg/ml for TSLP (292, 322), 5 pg/ml for eotaxin-1/CCL11, 3 pg/ml for IL-8/CXCL8, 7.8 pg/ml for IP-10/CXCL10, 7.8 pg/ml for RANTES/CCL5 and 7.8 pg/ml for TARC/CCL17 (321). Each data point is a representative of readings from three separate assays.

3.7.0. LENTIVIRAL VECTOR TRANSDUCTION IN ASM CELLS

For short-hairpin RNA (shRNA)-induced gene silencing studies, pseudotyped lentiviral vectors expressing specific shRNA (Syk-shRNA clone IDs: V2LHS_2250 and V2LHS_153702; STAT3-shRNA clone ID: V2LHS_262105) were obtained from Open-Biosystems (Huntsville, AL) (321, 325, 326). In these vectors, microRNA-adapted shRNA (shRNAmir or shRNA) are cloned into pGIPZ lentiviral vector to create an extremely powerful RNAi trigger capable of producing RNAi in most cell types including primary and non-dividing cells. Human embryonic kidney (HEK) 293T cells used for virus production and titration were cultured in Iscove's modified Dulbecco's medium (IMDM) (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin/glutamate (PSG) (Gibco, Grand Island, NY). Lentiviruses were generated using 293T cell lines using calcium phosphate-mediated transfection, and used for transduction of ASM cells as described elsewhere (327). In brief, 17.5 $\times 10^6$ 293T cells were transfected with 5 μg of pCMV-VSV-G (envelope vector), 12.5 μg of pCMVΔR8.2DVPR (packaging vector), and 12.5 μg of the lentivirus expression vector pGIPZ (Open-Biosystems). Culture supernatant containing the lentiviruses was collected on 3rd day post-transfection, pooled, and filtered through a 0.22-μm-pore-size filter. Lentivirus particles were ultra-centrifuged at 17,000rpm for 90 minutes at 4^{o}C to concentrate to 1X and then diluted to required concentration. A control shRNA unrelated to target sequence (scramble shRNA) was used as a transduction control.

Lentivirus titers were determined by infection of 293T cells, followed by flow analysis of the percentages of turbo-green fluorescent protein (tGFP)+ cells on day 3 after infection. Briefly, 293T cells (5×10^4/well) were cultured in a 24-well plate overnight (reaching to 1x10^5 cells next day) and were infected with pseudotyped lentivirus particles by incubating the cells with 250μl of virus supernatant (at appropriate dilutions, usually undiluted [1X] and 1/10X) in the presence

of Polybrene (8 μg/ml) at 37°C for 2 h. The virus supernatant was then removed and replaced with 500μl of complete IMDM. The percentage of tGFP+ 293T cells was analyzed on day 3 after infection. The lentivirus titre was calculated as: (1 x10^5 293T cells [the input of cells]) x (%/100 Transfected cells [use as decimal]) x (4 [brings to 1 ml from original 250μl]) x (Dilution factor). This formula will provide the lentivirus titre in plaque forming units (PFU)/ml.

The multiplicity of infection (MOI) is defined as the number of transducing lentiviral particles per cell, and is calculated as: (total number of cells per well) x (desired MOI) = total infectious units (IU). The total IU is multiplied by lentivirus titre to get the volume of lentivirus needed for transduction. For silencing target protein expression, ASM cells were transduced at an MOI of 10 in the presence of polybrene (8 μg/ml). Briefly, cells were exposed to recombinant lentivirus for 2 hr at 37°C, medium replaced and cultured for additional 72 hrs. Transduced cells were selected with puromycin. The average transduction efficiency was determined by flow cytometry analysis using the tGFP as the marker for cell sorting, and found to be more than 95%. Viability of the transduced cells undergoing experiment was >98% as assessed by trypan blue dye after completion of the experiment.

3.8.0. PROMOTER LUCIFERASE REPORTER ASSAY

ASM cells (4 x 10^4) were plated into 12-well culture plates in fresh complete DMEM. At 50-70% confluency, cells were transfected with plasmid constructs containing mutated or wild-type promoter sequences for human gene of interest, such as TSLP, eotaxin/CCL11, IL-8/CXCL8, IP-10/CXCL10 or RANTES/CCL5. Transient transfection of ASM cells was performed using ExGen 500 or TurboFect^{TM} *in vitro* transfection reagent (MBI Fermentas, ON,

Canada) according to the manufacturer's instructions. In each well, 1.6 μg of wild-type or transcription factor binding site-mutated gene promoter DNA and 0.4 μg of *Renilla* luciferase reporter vector pRL-TK (Promega, Madison, WI) were co-transfected for 24 h. The cells were washed, medium was changed to Ham's F12 supplemented with 100μg/ml streptomycin, 100U/ml penicillin, 5μg/ml insulin, 5μg/ml transferrin, and 5ng/ml selenium, and cells stimulated with human IgE or cytokines such as TNF (10ng/ml), IgE (10 μg/ml), IL-1β (10 ng/ml) or mouse IgG1 (mIgG1-MOPC21) (10 μg/ml). Since IL-1β is known to induce multiple cytokines/ chemokines gene expression in human ASM cells (323, 328-330), it was used as a positive control for promoter activity assays in some experiments. In some experiments, cells were pretreated for 40 minutes with R112 (2μmol/L), an ATP-competitive Syk inhibitor (inhibitory constant [K(i)] = 96 nmol/L) (kindly provided by the Department of Chemistry of Rigel Pharmaceuticals, Inc.) (331) or with DMSO vehicle before stimulation with IgE (10 μg/ml) for 12h. We also used another related Syk inhibitor, R406, which is more potent than R112 and has shown efficacy in animal models of allergic asthma (332) (data not shown). Cells were then harvested in passive lysis buffer, and the luciferase activity was measured by the Dual-Luciferase Assay System kit (Promega, Madison, WI) using a luminometer (model LB9501; Berthold Bad Wildbad, Germany). Briefly, 20 μl of cell lysate was mixed with 100 μl of Luciferase Assay Reagent II and firefly luciferase activity was first recorded. Then, 100 μl of Stop-and-Glo Reagent was added, and *Renilla* luciferase activity was measured. All values were normalized to *Renilla* luciferase activity and expressed relative to the control transfected non-stimulated cells.

3.9.0. ANALYSIS OF CELL SIGNALING PATHWAYS

3.9.1. Phospho-Mitogen activated protein (MAP) kinase array

Forty eight hour serum-starved HASM cells were incubated with IgE (10 μg/ml) for 0, 5, 15, and 30 minutes, and lysates were collected. For the parallel determination of the relative levels of phosphorylation of MAPK and other serine/threonine kinases, human Phospho-MAPK array kit (Proteome Profiler^{TM}, R&D Systems) was used according to the manufacturer's instructions. Levels of proteins were visualized by chemiluminiscence and quantified by densitometry analysis using AlphaEaseFC (FluorChem 8800) (Genetic Technologies, Inc., Miami, FL, USA) for Windows.

3.9.2. Western blotting to assess MAPK and STAT3 phosphorylation

To validate the MAPK array phosphorylation data, IgE-induced ASM signaling pathways were studied by performing Western blotting for phosphorylated MAPK and STAT3, as described earlier (324). Briefly, nearly confluent HASM cells were serum starved and were then stimulated in fresh FBS-free medium with IgE (10 μg/ml) or medium alone. At indicated time points, HASM cells were washed once with ice-cold PBS, and total proteins were extracted with lysis buffer. Harvested lysates were centrifuged for 10 min at 4°C to remove cellular debris. The supernatants were collected and stored at –70°C. Protein lysate (10 μg) was loaded onto 10% SDS-PAGE, followed by transfer to PVDF membranes (Amersham Pharmacia, Baie D'Urfe, Quebec, Canada). The blots were then blocked with 5% nonfat dry milk in TBS/0.1% Tween 20 (TBST) for 1 h at RT, and then incubated at 4°C overnight with Abs specific for phosphorylated ERK1/2, p38, JNK, Akt and STAT3. After washing with TBST, the blots were subjected to incubation with goat anti-mouse or goat anti-rabbit HRP-conjugated secondary Abs and bands were revealed with ECL reagents (Amersham Pharmacea). After stripping, total anti- ERK, -p38,

-JNK, -Akt, and -STAT3 were used as loading controls.

3.10.0. ASM CELL PROLIFERATION ASSAYS

3.10.1. Hemocytometer-based counting

Equal numbers of HASM cells were seeded in 6-well plates up to ~70% confluency. Cells were serum-deprived in Ham's F12 containing 1X ITS media for 48 h to growth-arrest and synchronize them. Fresh F12 containing 1X ITS was added and cells were stimulated with graded doses of IgE and other mitogens for 40 h. 10% FBS or platelet-derived growth factor- BB (PDGF-BB, R&D Systems) was used at 10ng/ml concentration (following optimization) as a positive control. Cells were trypsinized after 40h and subjected to hemocytometer-based cell counting.

3.10.2. Thymidine (^{3}H)-incorporation Assay

Tritiated thymidine (^{3}H)-incorporation assay was performed to assess the IgE-induced DNA synthesis as a surrogate marker of ASM proliferation by following the method of Goncharova and colleagues (333) with minor modifications. Briefly, ASM cells were seeded in 24-well tissue culture plates (3 x 10^4 cells/well) and allowed to grow for 2-3 days in complete DMEM to reach about 70% confluency in a 37^{o}C humidified 5% CO_2 incubator. Cells were serum-deprived in Ham's F12 containing 1X ITS media for 48 h to growth-arrest them in G_0/G_1 phase of the cell cycle. Fresh F12 containing 1X ITS was added and cells were stimulated with graded doses of IgE and other mitogens for 16 h. Platelet-derived growth factor- BB (PDGF-BB, R&D Systems) was used at 10ng/ml concentration (following optimization) as a positive control inducer of ASM DNA synthesis. After 16 h, *methyl*-^{3}H-thymidine (Specific activity 20Ci [740

GBq]/mMole, >97%; Perkin Elmer, Woodbridge, ON, Canada) was added at a final concentration of 2μCi/ml and cells were incubated at 37^{o}C for 24 h. Subsequently, radioactive media was discarded; ASM cells were rinsed in 1ml PBS three times before adding 0.1 ml 0.05% trypsin-EDTA for 15 minutes at 37^{o}C for lysis, followed by addition of 0.1 ml ice-cold 20% trichloroacetic acid (TCA) for 20 minutes at 4^{o}C to precipitate the DNA. Precipitated DNA was then carefully transferred to 96-well plates to facilitate its absorption on 96-well format glass fibre filter mats (Wallac, Turku, Finland) using Tomtec Harvestor 96 (Model-Mach III; Tomtec Inc., Hamden, CT, USA). Filter mats were air-dried and counted in liquid scintillation counter by one of these means: (i) by placing the filter mat in sealer bags with 5 ml scintillation fluid and counting directly in a 96-well format beta scintillation counter Wallac 1450 Microbeta Trilux (Perkin Elmer, Waltham, MA, USA); or by carefully picking individual dried pieces from 96-well filter mat and placing in single scintillation vials with 2-5 ml scintillation fluid, followed by counting in (ii) a multi-purpose scintillation counter LS6500 (Beckman Coulter Canada, Inc., Mississauga, ON), or in (iii) Wallac 1414 Microbeta Trilux (Perkin Elmer) all-purpose digital liquid scintillation counter. Experiments were performed in triplicate and the data was presented as mean $\pm$ SEM of counts per minute (cpm).

3.11.0. STATISTICAL ANALYSIS

All the data were obtained from experiments performed three or more times. Statistical analysis was performed by using GraphPad Prism Software Version 3.02 for Windows (GraphPad Software Software, San Diego, CA, USA). Association between data from subgroups was studied by using Mann-Whitney U test, and Student's unpaired *t* test. P values <0.05 were considered statistically significant.

4.0. CHAPTER 4

PROINFLAMMATORY AND TH2 CYTOKINES REGULATE THE HIGH AFFINITY IgE RECEPTOR (FcεRI) AND IgE-DEPENDANT ACTIVATION OF HUMAN AIRWAY SMOOTH MUSCLE CELLS

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N.S.R. drafted and generated 70% of the data required for the realization of this manuscript.

4.1.0. INTRODUCTION AND RATIONALE

Classically, TNF-α and IL-1β are prototype pro-inflammatory cytokines, which have been shown to play a central role in lung inflammation both in animal models and allergic patients (334-337). Th-2 cytokines are also known to play a critical role in pathogenesis of allergic disorders, and have been investigated extensively in strategies targeting respiratory diseases (338). In particular, IL-4, in combination with IL-13 is known to induce Th-2 humoral immune responses leading to IgE production by B-cells (12).

We have previously demonstrated that HASM cells express the tetrameric (αβγ2) FcεRI and its activation leads to transient increases in intracellular $Ca2^{+}$ concentration, and release of IL-4, IL-5, IL-13 and the CC chemokine eotaxin-1/CCL11 (98). FcεRI-α-chain, a member of the immunoglobulin superfamily, contains the binding site for its ligand (IgE) while both β and γ chains are responsible for the downstream signal propagation through the phosphorylation of their immunoreceptor tyrosine-based activation motif (ITAM) (53). Extensive studies in inflammatory cells suggest that the amount of FcεRI-α present on cell surface determines the effector functions of these cells and thus the intensity of allergic reaction (248). Besides the ligand IgE itself, various other factors control the regulation of FcεRI-α expression. These include β- and γ-subunits of FcεRI, cytokines, and growth factors present in local milieu (64, 248). ***Taking into account the proinflammatory and Th-2 cytokine milieu in allergic inflammation and expression of FcεRI on ASM, we hypothesized that the expression of FcεRI is regulated by local inflammatory factors which may also regulate the IgE-mediated synthetic function of human ASM cells.***

Here we demonstrate that TNF-α, IL-1β, and IL-4 induce the FcεRI-α mRNA and protein expression in human bronchial/ tracheal smooth muscle (B/TSM, referred collectively as

HASM for ease) cells. The functional studies demonstrated that the IgE stimulation of cytokine pre-sensitized HASM cells significantly augmented the selective CC and CXC chemokine expression. Interestingly, Lentivirus mediated spleen tyrosine kinase (Syk) silencing abrogated the IgE sensitization-induced transcription of selective CC and CXC chemokines at promoter level. Cumulatively, these results suggest the potentially novel mechanisms of FcεRI regulation, portraying the critical role of HASM associated IgE/ FcεRI complex in allergic airway inflammation.

4.2.0. RESULTS

4.2.1. TNF-α, IL-1β, and IL-4 regulate the FcεRI-α chain mRNA Expression in HASM cells

We previously speculated the probable modulation of FcεRI expression in ASM cells by proinflammatory and Th2 cytokines (97, 98). In the present study, HASM cells stimulated with IL-1β, TNF-α, or IL-4 showed significantly enhanced FcεRI-α mRNA expression (Figure 4.1A). In contrast to cytokine stimulation, the basal FcεRI-α mRNA expression was uniform and unaffected by time of culture. FcεRI-α mRNA increased expression was then confirmed by quantitative real-time RT-PCR analysis. As shown in Figure 4.1B, HASMCs stimulated with TNF-α, and IL-1β upregulated the FcεRI-α transcript expression by 45.0 ± 4.5-, and 28.2 ± 3.8-fold, respectively, compared to unstimulated cells at 2 h. Interestingly, the mRNA expression was downregulated at 6 h but again gained peak at 20 h; whereas TNF-α inducing the maximum expression (32.4 ± 2.1- fold). Notably, IL-4 stimulation steadily upregulated the FcεRI-α mRNA expression (2.44 ± 0.28- fold, 2h; 2.43 ± 0.29-fold, 6h; and 2.79 ± 0.7-fold, 20h) compared to the control in HASM cells (Figure 4.1B). On the other hand, the mRNA expression for FcεRI-γ chain was upregulated by TNFα only at 2h (Figure 4.1A). Collectively, this data suggest that

proinflammatory and Th-2 cytokines can potentially regulate the transcription of FcεRI in HASM cells.

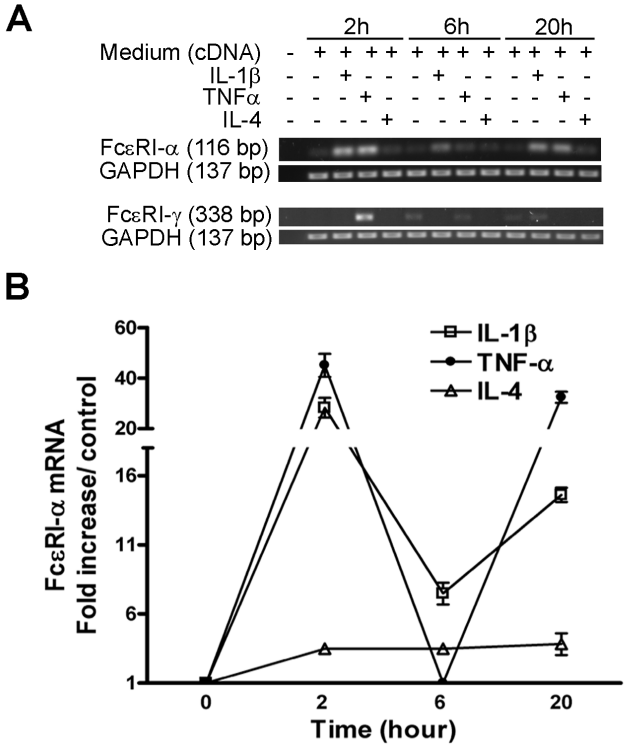

Figure 4.1. Proinflammatory and Th-2 cytokines upregulate the FcεRI mRNA expression in HASM cells. HASM FcεRI-α and –γ mRNA expression was analysed by (A) RT-PCR and (B) by Real-time RT-PCR for FcεRI-α chain. GAPDH was used as internal control for (A) and to normalize the FcεRI-α copy number for (B) as described in *Materials and Methods*. Each data point (except for TNF-α at 6 h) represents a significant ($p<0.05$, n=3) increase in copy no. over unstimulated control. P values were calculated using Mann Whitney U test.

4.2.2. TNF-α, IL-4 upregulate the FcεRI-α protein expression in HASMCs

To investigate the effect of IL-1β, TNF-α, and IL-4 stimulation on FcεRI-α and -γ protein expression, serum-deprived HASM cells were stimulated with TNF-α, IL-1β, or IL-4 and subjected to immunoprecipitation and Western blot. As demonstrated in Figure 4.2A, TNF-α and IL-4 stimulation enhanced the ~45 KDa (referred to be the intracellular) (53, 64) FcεRI-α protein expression at 24 h compared to unstimulated or IL-1β-stimulated HASMCs.

Immunoprecipitation from basophilic cell line KU812 also revealed a positive band at ~45 KDa (Figure 4.2A).

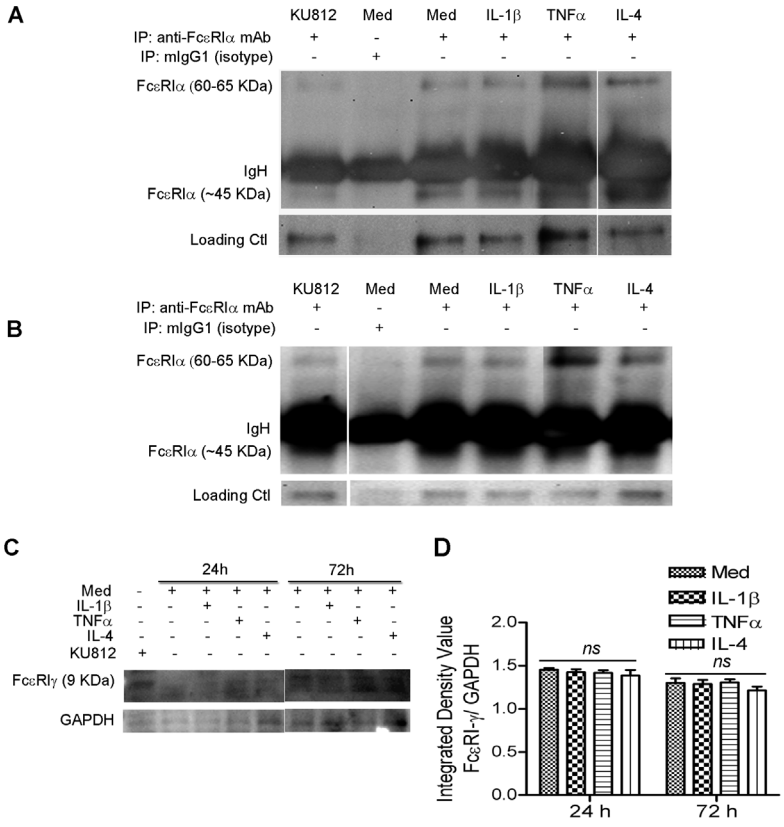

Figure 4.2. FcεRI-α protein expression (24, 72h) is upregulated by proinflammatory and Th-2 cytokines. Two-day serum-deprived primary HASM cells were cultured in presence or absence of IL-1β, TNF-α or IL-4. FcεRI-α protein from (A) 24 h and (B) 72 h culture cell lysates was Immunoprecipitated (IP) followed by Western blotting, as described in *Materials and Methods* section. FcεRI-α protein was immunoprecipitated with either anti-FcεRIα mAb 15/1 or with isotype antibody mouse IgG1 (MOPC21) for negative control. Non-specific bands from the same gels were used as loading control. (C) FcεRI-γ protein was analyzed by Western blotting and (D) presented as the ratio of 9 KDa FcεRI-γ-specific protein bands intensity over GAPDH (as internal control). Human basophilic KU812 cells were used as a positive control. Gel lanes were rearranged to bring in order from the same gel. *ns*, non-significant ($p>0.05$).

We then explored the effect of chronic (72 h) cytokine stimulation on FcεRI-α protein expression. As shown in Figure 4.2B, TNF-α, and IL-4 stimulation for 72 h led to the upregulation of FcεRI-α protein in HASM cells. TNF-α stimulation remarkably augmented the

~45 KDa intracellular and 60-65 KDa (referred to be as surface chain) (339) FcεRI-α protein compared to unstimulated HASM cells (Figure 4.2B). Chronic IL-4 stimulation also augmented the FcεRI-α protein expression, both for ~45 KDa and ~60-65 KDa bands (Figure 4.2A and B). In contrast to FcεRI-α and TNF-mediated FcεRI-γ mRNA upregulation, no significant upregulation in protein was observed (Figure 4.2C and D). As expected, KU812 cell lysate revealed both FcεRIα intracellular and surface, and FcεRI-γ protein bands and served as a positive control. Importantly, immunoprecipitation with isotype control antibody mIgG1-MOPC21 did not show any conspicuous bands of FcεRI-α either of ~45 KDa or 60-65 KDa at 24 or 72 h stimulation. Collectively, our data demonstrate that TNF-α and IL-4 upregulate the FcεRI-α chain protein expression in HASM cells.

4.2.3. TNF-α pre-sensitization augments the eotaxin-1/CCL11, IL-8/CXCL8, IP-10/CXCL10, RANTES/CCL5 but not TARC/CCL17 release in HASM following IgE stimulation

We then investigated the functional consequences of FcεRI-α protein upregulation by TNF-α, or IL-4 in HASM cells. Since we have previously demonstrated that IgE-dependent activation of HASM cells induces the CCL11 release (98), it was enticing to assess whether TNF-α or IL-4-mediated FcεRI-α protein upregulation followed by IgE stimulation can augment the chemokines release by HASM cells. Therefore, HASM cells were first sensitized with TNF-α, IL-1β, or IL-4 for 48 h, washed and then stimulated with IgE, mIgG1 (MOPC21) or left unstimulated for 24 h in fresh medium. CC (CCL11, CCL5, and CCL17) and CXC (CXCL8 and CXCL10) chemokines released in supernatants were then measured by ELISA. Since IL-1β did not induce a marked FcεRI-α protein expression (Figure 4.2A and B), it is plausible that it would not affect the subsequent chemokine release following IgE stimulation. Interestingly, TNF-α pre-

sensitized HASM cells released significantly elevated ($p<0.05$, n=3) levels of eotaxin-1/CCL11, IL-8/CXCL8, IP-10/CXCL10, and RANTES/CCL5 following IgE stimulation compared to IgE-unstimulated cells (Figure 4.3 A, B, C and D). Moreover, IL-4 pre-sensitization followed by IgE stimulation also enhanced the eotaxin-1/CCL11 release significantly ($p<0.01$, n=3, Figure 4.3A). We, however, did not see any significant change in TARC/ CCL17 release in TNF-α, IL-1β, or IL-4 pre-sensitized and IgE stimulated HASM cells (data not shown), suggesting the selective nature of FcεRI activation-induced chemokine expression. These results, therefore, suggest that TNF-α, in particular, upregulates the FcεRI-α expression as considerably that subsequent stimulation of HASM with IgE engages and activates the FcεRI strongly, leading to enhanced selective chemokine release.

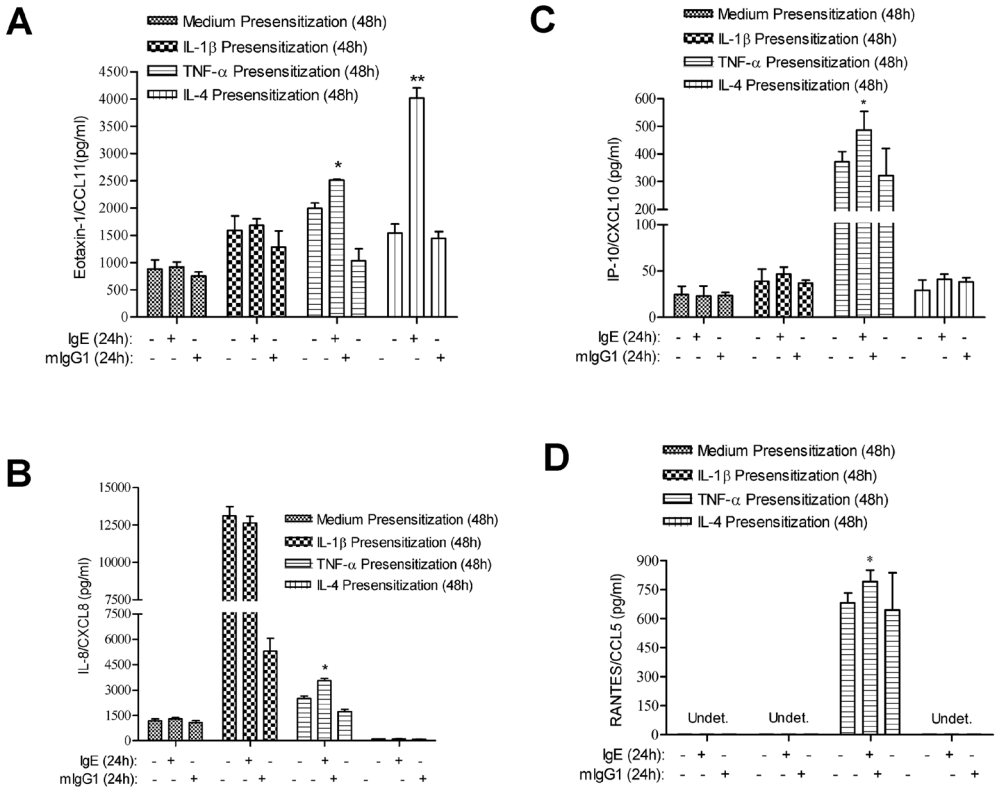

Figure 4.3. TNF-α pre-sensitization augments multiple CC and CXC chemokines release following IgE stimulation. Primary HASM cells were cultured in presence of 10ng/ml each of

TNF-α, IL-1β, or IL-4 for 48 h. Cells were then washed twice, and stimulated with either IgE (1μg/ml), mIgG1-MOPC21 (1 μg/ml) or left unstimulated (medium alone) for another 24 h. Culture supernatants were used for (A) eotaxin-1/ CCL11, (B) IL-8/CXCL8, (C) IP-10/CXCL10, and (D) RANTES/CCL5 measurement by ELISA. Data represents mean ± SD of three independent experiments performed under the same conditions. Mann-Whitney *U* test was performed to analyze the differences between the samples. **P*<0.05, ***P*<0.01.

4.2.4. IgE sensitization induces eotaxin-1/CCL11, IL-8/CXCL8, IP-10/CXCL10, and RANTES/CCL5 promoter activation in HASM cells

To unravel the mechanism by which IgE engagement of FcεRI on cytokine pre-sensitized HASM leads to enhanced CC and CXC chemokine release, we then tested whether IgE alone can induce promoter activity of CCL11, CXCL8, CXCL10 and CCL5 in HASM cells. HASM cells were transiently transfected with the luciferase reporter constructs driven by respective wild-type chemokine promoters. As shown in Figure 4.4, IgE stimulation of HASM cells significantly enhanced the promoter activity of eotaxin-1/CCL11, 1.42 ± 0.13-fold; IL-8/CXCL-8, 1.46 ± 0.11-fold; IP-10/CXCL10, 1.33 ± 0.05-fold; and RANTES/CCL5, 1.32 ± 0.09-fold, (n=3 $p<0.05$) compared to the unstimulated control. Murine isotype mIgG1 (MOPC21) stimulation failed to induce the promoter activity for any of the chemokines tested. Furthermore, as reported earlier in structural cells including ASMC (323, 328-330), IL-1β stimulation strongly induced the promoter activity of eotaxin-1/CCL11, 2.17 ± 0.17-fold; IL-8/CXCL-8, 3.26 ± 0.30-fold (n=3, $p<0.01$); IP-10/CXCL10, 1.65 ± 0.03-fold; and RANTES/CCL5, 1.49 ± 0.02-fold, (n=3, $p<0.05$) compared to the unstimulated control (Figure 4.4). Altogether, these results demonstrate that the IgE induces CC and CXC chemokine gene expression in HASM cells by acting at least at the promoter level.

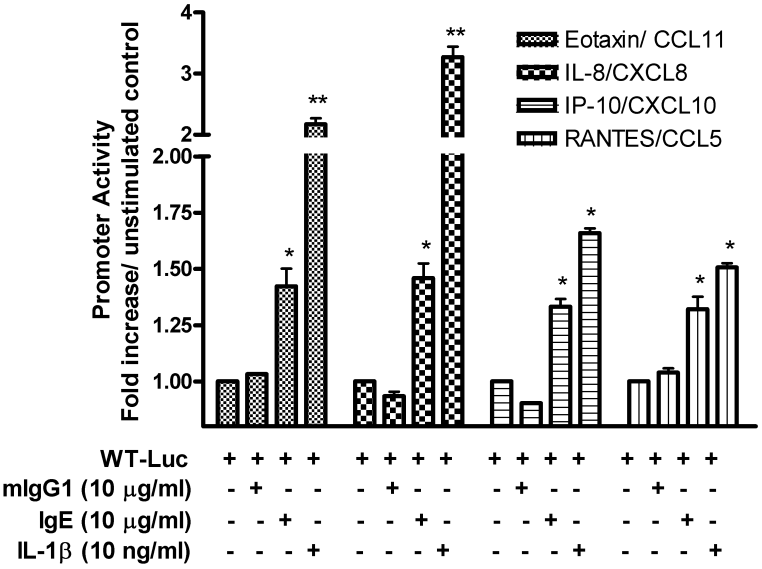

Figure 4.4. IgE-sensitization induces the chemokines promoter activity in HASM cells. Cultured HASM cells were transiently transfected with luciferase reporter constructs driven by wild-type promoters for human eotaxin-1/ CCL11, IL-8/CXCL8, IP-10/CXCL10, and RANTES/CCL5; stimulated with mIgG1-MOPC21, IgE, or IL-1β; and respective chemokine promoter activity was measured as mentioned in *Materials and Methods*. Data were normalized according to the *Renilla* luciferase activity, and presented as fold-increase over unstimulated control. Data represents the mean ± SD of three separate experiments. Mann-Whitney *U* test was performed to analyze the differences between the samples. **P*<0.05, ***P*<0.01 compared to unstimulated control.

4.2.5. Lentivirus mediated Syk-silencing abrogates the IgE-induced transcriptional activation of human eotaxin-1/CCL11, IL-8/CXCL8, IP-10/CXCL10, and RANTES/CCL5 promoters in HASM cells

In light of the above findings, we further investigated whether IgE-induced chemokine expression involved the FcεRI activation. In inflammatory cells such as mast cells, FcεRI activation triggers many signaling pathways including the phosphorylation of FcεRI-β and -γ by Lyn kinase, followed by the activation of Syk through its recruitment to FcεRI (53). Activation of Syk is crucial for FcεRI downstream signals propagation including phosphorylation of phospholipase Cγ, calcium mobilization, degranulation, and proinflammatory

cytokine/chemokine release (53, 97). These observations suggest that blocking/ silencing the Syk expression might be a useful strategy to investigate the FcεRI activation in HASM cells. To inhibit the Syk expression, HASM cells were transduced with a pseudotyped lentiviral vector expressing specific Syk-shRNA. Mock and scramble sequence were used as negative controls. As shown in Figure 4.5A, more than 95% of the lentivirus-transduced cells were tGFP positive by FACS analysis. Transduction of cells with Syk-shRNA clone resulted in a highly significant and reproducible decrease in Syk expression, as shown by Western blotting (Figure 4.5B). However, transduction with the control scramble shRNA failed to reduce Syk expression in HASM cells (Figure 4.5B). To determine if the transcriptional activation of chemokine expression by IgE is affected in the absence of Syk, stably Syk-silenced HASM cells were transiently transfected with wild-type promoters for earlier studied CC and CXC chemokines and stimulated with IgE, IL-1β, mIgG1 or left unstimulated. As shown in Figure 4.5C, D, E and F, IgE-induced chemokines promoter activity over unstimulated control was completely abrogated (eotaxin-1/CCL11, 1.30 $\pm$ 0.01 to 0.94 $\pm$ 0.14-fold; IL-8/CXCL8, 1.21 $\pm$ 0.01 to 1.08 $\pm$ 0.07-fold; IP-10/CXCL10, 1.22 $\pm$ 0.01 to 1.01 $\pm$ 0.07-fold, and RANTES/CCL5, 1.31 $\pm$ 0.02 to 0.94 $\pm$ 0.09-fold) in Syk-shRNA-transduced compared to the scramble-shRNA-transduced HASM cells. However, in contrast, lentivirus-mediated Syk-silencing in HASM cells did not affect the IL-1β-induced promoter activity for IL-8/CXCL8 and IP-10/CXCL10 (Figure 4.5D and E). Moreover, although eotaxin-1/CCL11 and RANTES/CCL5 promoter activity was slightly decreased, it was still significant compared to the unstimulated control in IL-1β-stimulated Syk-silenced HASM cells (Figure 4.5C and F). The isotype mIgG1 (MOPC21) stimulation did not affect the promoter activity for any of the chemokines in either Syk-shRNA or scramble-shRNA–transduced HASM cells (Figure 4.5). Taken together, these results suggest that the IgE-sensitization of HASM cells

induce the chemokine expression at least via involving Syk activity and the respective chemokine promoter activation.

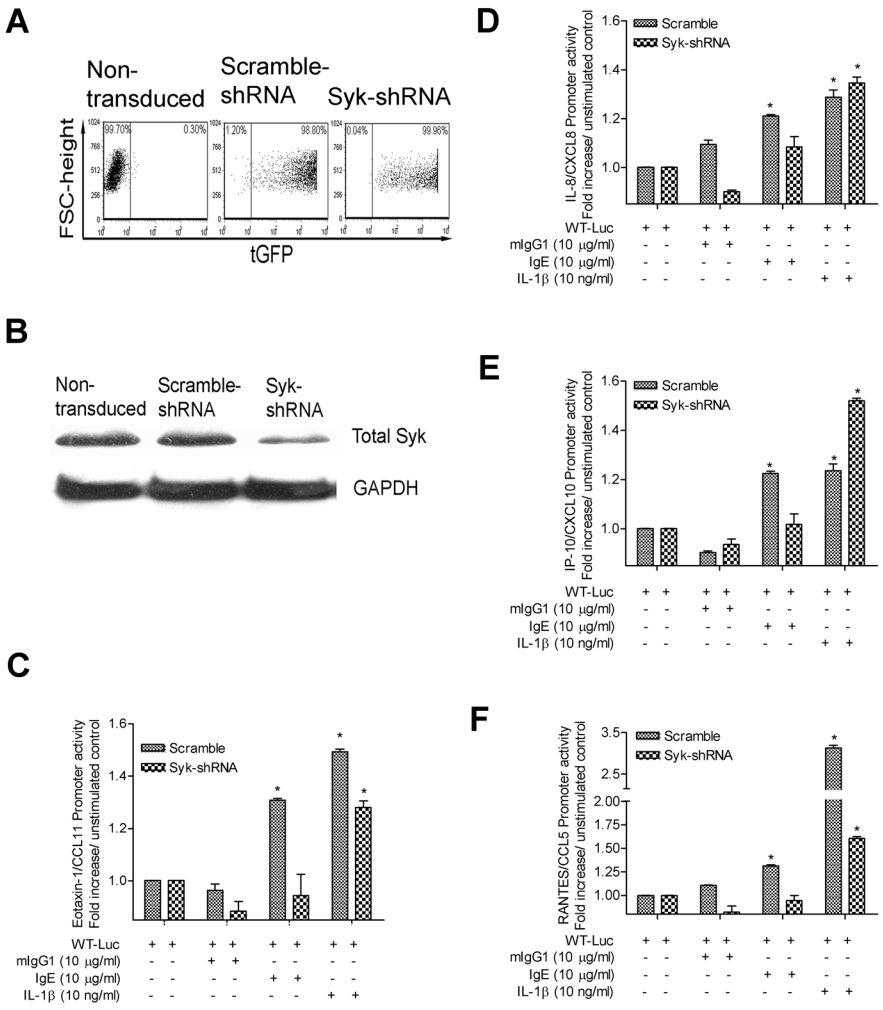

Figure 4.5. Lentivirus-mediated Syk shutdown abrogates the IgE-induced chemokines promoter activity in HASM cells. HASM cells were first transduced with a pseudotyped Lentiviral vector expressing specific Syk-shRNA or a non-specific scramble-shRNA. (A) The Lentiviral transduction efficiency was more than 95% for both scramble-shRNA and Syk-shRNA as determined by FACS using the tGFP as the marker for cell sorting. (B) Lentiviral Syk-shRNA transduction in HASM cells silenced the Syk expression efficiently as observed by Western blotting. Syk- and scramble-transduced HASM cells were then transiently transfected with luciferase reporter constructs driven by wild-type promoters for human (C) eotaxin-1/ CCL11, (D) IL-8/CXCL8, (E) IP-10/CXCL10, or (F) RANTES/CCL5; stimulated with mIgG1-MOPC21, IgE, or IL-1β for 12 h; and respective chemokine promoter activity was measured as mentioned in *Materials and Methods*. Data were normalized according to the *Renilla* luciferase activity, and presented as fold-increase over unstimulated control. Data represents the mean $\pm$ SD of three separate experiments. Mann-Whitney *U* test was performed to analyze the differences between the samples. **P*<0.05, ***P*<0.01 compared to unstimulated control.

4.3.0. DISCUSSION

Several studies have investigated the regulation of high affinity IgE receptor (FcεRI) expression in inflammatory cells both *in vivo* and *in vitro,* and a correlation has been established between the serum IgE levels and the FcεRI expression on cell surface (248, 340). However, IgE is not the only factor governing FcεRI expression, since mast cells from IgE-deficient ($IgE^{-/-}$) mice express low levels of FcεRI (252). Therefore, it is indeed apparent that the basal FcεRI expression is under the control of some other regulatory mechanisms. Moreover, although the ASM cells were recently shown to respond to IgE through the expression of a tetrameric FcεRI complex (αβγ2) (98), factors that modulate the FcεRI expression by ASM remain unknown.

In the present study, we demonstrated that proinflammatory (TNF-α) and Th2 (IL-4) cytokines upregulate the FcεRI-α expression in cultured HASM cells both at transcript and protein level. This was a novel finding as despite being central proinflammatory mediators in allergic inflammation (334-337), TNF-α and IL-1β have not previously been investigated in the context of FcεRI modulation in any cell type. Interestingly, IgE engagement of FcεRI on TNF-α pre-sensitized HASM cells significantly augmented the eotaxin-1/CCL11, IL-8/CXCL8, IP-10/CXCL10, and RANTES/CCL5 but not TARC/CCL17 release. In addition, IgE sensitization of HASM cells induced the gene expression for eotaxin-1/CCL11, IL-8/CXCL8, IP-10/CXCL10, and RANTES/CCL5 at promoter level, which was completely abrogated upon lentivirus-mediated Syk silencing. Our data underline the fact that proinflammatory and Th2 cytokine-induced FcεRI regulation in HASM cells could significantly contribute to the airway inflammation via potentially novel mechanisms involving IgE/ FcεRI complex.

TNF-α is a prototype proinflammatory cytokine which has been proposed to exert deleterious effects directly on airway smooth muscle, including its most recently explored roles

in (i) activation of transient receptor potential channel 3 (TRPC3) leading to abnormal store-operated calcium influx, and (ii) upregulation of CD38 which regulates intracellular calcium and plays a role in airway hyperresponsiveness (341, 342). Both TNF-α and IL-1β, independently, are known to induce IL-8/CXCL8 and RANTES/CCL5 release in ASM (343). IL-8/CXCL8 is a chemoattractant for neutrophils, eosinophils, and to a lesser extent, T-lymphocytes, while RANTES/CCL5 acts as a chemoattractant for T-cell, eosinophils and monocytes. TNF-α also induced the release of IP-10/CXCL-10, a potent chemokine for activated T cells, NK cells and mast cells; and its expression is differentially modulated by vitamin D in human ASM cells (344). In the present study, TNF-α stimulation augmented both FcεRI-α transcript and protein expression in HASM cells. Of note, we did not incubate the cultured HASM cells with cytokines and IgE simultaneously as it could mask the effect of IgE. Indeed, we first pre-sensitized the HASM cells with cytokines and then engaged the receptor with IgE stimulation. Interestingly, TNF-α pre-sensitization (and thus FcεRI upregulation) followed by IgE stimulation augmented the selective eotaxin-1/CCL11, IL-8/CXCL8, IP-10/CXCL10, and RANTES/CCL5 chemokines release, compared to IgE non-stimulated control. This was quite a plausible observation at functional level since IgE could engage the TNF-upregulated FcεRI by a higher magnitude than basal FcεRI expressed on HASM cells. Surprisingly, IL-1β enhanced the FcεRI-α mRNA expression but had no significant effect on FcεRI-α protein upregulation. In accordance with this, IgE stimulation following IL-1β pre-sensitization did not augment the chemokine release compared to IgE-unstimulated cells. This may be explained by the involvement of post-transcriptional regulatory mechanisms in FcεRI protein expression in response to IL-1β stimulation, and thus low magnitude of IgE-mediated signaling. Taken together, our observations essentially provide another mechanism by which TNF-α contribute to airway inflammation,

mainly amplifying the FcεRI-mediated ASM activation, and ultimately recruiting inflammatory cells through multiple chemokine expression in airways.

FcεRI-γ chain was shown to be downregulated along with FcεRI-α chain by TGF-β1 in bone marrow derived mast cells (BMMC) (345), and has been demonstrated to be the limiting factor governing FcεRIα surface expression in dendritic cells (DCs) (253). However, we found that only FcεRI-γ transcript but not protein was upregulated by TNF-α. Lack of FcεRI-γ protein upregulation by TNF, IL-1 β or IL-4 may suggest some post-transcriptional regulatory mechanisms modulating the FcεRI-γ protein translation. Since HASM express all FcεRI subunits (αβγ2) (98), possibility of different regulatory mechanisms in HASM FcεRI than trimeric DC (αγ2) (253) cannot be denied. Moreover, since FcεRI-γ chain is also shared by Fc gamma receptor subtypes expressed by HASM (96), additional regulatory mechanisms may be in force in controlling FcεRI-γ expression. Detailed studies are therefore required to delineate the regulation and role of FcRγ in amplification or stabilization, if any, of FcεRI surface expression in HASM cells.

IL-4 on the other hand, is a prototype Th2 cytokine which in combination with IL-13, induce the class switch-recombination from IgG to IgE by B-cells (12). IL-4 plays a critical role in atopic diseases and is also known to cause a marked increase in eotaxin-1/CCL11 release in ASM cells (346, 347). In our present study, interestingly, we found that IL-4 significantly enhances the surface and intracellular FcεRI-α protein expression in HASM cells. This was quite interesting as there is a steady-state FcεRI-α mRNA expression under IL-4 stimulation, suggesting the coherent translation of FcεRI-α transcript into the protein at 24 h, 72 h after stimulation (Figure 4.1B, 4.2A and B). Our data, therefore, strongly supports the previously observed positive role of IL-4 in the transcription of FcεRIα-chain in human mast cells,

eosinophils from atopic dermatitis patients, human dendritic cells, and human neutrophils (64, 257, 348, 349).

Classical paradigm entails the IgE binding to FcεRI as a 'passive sensitization' step in the mast cell activation and requires the multivalent antigens for cross-linking of FcεRI-bound IgE (53). However, recent reports highlight the IgE-mediated spectrum of effects including the pro-survival effects on mast cells, monocytes, and asthmatic neutrophils through binding to FcεRI (230, 231, 241, 242). Moreover, IgE alone (i.e. sensitization) induced the expression of multiple cytokines (e.g. IL-6, TNF-α, IL-4 and IL-13) and activated signaling pathways by phosphorylation of several kinases such as Erks, p38, JNK and PKB in normal murine BMMC (231). In this line, after exploring the FcεRI regulation by proinflammatory and Th-2 cytokines and subsequent IgE-mediated HASM activation, we tested our hypothesis of effect of IgE sensitization alone on ASM synthetic function. Interestingly, we found that IgE sensitization induces multiple, selective CC (eotaxin-1/ CCL11, RANTES/CCL5) and CXC (IL-8/CXCL8, IP-10/CXCL10) chemokines expression in HASM cells at least at promoter level.

In inflammatory cells, activation of Syk is crucial for IgE cross-linking-induced FcεRI downstream signals propagation including phosphorylation of phospholipase Cγ, calcium mobilization, and degranulation (53, 97). Although details still remain to be investigated, the initial signaling events in non cross-linking model (i.e. IgE sensitization alone) of FcεRI activation include the activation of Lyn and Syk leading to the activation of ERK in mast cells (240, 245). Interestingly, in our study, lentivirus-mediated Syk shutdown in HASM cells completely abrogated the CC and CXC chemokine promoter activity, suggesting the involvement and requirement of Syk activity in IgE sensitization-induced chemokine expression. These results essentially provide a proof-of-principle and increase our understanding that at least initial

signaling events of IgE sensitization-induced FcεRI activation are similar, if not the same, in ASM and mast cells. Altogether, our data suggest that blocking or silencing Syk, among others, could present a useful therapeutic strategy for allergic inflammatory disorders.

Collectively, our data demonstrate that proinflammatory (TNF-α) and Th2 (IL-4) cytokines modulate the FcεRI expression in HASM, which can further augment the selective CC and CXC chemokine release following IgE exposure. Moreover, IgE sensitization of HASM cells induces multiple chemokine gene expression, via pathways involving at least Syk activity and the respective gene promoter activation. Our data, therefore, highlight the fact that proinflammatory and Th2 cytokines directly or indirectly manifest the allergic inflammation, also by regulating FcεRI expression, hence amplifying the IgE/ FcεRI associated reactions.

5.0. CHAPTER 5

ESSENTIAL ROLE OF NF-κB AND AP-1 TRANSCRIPTION FACTORS IN TNF-INDUCED TSLP EXPRESSION IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

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N.S.R. drafted and contributed to 70% of data collection and analysis for the realization of this manuscript.

5.1.0. INTRODUCTION AND RATIONALE

Tumor necrosis factor alpha (TNF-α), a proinflammatory cytokine, has been demonstrated to play a central role in asthma pathogenesis through direct immunomodulatory actions on ASM (116). TNF-α by itself or in combination with other cytokines such as IL-1β or IL-13, augments the expression of various 'pro-asthmatic' mediators such as cytokines (IL-6, GM-CSF), chemokines (eotaxin-1, eotaxin-3, RANTES, TARC, IL-8/CXCL8), adhesion molecules (VCAM-1, ICAM-1), and other molecules (cyclooxygenase-2/COX-2: generates proinflammatory prostaglandins) (93, 116). Notably, TNF-α is one of the most potent cytokine activators of NF-κB, an ubiquitously expressed transcription factor that modulates the expression of a number of cellular genes involved in immune inflammatory processes (350). In a recent mechanistic study, TNF-α was shown to induce the expression of multiple NF-κB-sensitive genes, including IL-6, IL-8/CXCL-8, and eotaxin-1 via NF-κB acetylation, transactivation, and histone acetyl transferase (HAT) function in human ASM that could potently be inhibited by IFN-γ treatment (351). Taken together, TNF-α-mediated ASM activation has emerged as a detrimental element in asthma pathogenesis.

We and others have recently demonstrated that human ASM cells are capable of expressing constitutive TSLP *in vitro* and *in vivo* (288, 292, 310). We reported that TNF-α and IL-1β, alone or in combination, considerably upregulated the HASM TSLP expression. Due to increasing interest in role of TSLP in pathophysiology and hunt for novel therapeutics of allergic inflammatory disorders such as atopic dermatitis (AD), asthma and COPD, it is of utmost importance to decipher the triggers and associated mechanisms of TSLP expression. In this report, based on our previous findings and emerging information on NF-κB control of ASM gene

expression, ***we hypothesized that NF-κB may be a critical transcriptional regulator of TNF-induced TSLP expression in HASM.***

5.2.0. RESULTS

5.2.1. TNF-α upregulates the TSLP expression at mRNA and protein level

We have earlier reported that TNF-α upregulates the TSLP expression in HASM cells (292). In this report, we first ascertained that HASM cells express TSLP constitutively and TNF-α enhances the TSLP expression at mRNA level (Fig. 5.1A). TSLP measurement by ELISA confirmed that TNF-α stimulation enhanced the TSLP protein release compared to the control in a time-dependent manner (Fig. 5.1B). Collectively, our previous and current data establish the fact that TNF-α upregulates the TSLP expression in HASM cells.

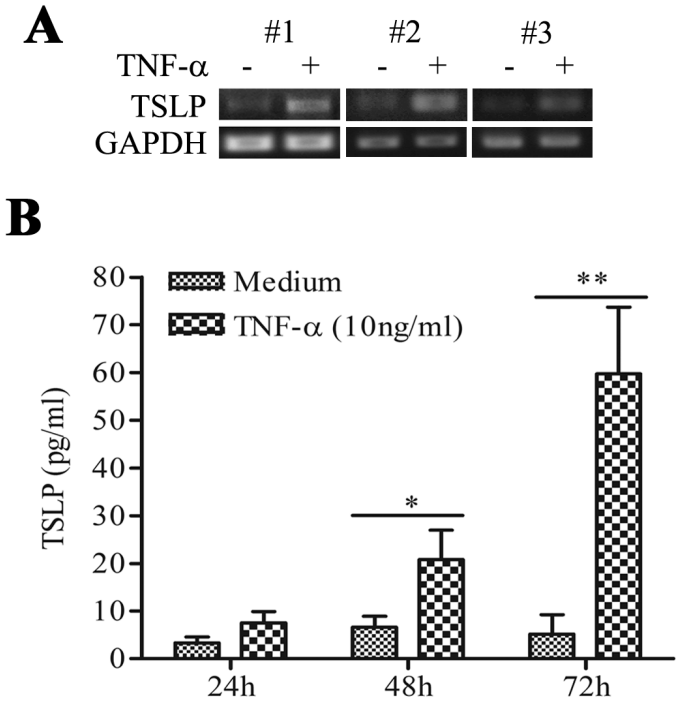

Figure 5.1. TNF-α upregulates TSLP mRNA and protein expression in HASM cells. Serum-deprived HASM cells from three donors were cultured in presence of TNF-α (10ng/ml) for 2 h,

lysed in TRIZOL™, total mRNA was extracted and reverse transcription-PCR was performed using specific TSLP primers (A). TSLP protein release from TNF-α-stimulated HASM cells at 24h, 48h and 72h was measured by ELISA (B). Figures represent at least three independent experiments performed under similar conditions. *$P<0.05$, **$P<0.01$ compared to the respective unstimulated control.

5.2.2. TNF-α-induced TSLP transcriptional activation involves NF-κB and AP1

To investigate the underlying mechanisms in TNF-α-induced TSLP expression, we employed the transient transfection of sub-confluent HASM cells with the proximal TSLP promoter fused to a luciferase reporter gene, and the subsequent effect of TNF-α stimulation was assessed on TSLP promoter activation. In contrast to the control, TNF-α stimulation induced a 3.702 ± 0.844-fold increase in luciferase reporter activity compared to control ($p<0.05$, $n=3$, Fig. 5.2). This suggests that the TNF-α induces TSLP gene expression in HASM cells, at least via modulating the TSLP promoter activity.

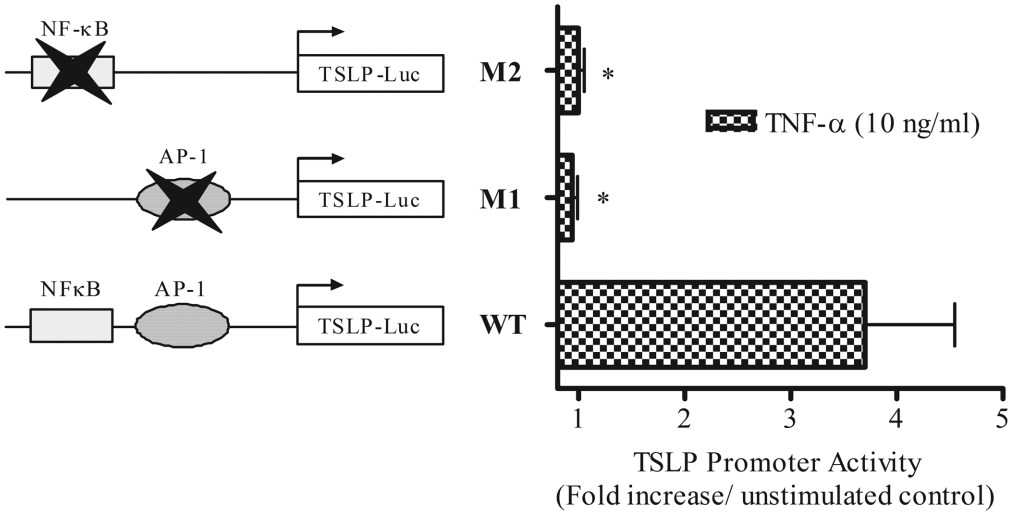

Figure 5.2. TNF-α-induced TSLP promoter activity is mediated by NF-κB and AP-1. Serum-fed HASM cells were transfected with wild-type or NF-κB and AP-1 mutant constructs of TSLP promoter. Cells were then stimulated with TNF-α (10ng/ml) or left unstimulated for 12h and luciferase reporter activity was measured, as described in *Materials and Methods*. Data represents at least three independent experiments. Data are normalized according to the *Renilla* luciferase activity. *$P< 0.05$ compared to stimulated wild-type (WT) control.

To further characterize the promoter region(s) involved in TSLP transcriptional activation by TNF-α exposure, we employed the reporter plasmids (-4.0 Kb) containing TSLP promoter with mutations in NF-κB or AP1-like transcription factors. These mutated binding sites span the -3.86 to -3.74 Kb region (~120bp) of human TSLP promoter gene, and were previously used in human bronchial epithelial cells (HBEC) (290). Transfection with TSLP promoter harboring mutated NF-κB or AP-1 binding sites completely abrogated the TSLP promoter activity ($p<0.05$, n=3, Fig. 5.2) in HASM. Altogether, this data indicates that the NF-κB and AP-1 transcription factors are important in TNF-α-induced TSLP expression.

5.2.3. TNF-α regulates the TSLP transcriptional activation via IKK pathway of NF-κB

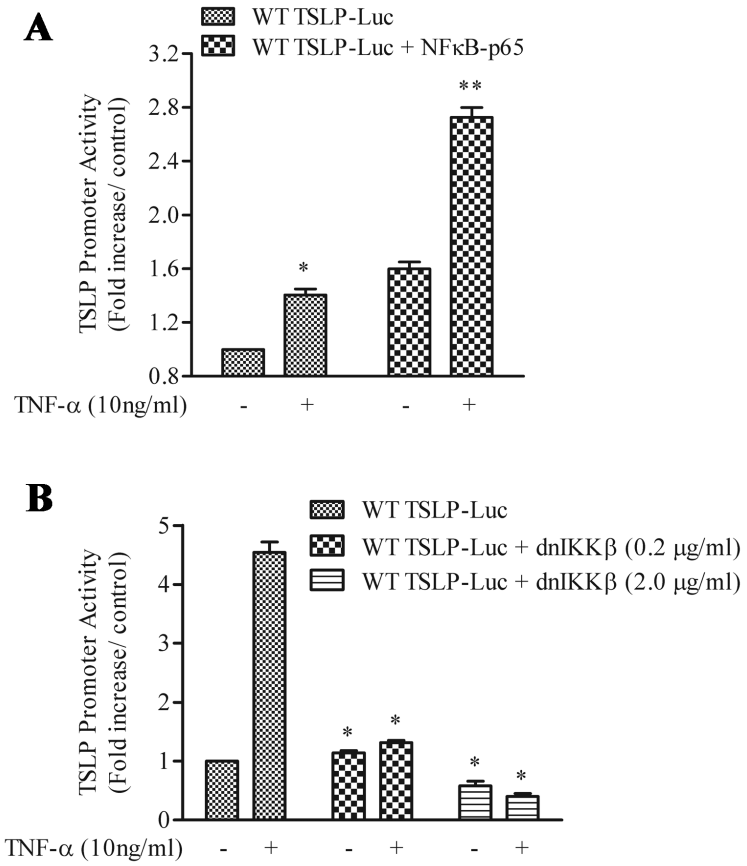

Figure 5.3. TNF-α regulates TSLP promoter activation in HASM via IKK pathway.

(A) HASM cells were transfected with luciferase reporter constructs containing human TSLP promoter and 1.5 μg of NF-κB/p65 subunit over-expressing plasmid and stimulated with TNF-α (10 ng/ml). *P< 0.05, **P<0.01 compared to respective unstimulated control. (B) HASM cells were transfected with wild-type human TSLP promoter and 0.2 μg or 2.0 μg of dominant-negative mutant plasmid of IKKβ (dnIKKβ). Cells were then stimulated with TNF-α and luciferase reporter activity was measured. Data are normalized according to the *Renilla* luciferase activity and represent at least three independent experiments performed under the same conditions. *P< 0.05 compared to the stimulated control.

To further establish the role of NF-κB pathway in TNF-α-induced TSLP expression in HASM, we looked back at the NF-κB activation pathway. NF-κB consists of either homo- or hetero-dimers of the NF-κB/ Rel family member subunits, and these subunits contribute to the differential gene transcriptional activation (350). An essential step in NF-κB canonical pathway is the phosphorylation of inhibitor of NF-κB (IκB) proteins by IκB kinases (IKKs), followed by the migration of NF-κB dimers from cytoplasm to nucleus (350, 352). To conclude whether this pathway is involved in TNF-α-induced TSLP transcription, we investigated the effect of over-expressing wild-type NF-κB/ p65 subunit or expression of dnIKKβ in HASM cells (290). Interestingly, the overexpression of wild-type NF-κB/p65 domain in HASM enhanced the basal TSLP promoter activity that was significantly upregulated following TNF-α stimulation (Fig. 5.3A; $p<0.01$, $n=3$). Moreover, the expression of dnIKKβ significantly downregulated the TSLP promoter activity (Fig. 5.3B; $p<0.05$, $n=3$); and also downregulated the TSLP release following TNF stimulation (Fig. 5.4C). Altogether, this data establishes an essential role for IKK pathway of NF-κB activation in TNF-α-induced TSLP expression in HASM.

5.2.4. IκB kinase and NF-κB inhibitors suppress the TNF-α-induced TSLP release in HASM cells

We then examined whether TNF-α-induced TSLP protein release is inhibited by peptide inhibitors of IκB kinase and NF-κB. As shown in Fig. 5.4A, TNF-α-induced TSLP protein release was significantly suppressed ($p<0.05$, $p=3$) by IκB kinase inhibitory peptide (P1-10μg/ml) compared to the inactive control peptide P2 (Fig. 5.4A); and so was true when we used NF-κB inhibitory peptide SN50 (P3- 10μg/ml) that inhibited the TNF-α-induced TSLP release ($p<0.05$, $n=3$) compared to the control peptide P4 (Fig. 5.4B). Furthermore, the expression of dnIKKβ also downregulated the TSLP release following TNF-α stimulation (Fig. 5.4C). Taken together, this data substantiate the other results that NF-κB activation is critical in TNF-α-induced TSLP release in HASM cells.

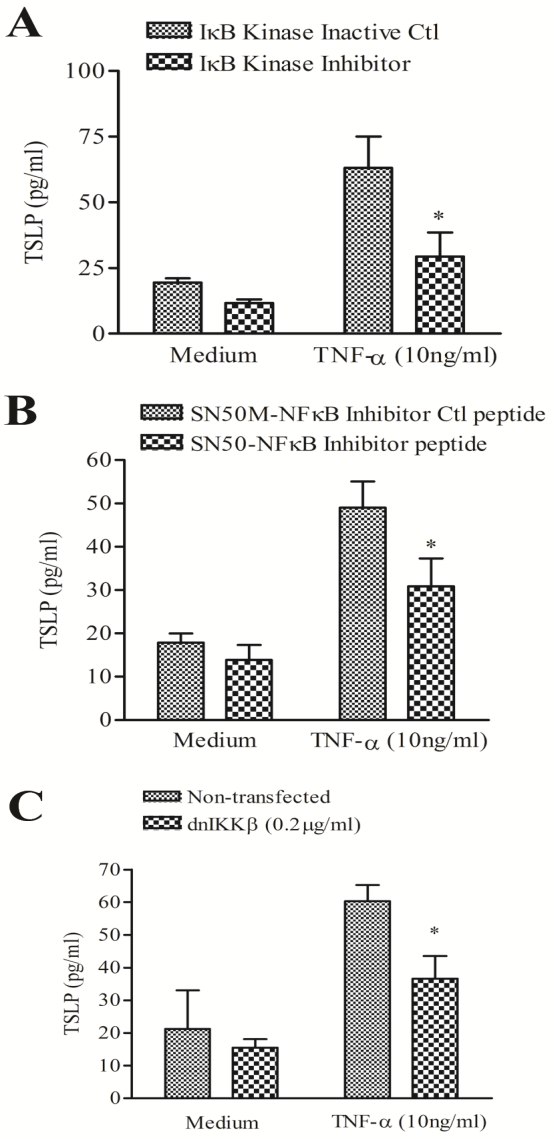

Figure 5.4. IκB kinase and NF-κB peptide inhibitors diminish the TNF-α-induced TSLP release. Serum-starved HASM cells were incubated with cell-permeable peptide inhibitors (10μg/ml each) of (A) IκB kinase or an inactive control; and (B) NF-κB (SN50) or an inactive control (SN50M), for one hour prior to stimulation with TNF-α (10ng/ml, 24h) as described in *Materials and Methods* section. (C) Serum-fed HASM cells were transfected with 0.2 μg/ml of dnIKKβ construct or control vector (pRLTK) and stimulated with TNF for 24h, Cell supernatants were collected and TSLP protein release was measured by ELISA. Figures represent three independent experiments. **P*< 0.05 compared to the stimulated control.

5.2.5. NF-κB binding site is critical for the efficacy of NF-kB inhibitory peptides in abrogating the TNF-α-induced TSLP promoter activation

Finally, we confirmed the importance of an intact NF-κB binding site by transfecting the HASM cells with wild-type and mutated TSLP promoter constructs, followed by incubation with NF-κB inhibitory peptides and further stimulation with TNF-α. As shown in Fig. 5.5A, B, TNF-α-induced TSLP promoter activity in HASM cells was significantly reduced ($p<0.05$, $n=3$) by both NF-κB (SN50) and IκB kinase inhibitory peptides. However, both inhibitory peptides did not change the NF-κB-mutated TSLP promoter activity in HASM cells, suggesting the critical requirement of intact NF-κB binding site in TNF-α-induced TSLP expression.

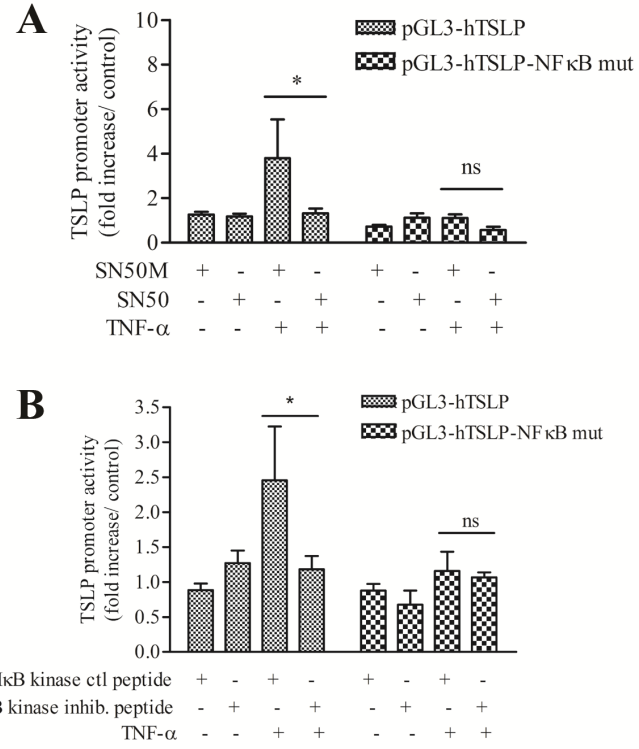

Figure 5.5. An intact NF-κB binding site is crucial for inhibitory peptide-mediated abrogation of TSLP promoter activity. Serum-fed HASM cells were transfected with wild-type or NF-κB mutant constructs of TSLP promoter. Cells were then pre-incubated with NF-κB (A) and IκB kinase inhibitory peptides (B) or respective inactive control peptides for 1h before stimulation with TNF-α (10ng/ml) for 10-12h, and luciferase reporter activity was measured, as described in *Materials and Methods*. **P*<0.05 compared to TNF-α-stimulated control, ns, non significant.

5.3.0. DISCUSSION

TSLP has recently emerged as a critical pro-allergic cytokine that is necessary and sufficient to initiate or perpetuate the Th-2 type allergic inflammation. We show here that the proinflammatory cytokine TNF-α induces TSLP expression in HASM via a transcriptional mechanism regulated by NF-κB and AP-1. NF-κB is a ubiquitously expressed transcription factor known to mediate the expression of many inflammatory mediators including cytokines, adhesion molecules, chemokines, and growth factors. An essential step in the 'canonical pathway of NF-κB activation' is the phosphorylation of inhibitor of NF-κB (IκB) proteins by IκB kinases (IKKs), followed by the migration of NF-κB dimers from cytoplasm to the nucleus where they mediate the gene transcription (350, 352). NF-κB-dependent pro-inflammatory genes are implicated to play a critical role in airway inflammatory diseases such as asthma. Increased NF-κB activation and resultant inflammatory milieu has been demonstrated in the airways of asthmatic subjects as well as in rodent models of asthma (353). This has kept NF-κB on the list of attractive targets for asthma and inflammatory airway disease therapy. Indeed, the strategies targeting NF-κB pathway have shown promising results in inhibiting multiple aspects of the allergic inflammation in animal models of asthma (353).

With respect to ASM, various studies have demonstrated the NF-κB-mediated inflammatory gene expression such as IL-17-induced IL-8/CXCL8 release (328), neutrophil elastase-induced TGFβ release, and IL-1β and TNF-α-induced GRO-α release (354). Different components of NF-κB signaling pathway have been targeted to develop therapeutic strategies (353). IKK complex is one of the most critical among these and has been investigated extensively (352). IKK consists of three subunits, including two catalytic subunits IKK-α and –β, and one regulatory subunit IKK-γ (also known as NEMO) (350, 352). IKKβ (considered as the most critical for NF-κB activation) has been subjected to the development of pharmacological

and genetic inhibitors for therapeutic intervention (355). Interestingly, we also found that the expression of a dnIKKβ mutant inhibits the TNF-α-induced TSLP expression in HASM cells. Moreover, the increase in NF-κB/p65 overexpression-mediated TSLP transcription in our study confirmed the significance of this critical pathway in NF-κB activation, as also demonstrated in studies where TNF-α was shown to phosphorylate both NF-κB/p65 (356) and IKKβ (351) in HASM. Various small molecule inhibitors such as TPCA-1, PS-1145 and ML120B, or molecular intervention using genetic IKKβ-knockdown demonstrate a role for this kinase in the expression of multiple inflammatory gene expression in ASM (357, 358), reviewed in (353). Likewise, IκB kinase- (314) or NF-κB peptide inhibitor (SN50) (315)-mediated and dnIKKβ expression-directed reduction in TNF-α-induced TSLP expression in our study insists on considering NF-κB as a potential target for therapeutic interventions to suppress the allergic inflammation in airway diseases such as asthma.

TNF-α exerts its pleiotropic actions by binding to two receptors designated as p55 (TNFR1) and p75 (TNFR2), both shown to be co-expressed on most cell types including HASM (123). Although ASM express both of these receptors, TNF-α exerts its effects on ASM primarily via activation of TNFR1, including synthetic functions (IL-6, RANTES) (359), augmentation of agonist-induced calcium signals, cell proliferation, and expression of adhesion molecule ICAM-1 (360). Of note, TNF-α has been well documented as critical factor in modulating mitogen-activated protein kinases (MAPKs) activation in ASM (359). The MAPKs play an essential role in modulating contractile, proliferative, and synthetic responses in HASM (153).

MAPK-mediated transcriptional regulation has also been shown to involve transcription factors such as NF-κB and AP-1. Specifically, TNF-α-induced MAPK activation can mediate the

nuclear translocation and enhancement of the transcriptional activity of NF-κB and c-Jun (361), later being known as AP-1 following heterodimerization with c-fos. In cardiac myocytes, p38 MAPK regulates the NF-κB activation to mediate TNF-α-induced IL-6 expression (362). A recent study in HASM demonstrates the regulation of CD38 gene expression through p38 and JNK MAPKs involving NF-κB and AP-1 activation (363). Similarly, IL-8/CXCL8 expression in HBEC involved p38 and JNK MAPKs, besides NF-κB and AP-1-like transcription factors activation (364). Earlier, we have also demonstrated that IL-17 enhances IL-1β-mediated IL-8/CXCL8 expression that is regulated by p38, ERK1/2 MAPKs, and PI3K pathways and is dependent on the cooperation of the AP-1 and NF-κB transcription factors upstream of the IL-8/CXCL8 gene (328). These studies provide the potential evidence for cross-talk between the MAPK signaling pathways and transcription factors (e.g. NF-κB, AP-1) associated with the regulation of gene expression. Indeed, in one of our previous reports, pharmacologic inhibitors of MAPK (p38 and p42/p44 ERK) but not PI3K abolished the TNF-α-induced TSLP expression in HASM (292). Ablation of TNF-α-induced TSLP promoter activity and TSLP release following pharmacologic inhibition of IκB kinase or NF-κB (SN50 peptide) in our current study may further strengthen the possibility of cross-talk between MAPKs and NF-κB.

TSLP is known to be produced by epithelial cells in the lungs, gut, skin, fibroblasts, airway smooth muscle, and mast cells (288-292). Besides activating mDCs and inducing proallergic CD4+ or CD8+ responses, TSLP can also synergize with IL-1β and TNF-α to induce Th-2 cytokine and chemokine (IL-5, IL-13, IL-6, GM-CSF, CXCL8, and CCL1) expression in mast cells (289). TSLP expression is under the control of differential regulatory mechanisms under different stimulations such as p38 and Jun kinase (JNK) in response to respiratory syncytial virus (RSV) (365); cooperation between NF-κB and JAK/STAT following combination

of TNF-α and Th-2 cytokines (366); IL-1β- and TNF-α-induced NF-κB activation in HBEC (290); and IL-1β- and TNF-α-induced MAPK (p38 and p42/p44 ERK) activation in HASM (292). Notably, the NF-κB activation has been reported to be involved in TSLP expression in airway epithelial cells (290), synovial fibroblasts (365), intervertebral disc cells (294), and human corneal epithelial cells (367). Our data is in agreement with these findings and suggests a critical role of NF-κB in TNF-α-induced TSLP expression in human ASM cells. In one of our experimental strategies, we also performed electrophoretic mobility shift assay (EMSA) to demonstrate the NF-κB and AP-1 binding to their respective sites on TSLP promoter. Our data revealed a weak binding signal (data not shown) suggesting the involvement of other transcription factors in regulating NF-κB and AP-1 binding to TSLP promoter. Notably, human TSLP promoter has other binding sites such as IRF-1 and Opaque 2 (290) that may have a negative regulatory effect on NF-κB and AP1 binding.

Collectively, we provide multiple lines of evidence to establish the involvement of NF-κB activation in TNF-α-induced TSLP expression in HASM; i) mutation in NF-κB binding site completely abrogated the TNF-α-induced TSLP promoter activity (Fig. 5.2), ii) over-expression of NF-κB/p65 subunit enhanced the TSLP promoter activity (Fig. 5.3A), iii) expression of dnIKKβ abrogates the TSLP promoter activation and protein release (Fig. 5.3B, 5.4C), iv) peptide inhibitors of IκB kinase or NF-κB inhibit the TNF-α-induced TSLP protein release (Fig. 5.4), and v) NF-κB or IκB kinase inhibitory peptides abrogate the TSLP promoter activity when NF-κB binding site is intact (Fig. 5.5). Altogether, NF-κB appears to be a critical factor in HASM TSLP expression and warrants potential therapeutic interventions in allergic airway inflammatory diseases such as asthma.

5.4.0. ACKNOWLEDGEMENTS

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6.0. CHAPTER 6

IgE INDUCES TRANSCRIPTIONAL REGULATION OF THYMIC STROMAL LYMPHOPOIETIN (TSLP) IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

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*N.S.R. and A.S. contributed equally to this work. N.S.R. drafted the manuscript, collected 40% of the data and performed data analysis. A.S. generated 60% of the data required for realization of this manuscript.

6.1.0. CAPSULE SUMMARY

IgE sensitization induces the pro-allergic thymic stromal lymphopoietin (TSLP) expression in human airway smooth muscle cells, which could contribute to the 'atopic' or 'pro-allergic' state of human ASM that occurs in the absence of allergen.

6.2.0. LETTER TO THE EDITOR

Human airway smooth muscle cells (ASMC) are considered as 'inflammatory-like cells' by virtue of expressing cell adhesion, costimulatory molecules, and secretion of cytokines and chemokines that may initiate or perpetuate the airway inflammation and the development of airway remodeling(117). Recent data suggest that ASMC are capable of producing and responding to the pro-allergic thymic stromal lymphopoietin (TSLP) in asthma and COPD (292, 324). In this report, we demonstrate that IgE induces TSLP in ASMC via a transcriptional mechanism. These effects are dependent on spleen tyrosine kinase (Syk), NF-κB and AP-1 transcription factors.

TSLP has emerged as a key player in the pathogenesis of allergic diseases such as asthma, atopic dermatitis, and allergic rhinitis (312). TSLP activates the myeloid dendritic cells to induce pro-allergic CD4+ and CD8+ T cell responses; and activates the mast cells to facilitate their cross-talk with ASMC (288). In human ASMC, IL-1β, TNF-α, and cigarette smoke extract can induce elevated levels of TSLP (288, 292, 324). Interestingly, β_2-agonists enhanced the cytokine-induced TSLP in ASMC, an effect that was abolished by concurrent use of glucocorticoids; suggesting a novel mechanism of adverse effects associated with prolonged use of β_2-agonists in absence of glucocorticoids in asthma (296). Traditionally, bronchial hyperresponsiveness has been shown to be correlated with serum IgE levels, was shown and to

be transferable from asthmatic to non-asthmatic subjects by IgE-rich serum. Indeed, IgE-rich serum alters the contractile and synthetic function of ASM. We and others have demonstrated the expression of the high affinity IgE receptor (FcεRI) in human ASMC *in vitro* and *in vivo (98, 278)*. FcεRI activation on ASM induced rapid and transient intracellular Ca^{2+} mobilization, Th2 (IL-4, IL-5, IL-13) cytokines, and chemokine (eotaxin-1/CCL11) release (98).

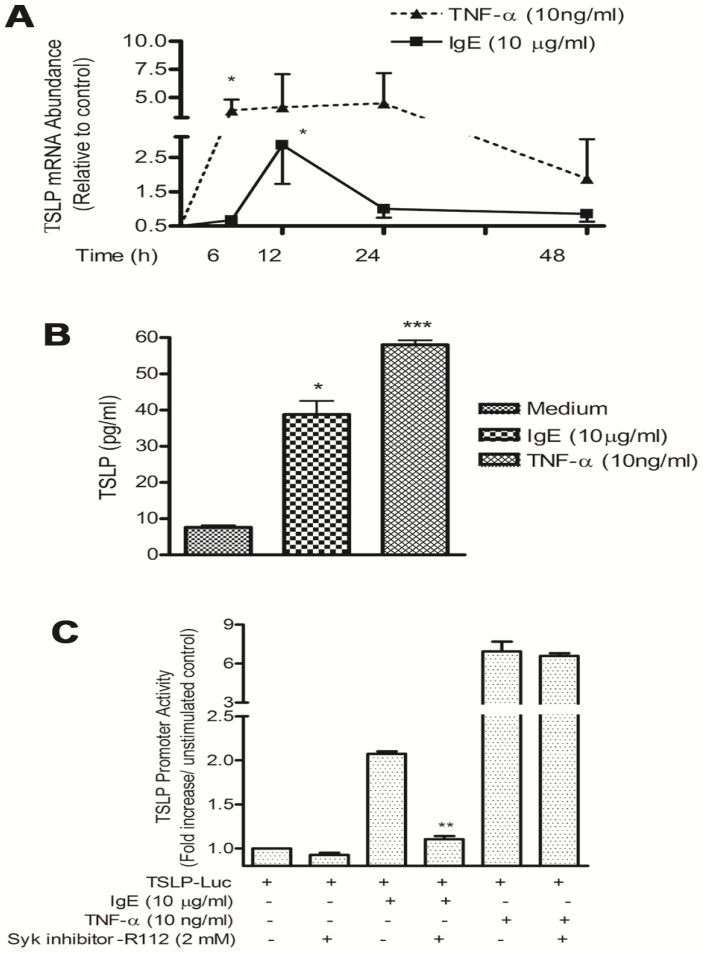

Figure 6.1. IgE induces Syk expression in ASM cells. ***A,*** TSLP mRNA, and ***B*,** protein expression were measured from ASM cells stimulated with IgE or TNF-α, by real-time RT-PCR and ELISA, respectively. ***C***, ASM cells were transiently transfected with human TSLP promoter luciferase construct, and stimulated with IgE or TNF-α in presence of a Syk inhibitor, R112 or vehicle (DMSO). TSLP promoter activity was measured. n=4, **P*< 0.05, ***P<0.01,* ****P<0.001* compared with the unstimulated (***A,*** *Mann Whitney test;* ***B****, unpaired t-test*) or stimulated (***C,*** *unpaired t-test*) control.

In current report, we found that the IgE stimulation (10μg/ml) of cultured primary human ASMC induced a significant increase in TSLP transcript at 12 hours (p=0.017, n=4, Fig 6.1, ***A***), and also enhanced the TSLP protein release at 24 hours (p=0.014, Fig 6.1, ***B***). In corroboration with earlier reports (288, 292), TNF-α induced a stronger and sustained TSLP expression in ASMC. Interestingly, IgE activated the human TSLP gene promoter and induced a 2.07$\pm$0.02-fold increase in luciferase reporter activity (p<0.001, n=4, Fig 6.1, ***C***). In inflammatory cells, Syk activation is an indispensable upstream signaling component known to facilitate the FcεRI activation-induced signaling events, and transcription factors activation. To test this possibility in ASM FcεRI activation, we employed a novel pharmacological Syk inhibitor, R112, which has been shown to completely abrogate the mast cell degranulation and mediator release following FcεRI cross-linking (331, 332). Incubation of ASM cells with R112 prior to IgE stimulation abrogated the IgE induction of TSLP promoter activity (n=3, p=0.002, Fig 6.1, ***C***). However, R112 did not affect the constitutive or TNFα-induced TSLP promoter activity in ASMC. We then knocked down the Syk expression in ASMC using lentiviral shRNA strategy (Fig 6.2). As revealed by FACS analysis, more than 95% of the lentivirus-transduced cells were turbo (t)-GFP positive (Fig 6.2, ***A***). Transduction of cells with Syk-shRNA clones resulted in a significant decrease in Syk expression (Fig 6.2, ***B***). We then tested the effect of IgE or TNF-α on ASMC transiently transfected with luciferase-encoding TSLP promoter constructs. As shown in Fig 6.2***C***, IgE-induced TSLP promoter activity was abrogated in Syk-shRNA-transduced cells but was unaffected in scramble-shRNA-transduced cells (p=0.019). In contrast, the TNF-α-induction of TSLP promoter activity was unaffected in lentivirus-mediated Syk-silenced ASMC (Fig 6.2 ***C***). Collectively, consistent with our pharmacological approach, these results reveal an essential role for Syk in IgE-induced TSLP gene promoter activation in ASMC (Fig 6.1***B***, 6.2***C***).

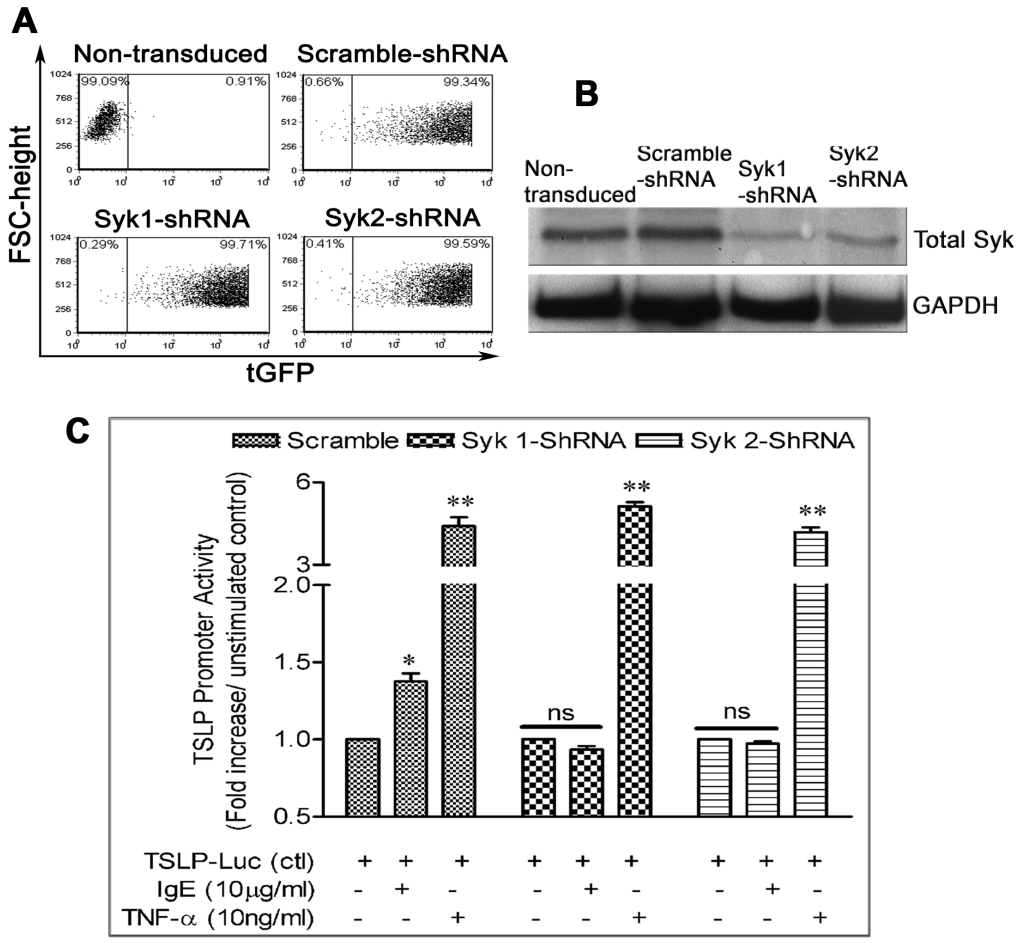

Figure 6.2. Syk is crucial for IgE-induced TSLP expression in ASMC. ***A,*** Lentiviral transduction efficiency in ASM for the Syk-shRNAs as determined by FACS using tGFP as marker. ***B,*** Lentiviral Syk-shRNA transduction silenced the ASM Syk expression efficiently as observed by Western blotting. ***C,*** Syk-silenced and scramble ASM cells were transfected with a TSLP promoter construct, stimulated with IgE or TNF-α for 12h, and TSLP promoter activity was measured. n=3, **P*< 0.05, ***P<0.01* (***C,*** *unpaired t test*) compared with the unstimulated control.

To identify the promoter region(s) involved in transcriptional activation by IgE, we employed reporter plasmids containing TSLP promoter with mutations in NF-κB or AP1-like transcription factors. Transient transfection of these constructs revealed a baseline reporter activity, confirming the constitutive expression of TSLP in ASMC. Compared to wild-type (WT) promoter constructs, IgE or TNF-α exposure resulted in a markedly lower activation of NF-κB

(M1) or AP-1 (M2) mutant luciferase reporter constructs (n=3, p=0.014), suggesting an important role for these transcription factors (Fig 6.3, ***A***). An essential step in the canonical pathway of NF-κB activation is the phosphorylation of the inhibitor of NF-κB (IκB) proteins by IκB kinases (IKKs), followed by the migration of NF-κB dimers from cytoplasm to nucleus. To determine the role of this pathway in current study, we investigated the effect of over-expressing wild-type NF-κB/p65 or dominant-negative mutant of IKKβ (referred to as dnIKKβ) in ASM cells (290, 322). Interestingly, overexpression of the pro-activity NF-κB/p65 subunit enhanced the basal transcriptional activity of TSLP promoter (Fig 6.3, ***B)***. Moreover, both IgE (p=0.045) and TNF-α (p=0.021) stimulation further augmented the TSLP promoter activity that was slightly improved upon wild-type NF-κB/p65 over-expression (Fig 6.3, ***B***). Importantly, Fig 6.3, ***C*** demonstrates a dose-dependent inhibition of the IgE- or TNF-α-mediated TSLP promoter activity following expression of the dnIKKβ subunit. Collectively, these results suggest that the IgE- or TNF-α-induced TSLP expression in ASM is mediated by NF-κB and AP-1 transcription factors activation (Fig 6.3).

Classically, IgE binding to FcεRI in inflammatory cells has been considered as a 'passive sensitization' step and requires multivalent antigens to promote cross-linking of FcεRI-bound IgE. However, in last decade, IgE sensitization alone has been shown to confer synthetic and pro-survival effects on mast cells, monocytes and asthmatic neutrophils (231, 241, 242). Along the same line, we have recently reported that FcεRI expression in ASM is regulated by proinflammatory and Th-2 cytokines; and IgE sensitization induces the expression of CC (CCL11, CCL5) and CXC (CXCL8, CXCL10) chemokines (321). In agreement with our data, IgE-induced *de novo* synthesis of IL-4, IL-6, TNF-α, and IL-8/CXCL8 in ASM was shown to be

abolished by the therapeutic anti-IgE monoclonal antibody, Omalizumab/Xolair™, without affecting FcεRI expression (278)

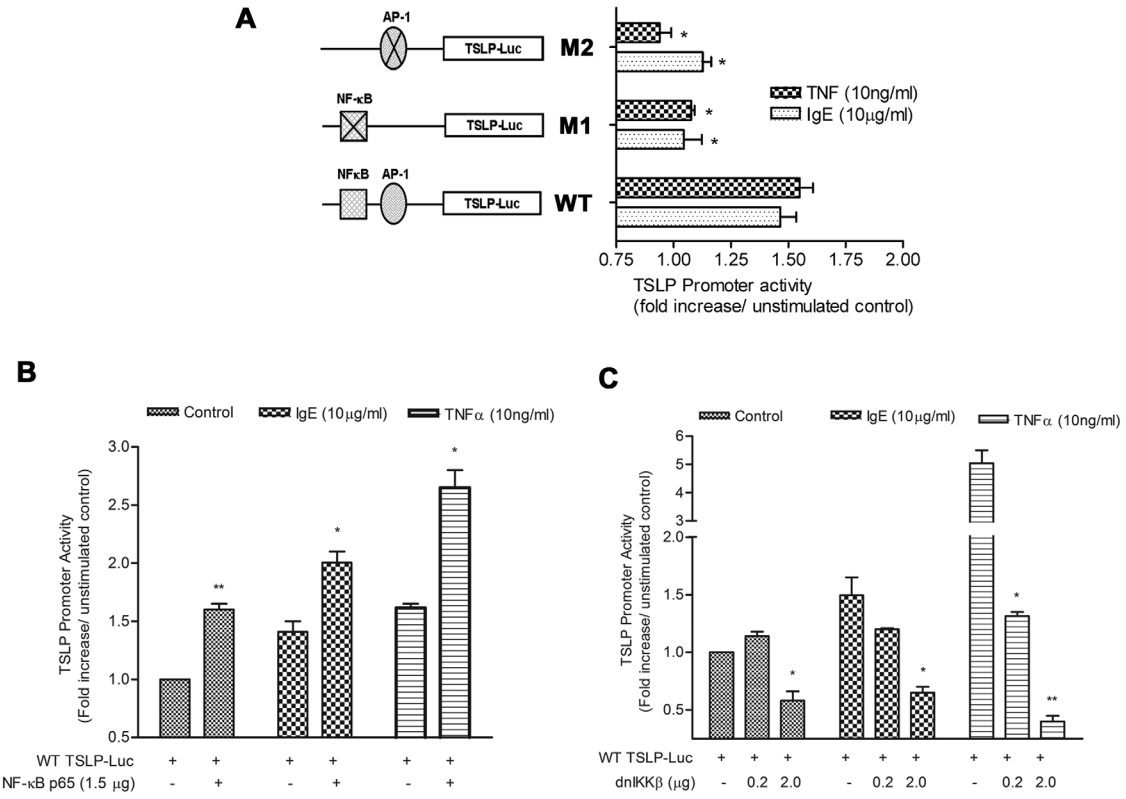

Figure 6.3. IgE-induced TSLP expression in ASMC requires NF-κB and AP-1 activity. Primary ASM cells were transfected ***A***, with mutant constructs of NF-κB (M1), AP-1 (M2) or wild-type TSLP promoter (WT); ***B,*** with WT TSLP promoter construct and NF-κB/p65 subunit over-expressing plasmid; and ***C,*** with WT TSLP promoter and dominant-negative mutant of IKKβ (dnIKKβ). Cells were stimulated with IgE or TNF-α for 12h and luciferase reporter activity was measured. n=3, **P*< 0.05, ***P*<0.01 (***A*****,** *Mann-Whitney test*; ***B, C,*** *unpaired t test*) compared with the stimulated wild-type control.

In vivo, the local IgE expression has been demonstrated in the airways of atopic subjects (11), which could modulate the ASM synthetic function leading to TSLP expression without allergen/antigen cross-linking. This ongoing TSLP expression via IgE/FcεRI pathway may in fact explain the 'atopic' or 'pro-allergic' state of ASM in allergic asthma that has been linked to the development of airway hyperresponsiveness in large epidemiological studies, particularly the

Tucson Cohort Study (368). In fact, early antigen sensitization of ASM without subsequent airway challenge or resensitization may confer a distinctive and persistent hypercontractile phenotype that can arise neonatally and persist into adult life.

6.3.0. FOOTNOTES

6.3.1. Acknowledgements

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7.0. CHAPTER 7

IgE INDUCES PROLIFERATION IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

This collaborative work is presented as a manuscript in preparation

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N.S.R. drafted the manuscript, generated 75% of the data and performed data analysis. L.S. and H.L.A. generated 25% of the data required for realization of this manuscript.

7.1.0. INTRODUCTION AND RATIONALE

Allergic or IgE-mediated asthma is characterized by the production of IgE antibodies against environmental allergens such as house dust mite, cockroach, animal danders, or tree pollens (179, 369). Recent studies, have established a new paradigm in which monomeric IgE binding alone (i.e. sensitization without subsequent allergen/anti-IgE cross-linking) can activate inflammatory cells with a spectrum of effects such as proinflammatory cytokine and chemokine release, inhibition of apoptosis or pro-survival effects though the activation of various signaling pathways. So far, monomeric IgE has credibly been shown to enhance the survival of mast cells, monocytes, and asthmatic neutrophils (230, 231, 241, 242).

Increased ASM mass is considered one of the hallmark features of airway remodeling in allergic asthma. Although the precise mechanisms remain yet to be established, an increased cell number (hyperplasia) is postulated to be one of the factors underlying this increase in ASM mass (73, 138). Indeed, ASM proliferation in severe asthma was shown to be a dynamic process *in vivo* recently (144). Molecular studies suggest the role of mitogen activated protein kinases (MAPK) and signal transducer and activator of transcription (STAT) family members, besides other pathways, in regulating the ASM cell proliferation under various contexts (370, 371). STATs reside in an inactivated state in cytoplasm, and become activated through tyrosine phosphorylation by growth factor receptor kinases, the JAKs, and the Src kinases. Phosphorylation results in STATs to undergo a conformational change, dimerization, and translocation to the nucleus where they bind to specific DNA-binding elements to regulate transcription (372).

Ours and others work shows that IgE sensitization induces multiple cytokines and

chemokines release in ASM (278, 321, 326). However, there is no clue as to whether IgE can affect ASM growth. Based on previous studies showing that IgE may modulate survival in multiple cell types; in current study, ***we hypothesized that IgE induces proliferation in ASM cells by activating mitogenic signaling pathways, which may eventually have profound consequences on airway remodeling in allergic asthma.***

7.2.0. RESULTS

7.2.1. IgE induces DNA synthesis and proliferation in HASM cells

IgE has been shown to induce pro-survival effects in mast cells, monocytes, and asthmatic neutrophils. To test the mitogenic potential of IgE on human ASM cells, we performed ^{3}H-thymidine incorporation assay. As shown in Fig. 7.1A, IgE induced new DNA synthesis in a dose-dependent manner in HASM cells of bronchial and tracheal origin derived from various donors ($n=5$, $p<0.05$). Total human IgG did not have any increase compared to control (data not shown), suggesting the specificity of IgE effect. As expected, PDGF induced a dramatic increase in DNA synthesis and served as a positive control in all our proliferation experiments, as also reported in previous reports (319, 373).

We then validated the IgE-induced ^{3}H-thymidine incorporation data by using hemocytometer-based cell counting method. IgE-induced thymidine incorporation was clearly translated into increase in cell number compared to control (Fig. 7.1B, $n=4$, $p<0.05$), suggesting that the IgE is able to induce DNA synthesis and subsequent proliferation in HASM cells (Fig. 7.1). Since IgE dose of 10 μg/ml showed a consistent increase in HASM cell proliferation (8625 $\pm$ 346.7 compared with 5231 $\pm$ 179.2 counts per minute [cpm] in control) and HASM cell synthetic function (278, 326), we used this concentration in following experiments.

7.2.2. Lentivirus-mediated Syk inhibition abrogates IgE-induced HASM proliferation

FcεRI activation leads to a spectrum of signaling events in inflammatory cells, starting with phosphorylation of Lyn kinase followed by recruitment and phosphorylation of Syk. Activation of Syk then serves as an indispensable mechanism of downstream propagation of

signals leading to the activation of various kinases, transcription factors, mediator release, and survival (53, 227, 229). This suggests that inhibition/silencing of Syk might be a useful strategy to understand the role of Syk and FcεRI pathway in IgE-induced HASM cell proliferation. To implement this, we utilized the lentiviral-mediated Syk inhibition strategy, which we have reported earlier in IgE-induced mediator release in HASM cells (321, 326). HASM cells were stably transduced with pseudotyped lentiviral vector expressing specific Syk-shRNA. Mock and scramble sequence were used as negative controls. As reported earlier (321, 326), more than 95% of HASM cells were transduced as shown by turbo-GFP signal positivity by FACS analysis (data not shown). Lentiviral-Syk-shRNA transduction resulted in a highly significant and reproducible decrease in Syk expression, as shown by Western blotting (Fig. 7.2A). However the control scramble-shRNA transduction did not affect the Syk expression in HASM cells (Fig. 7.2A).

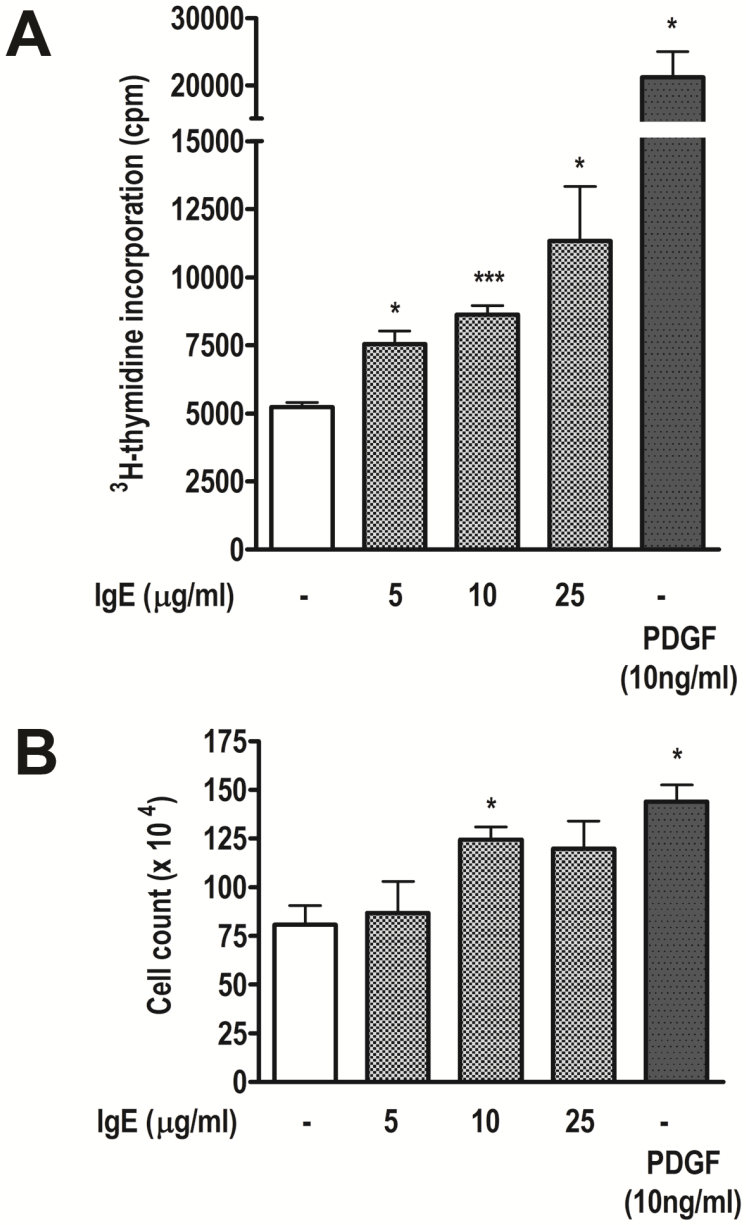

Figure 7.1. IgE induces DNA synthesis and cell proliferation in HASM cells. (A) Forty eight hour serum-starved HASM cells were stimulated with graded doses of IgE (5, 10, 25 μg/ml), PDGF-BB (10 ng/ml), or left unstimulated. After 16 h, 2 μCi/ml of *methyl*-^{3}H-thymidine was added for another 24 h. Cells were washed, trypsin-digested, and DNA was precipitated in TCA as described in *Materials and Methods*. Thymidine incorporation was measured by liquid scintillation counting and presented as cpm. (B) Serum-starved HASM cells were stimulated with IgE, PDGF-BB, or left unstimulated as in (A). Cells were trypsinized after 48 h and counted by hemocytometer. Data represents 4-5 experiments performed at least in duplicate. *$p<0.05$, ***$p<0.001$ compared to unstimulated control.

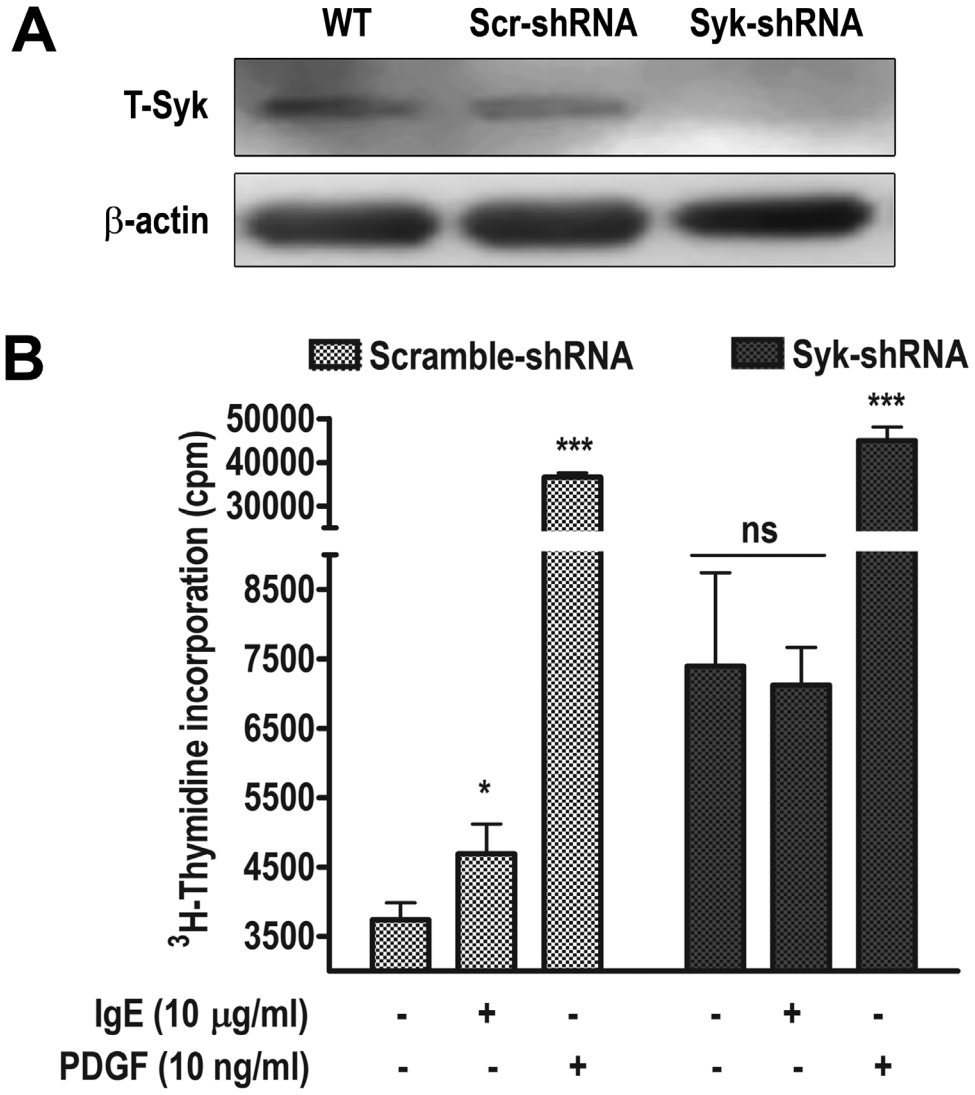

Figure 7.2. IgE-induced HASM cell proliferation requires Syk activity. (A) HASM cells were stably transduced with pseudotyped lentiviral vectors expressing Syk-shRNA or non-specific scramble-shRNA. Syk expression was assessed by Western blotting as described in *Material and Methods* section. (B) Syk- or scramble-shRNA-transduced HASM cells were stimulated with IgE (10 μg/ml), PDGF-BB (10 ng/ml), or left unstimulated. 3H-thymidine incorporation was measured as a marker of cell proliferation as in Fig. 7.6.1A. n=3, *$p<0.05$, ***$p<0.001$, and ns, non-significant compared to unstimulated control.

We then used these lentiviral-transduced cells and stimulated them with IgE and PDGF. As shown in Fig 2B, whereas scramble-shRNA-transduced HASM cells demonstrated a significant increase in thymidine incorporation ($p<0.05$, $n=3$) similar to the wild-type cells (Fig. 7.1A), Syk-shRNA-transduced cells lost the effect of IgE on thymidine incorporation ($p>0.05$, non-significant). PDGF consistently showed highly significant thymidine incorporation in both scramble and Syk-inhibited HASM cells (Fig. 7.2B). These results suggest that IgE-induced proliferation requires the function of Syk, besides confirming the IgE specificity to FcεRI activation, and validating an upstream target of this pathway in HASM cells.

7.2.3. IgE activates multiple signaling pathways in HASM cells

To understand the molecular signaling pathways involved in IgE-induced HASM cell proliferation, we assessed the activation of multiple signaling pathways by employing a high-throughput phospho-specific 'Human Phospho-MAPK Array kit, R&D Systems, Minneapolis, MN, USA'. HASM cells were treated with IgE (10 μg/ml) and cell signaling was studied in the corresponding cell lysates. As shown in Fig. 7.3A-B, IgE induced marked phosphorylation (p) of Erk 1/2, p38 MAPK, GSK-3α/β, and Akt 1 at 15 min which tend to decline at 30 min. The levels of phosphorylation ranged from nearly 2-fold (Erk 1/2) to ~3-5-fold (p38, Akt1, and GSK-3α/β). Phosphorylation of other kinases such as JNK, ribosomal protein S6 kinase, p90 (RSK) 1, RSK2, mitogen- and stress-activated kinase (MSK) 1, MSK2, or p70 S6 kinases on this platform was not noticeable.

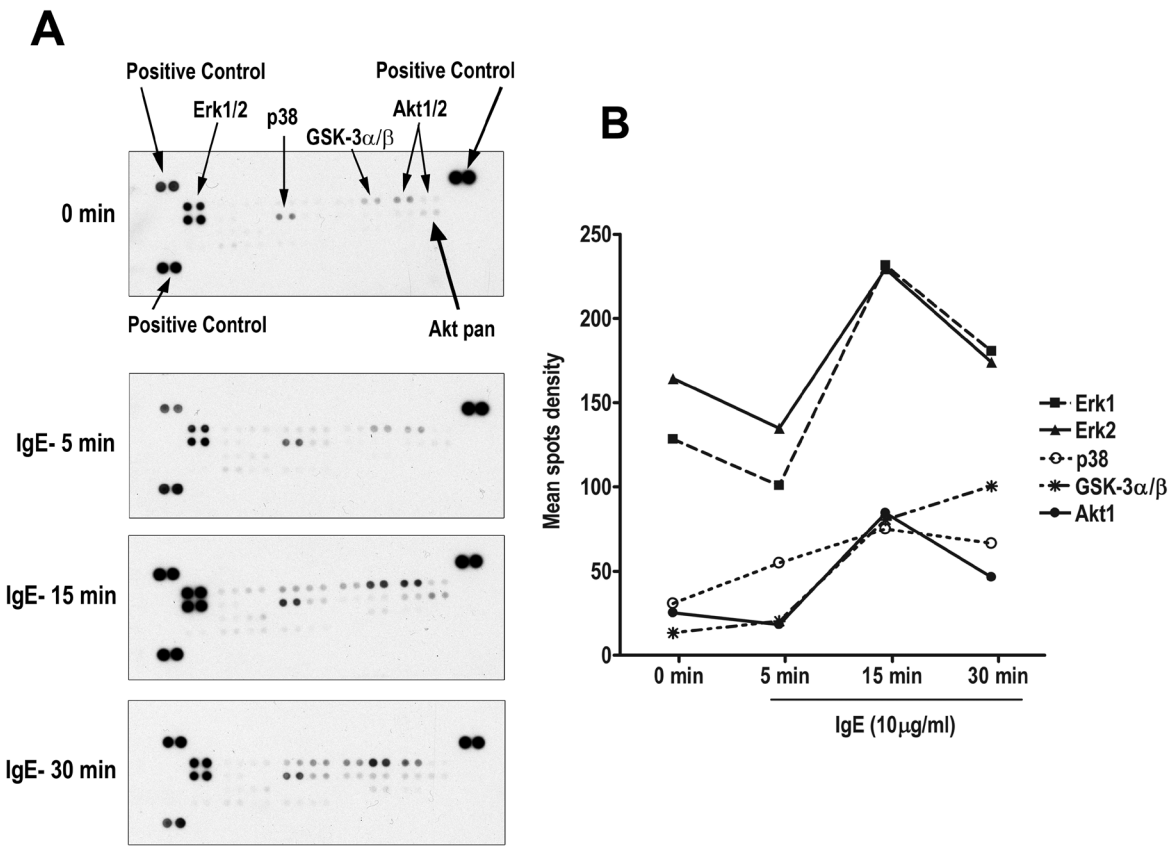

Figure 7.3. IgE induces phosphorylation of multiple kinases. (A) Serum-starved HASM cells were stimulated with IgE (10 μg/ml) for 0, 5, 15, and 30 min. Cells were washed and harvested in lysis buffer containing protease and phosphatase inhibitors cocktail. Phosphorylation of MAP kinases and Ser/Thr kinases was assessed by using human Phospho-MAPK Array kit (Proteome Profiler™, R&D Systems) by following the manufacturer's instructions. (B) Phosphorylation of MAP and other kinases was quantitated by densitometry and presented as mean spot density.

In order to validate the phospho-MAPK array data, we performed conventional Western blotting on HASM cell lysates stimulated with IgE over time point 0-120 min. Although JNK was not phosphorylated in phospho-MAPK array, Western blotting revealed a noticeable phosphorylation at 30-60 min. While Erk1/2, p38, and JNK phosphorylation occurred transiently between 5-60 min, Akt phosphorylation was interestingly sustained beyond two hour (Fig. 7.4). In summary, IgE phosphorylates multiple signaling kinases in HASM cells which may play a role in IgE-induced cell proliferation.

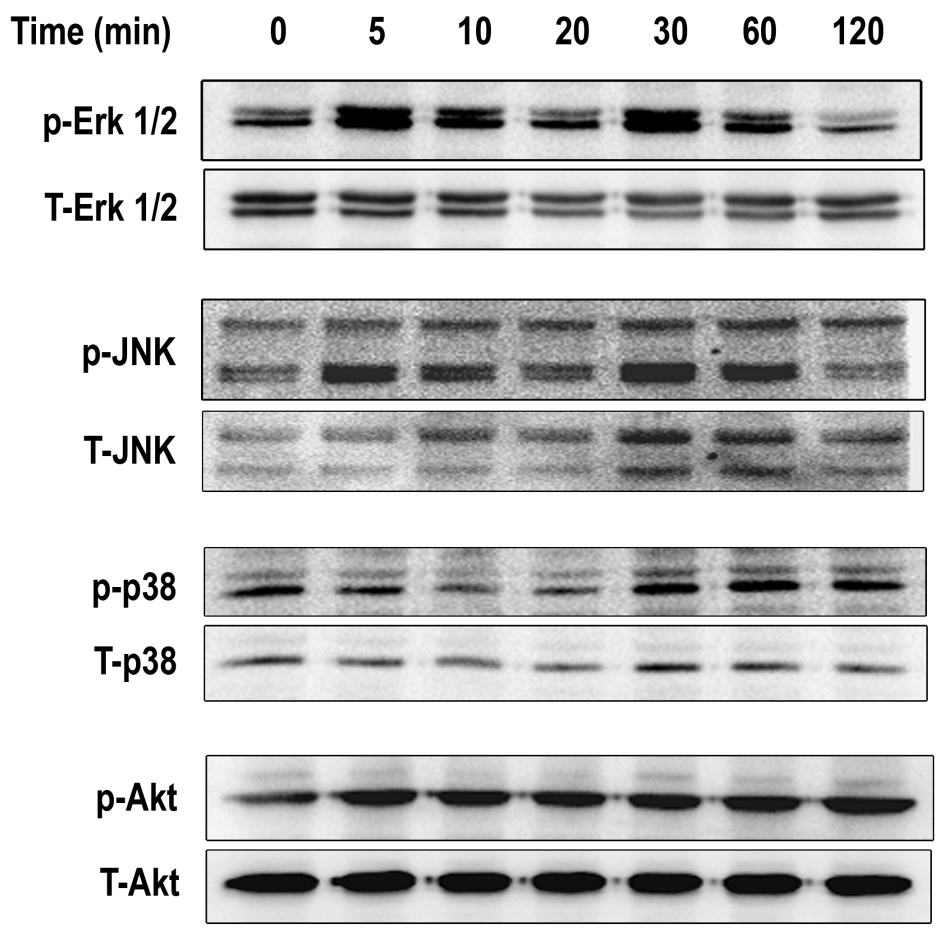

Figure 7.4. IgE induces transient Erk ½, p38, JNK MAPK and sustained Akt phosphorylation in HASM cells. Forty-eight-hour serum-starved HASM cells were stimulated with IgE (10 μg/ml) for designated times. Phospho-MAPKs (p-ERK1/2, p-p38, and p-JNK) and p-Akt were assessed by immunoblotting from total cell lysates. The same blots were stripped and reprobed with anti-total (T)-ERK1/2, anti–T-p38, anti–T-JNK, or anti–T-Akt Abs and used for loading control. The results represent one of similar results from three independent experiments.

7.2.4. MAPK inhibitors abrogate the IgE-induced HASM cell proliferation

We then confirmed the involvement of different MAPKs in IgE-induced HASM cell proliferation by using chemical and peptide inhibitors. The dose of various inhibitors was first optimized to find the dose that inhibits IgE-induced cell proliferation without having any noticeable cytotoxicity (data not shown). Figure 7.5A shows that the IgE-induced HASM cell proliferation was inhibited significantly ($p<0.05$, $n=3$), up to the basal control levels, upon pre-incubation for one hour with inhibitors of Erk1/2 (1 μM, U0126), JNK (10 nM, SP600125), p38 (10 μM, SB203580), Akt (10 μM, TAT-*Akt-in*), and GSK-3α/β (1 μM, TWS119). Incubation of HASM cells with these inhibitors did not have any effect on basal cell proliferation (data not shown). To rule out the effect of dimethyl sulfoxide (DMSO), HASM cells were stimulated with different concentrations of DMSO (the concentration range used in chemical inhibitors). However, no dose of DMSO showed any effect on IgE-induced HASM proliferation (Fig. 7.5B), suggesting the specificity of MAPK inhibitors. In conclusion, IgE-induced HASM cell proliferation requires the activation of Erk1/2, p38, JNK MAPK, GSK-3α/β, and Akt.

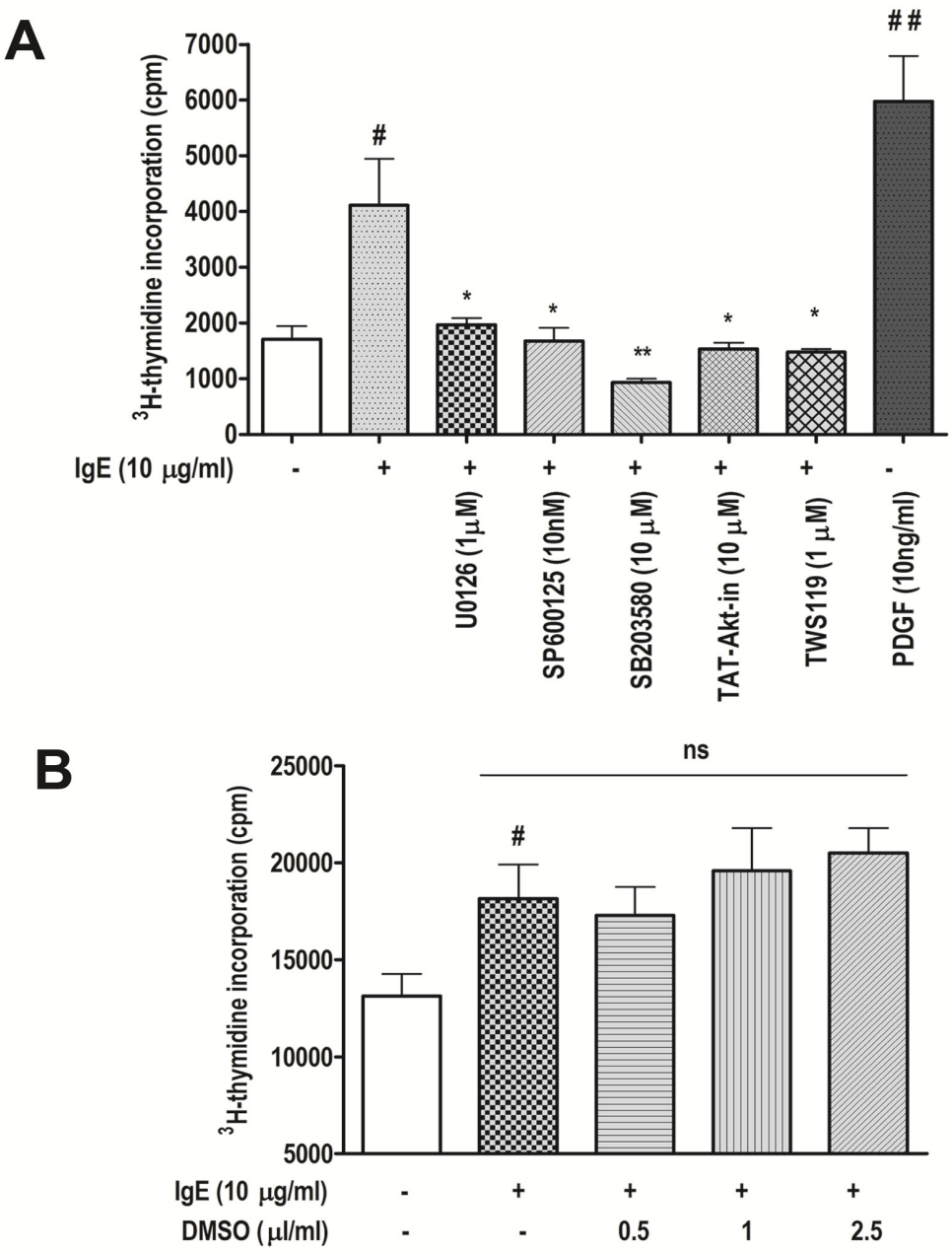

Figure 7.5. MAPK, Akt, and GSK-3α/β inhibitors abrogate the IgE-induced HASM cell proliferation. (A) Serum-starved HASM cells were stimulated with 10 μg/ml IgE for 24 h with or without pretreatment for 1 h with inhibitors of ERK 1/2 (1 μM, U0126), JNK (10 nM, SP600125), and p38 MAPK (10 μM, SB203580), Akt (10 μM, TAT-*Akt-in*), or GSK-3α/β (1 μM, TWS119). ^{3}H-thymidine incorporation was performed as described in *Materials and Methods*. (B) Effect of DMSO (vehicle control for inhibitors) was also determined by incubating serum-starved HASM cells with different concentrations (0.5, 1.0, 2.5 μl/ml) of DMSO (v/v) along with IgE (10 μg/ml). Thymidine incorporation was performed as described in *Materials and Methods*. Error bars represent means ± SEM of triplicate values from one of the three independent experiments. #p<0.05, ##p<0.01 compared with unstimulated control. *p<0.05, **p<0.01, ns, non-significant compared with IgE-stimulated cells.

7.2.5. STAT3 is critical in IgE-induced HASM cell proliferation

Besides MAP and Ser/Thr kinases, STAT3 has been shown to be critical in ASM cell mitogenesis. STAT3 activation is indispensable in HASM cell proliferation in response to PDGF (373); and monomeric IgE induces STAT3 phosphorylation in murine bone marrow-derived mast cells and rat basophilic leukemia cells, eventually regulating the transcription of genes important in cell survival (374). With these reports in consideration, we first sought to determine whether IgE is able to induce the STAT3 phosphorylation in HASM cells. Indeed, a representative Western blot depicts in Fig. 7.6A that IgE leads to a noticeable increase in phosphorylation of STAT3 in HASM cells, though the intensity and phosphorylation times varied among different donors. To confirm the role of STAT3 activation in IgE-induced HASM cell proliferation, we employed the lentiviral vector-mediated STAT3 inhibition approach, as we did for Syk inhibition in Fig. 7.2. The HASM cells were stably transduced with pseudotyped lentiviral vector encoding specific STAT3-shRNA. Mock and scramble sequence served as negative controls.

As we demonstrated earlier (324, 325), more than 95% of HASM cells were transduced as shown by turbo-GFP signal by FACS analysis (data not shown). Lentiviral-STAT3-shRNA transduction resulted in a conspicuous and reproducible decline in STAT3 expression, as shown by Western blotting (Fig. 7.6B). The control scramble-shRNA transduction showed no effect on the STAT3 expression in HASM cells (Fig. 7.6B).

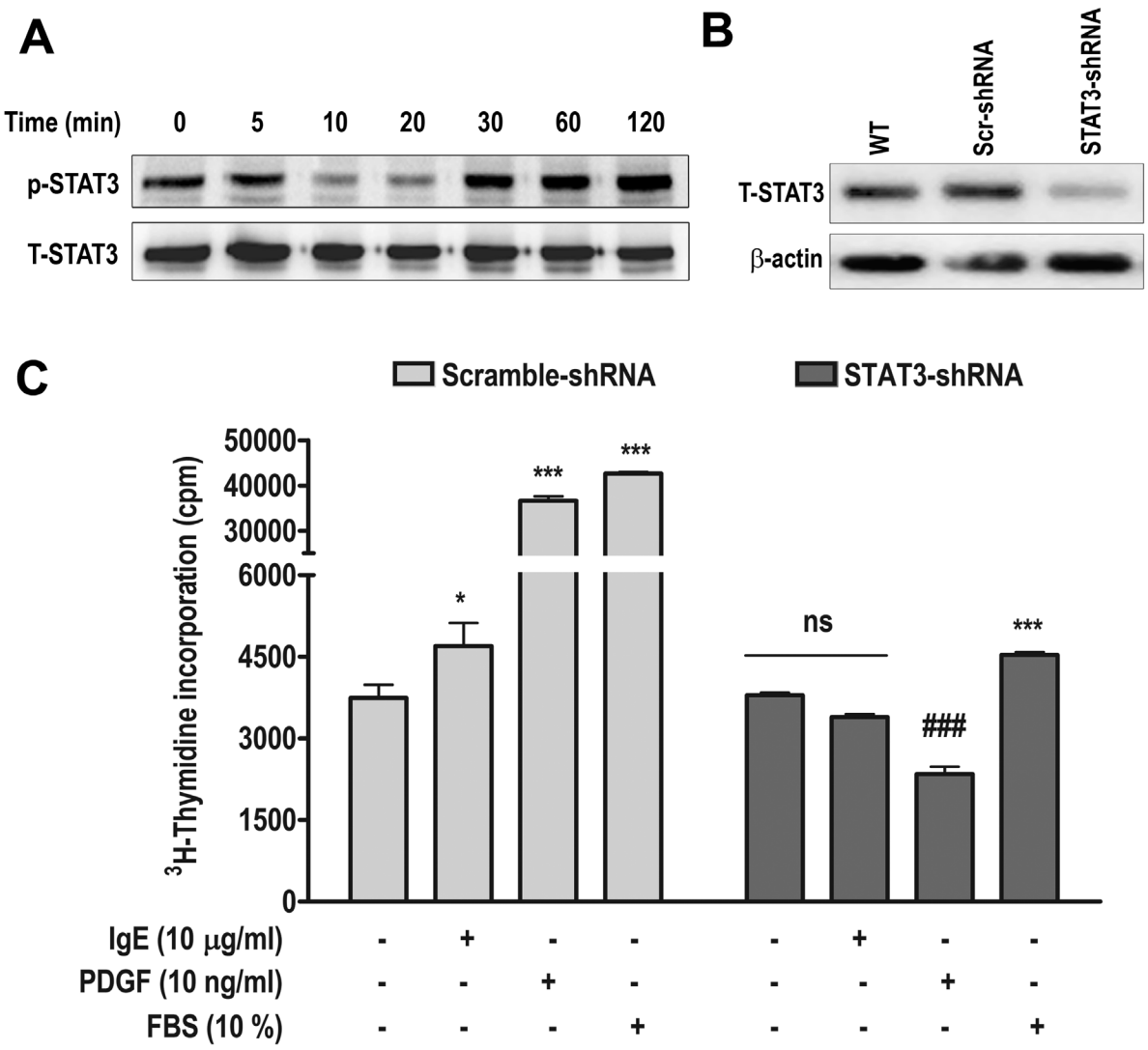

Figure 7.6. Lentivirus-mediated STAT3-inhibition abrogates IgE- and PDGF-induced proliferation in HASM cells. (A) IgE induces STAT3 phosphorylation. Forty-eight-hour serum-starved HASM cells were stimulated with IgE (10 μg/ml) for designated times and levels of phospho (p) - and total (T)-STAT3 were determined by Western blotting as described in *Materials and Methods* section. (B) Efficiency of STAT3-shRNA transduction. HASM cells were transduced by infecting with lentivirus containing scramble-shRNA or STAT3-shRNA sequence. Expression of total STAT3 in wild-type (WT or non-transduced), scramble-shRNA, and STAT3-shRNA–transduced HASM cells was analyzed by Western blotting. (C) Stably lentivirus-mediated STAT3- and scramble-shRNA–transduced HASM cells were stimulated with IgE (10 μg/ml), PDGF-BB (10 ng/ml), and 10% FBS. HASM cell thymidine incorporation was performed as described in *Materials and Methods*. The mean ± SEM of a representative experiment is shown (n=3). *p<0.05, ***p<0.001, ns, non-significant compared with respective unstimulated cells. ###p<0.001 compared with STAT3-shRNA-transduced unstimulated cells.

Both scramble-shRNA- and STAT3-shRNA-transduced HASM cells were stimulated with IgE and PDGF to analyze thymidine incorporation. Since PDGF-induced mitogenic signaling requires STAT3 expression and may not serve as a positive control, we used 10% FBS as an additional positive control in this experiment. As expected, scramble-shRNA-transduced HASM cells showed a normal and statistically significant response to IgE ($p<0.05$), PDGF, and 10% FBS ($p<0.001$) compared with unstimulated control (Fig. 7.6C). However, the effect of IgE was completely abrogated in STAT3-shRNA transduced cells, and so did the effect of PDGF, also confirming the previous reports (373) and strengthening our experimental approach. Indeed, the PDGF-stimulated STAT3-inhibited cells appeared to have negative regulation of proliferation (Fig. 7.6C), although we did not observe any visible sign of cell death. This observation may require further analysis to understand this phenomenon. On the other hand, although 10% FBS showed an increased thymidine incorporation in STAT3-shRNA-transduced cells, the effect was much less pronounced when compared with scramble-shRNA-transduced HASM cells (Fig. 7.6C). This is consistent with the observation by other groups (373), and suggests that the serum components may also require STAT3 activation to induce mitogenic signaling in HASM cells. In summary, it is evident that IgE-induced STAT3 activation plays a critical role in HASM cell proliferation.

7.3.0. DISCUSSION

We report in this study that IgE sensitization alone induces DNA synthesis and proliferation in HASM cells. IgE-induced HASM cell proliferation requires Syk activity as an upstream target of FcεRI activation; and Erk 1/2, p38, JNK MAPK, GSK-3α/β, and Akt kinase inhibitors abrogate the IgE-induced HASM cell proliferation. Furthermore, lentivirus-shRNA-mediated experiments showed that STAT3 activation is indispensable in IgE- or PDGF-induced HASM cell proliferation. Collectively, we show for the first time that the IgE sensitization plays an essential role in human ASM cell mitogenesis which may modulate the airway remodeling response observed in allergic asthma.

Clinically, majority of asthma patients demonstrate a significant increase in ASM bundles and increase in cell number in these bundles, collectively contributing to the airway remodeling response (73, 144, 375). Interestingly, studies in childhood asthma and a recent rhesus monkey asthma model suggest that the airway remodeling may occur as early as during pregnancy which is increased further up to first 6 years of life, mostly without any signs of inflammation (148, 375-379). Tissue remodeling due to increased ASM mass in allergic asthma is also known to correlate with bronchial hyperresponsiveness in some patients (142, 380). These studies suggest that ASM remodeling may in fact be a cause, instead of consequence of asthma pathophysiology (87, 279). Recent evidence further argues that the ASM proliferation is a dynamic process in severe asthma (144). Therefore, the mechanisms defining the ASM proliferation are of utmost priority to pursue novel asthma therapeutics.

Serum IgE levels have traditionally been shown to affect ASM cell function and correlate with bronchial hyperresponsiveness (166). Atopic (IgE-rich) serum sensitization of ASM cells led to increase in intracellular calcium and smooth muscle contractile response (171, 381). ASM

cells are known to express both low affinity FcεRII/CD23 (96) and high affinity FcεRI receptors (98). However, FcεRI binds to IgE with more than 1000-fold higher affinity than FcεRII/CD23 (382). Both constitutive and IL-13-inducible FcεRI expression has been noted in HASM cells (98, 277). FcεRI activation by IgE/anti-IgE incubation led to a marked release of pro-asthmatic Th2 cytokines IL-4, IL-5, IL-13, and eosinophil-attracting CCL11/eotaxin-1 chemokine; and a rapid and transient increase in ($[Ca^{2+}]i$) mobilization, altogether suggesting a critical role of this pathway in airway inflammation and hyperresponsiveness (97, 98). Mimicking of asthmatic airway milieu with proinflammatory and Th2 cytokines enhanced the expression and subsequently amplified the outcome of FcεRI activation on HASM cell release of chemokines such as CCL11/eotaxin-1, CCL5/RANTES, CXCL8/IL-8, and CXCL10/IP-10 (321). Another study showed that the IgE/anti-IgE treatment of HASM leads to modest levels of matrix metalloprotease 1 (MMP-1) production which may contribute to airway inflammatory and remodeling responses (383). Later, IgE was also shown to induce transcription and release of thymic stromal lymphopoietin (TSLP), a pro-allergic mediator known for its modulatory effects on dendritic cells which render naïve T cells to differentiate into Th2 cells (326). Finally, a clinically-proven anti-IgE monoclonal antibody, Omalizumab/Xolair^{TM} abrogated the IgE-induced mediators of asthma relevance such as IL-4, IL-6, IL-8, and TNF (278). The current study extends the function of IgE and FcεRI activation on HASM cells by suggesting a mitogenic effect which may have critical consequences on airway tissue remodeling. The *in vivo* relevance of our findings may be reflected in a murine model of asthma wherein anti-IgE treatment decreased the thickness of ASM layer compared with the ovalbumin (OVA)-challenged mice, indirectly implicating that IgE could be one of the factors inducing ASM remodeling *in vivo* (384).

There is sufficient evidence from past decade to convince that the 'IgE sensitization' of FcεRI on inflammatory cells itself can activate multiple signaling pathways; induce a plethora of proinflammatory mediators release and cell survival factors, and subsequent repression of apoptosis (240). Interestingly, IgE-induced survival or cytokine release does not necessarily require receptor aggregation and merely receptor occupancy can induce these effects (230, 231, 241, 245). Nonetheless, the role of FcεRI cross-linking in conferring pro-survival effect has been a matter of debate. While two initial reports (230, 231) suggested the lack of cross-linking, Xiang et al (385) argued for FcεRI cross-linking-mediated degranulation in mast cell survival. IgE induced monocyte survival in both instances (aggregation or no aggregation), while mast cells and asthmatic neutrophils showed IgE-mediated survival benefit without FcεRI cross-linking or aggregation (242, 245). These findings are supported by *in vivo* observations where IgE can promote immune sensitization to hapten in the skin, without the need of antigens (386). Not only monoclonal IgE, a recent report suggest that the polyclonal IgE from human atopic dermatitis patients can induce survival effects and cytokine release in human cord blood-derived mast cells, a finding that is clinically relevant (247). Of note, HASM cells have been shown to be activated by both 'sensitization alone' (278, 321, 326) and 'cross-linking' (98, 383) models. Whether the currently observed mitogenic effects of IgE on HASM cell require cross-linking/aggregation is not known. However, the cross-linking of FcεRI-bound IgE with anti-IgE antibodies from various sources did not further augment the HASM cell thymidine incorporation in our study (data not shown). Although the manufacturer of our IgE claims a single band of ~185KDa in non-reducing PAGE conditions, IgE aggregate formation due to storage and handling conditions may not be denied. However, other groups have ruled out such possibility when IgE is stored in PBS or culture medium (231). It is also worth noting here that the proliferation effect of IgE

varied among HASM cells of various donors, probably owing to the variability of FcεRI expression among different donors (98). In conclusion, although further studies are required to confirm, the mitogenic effect of IgE on HASM cells seem to occur through simple receptor occupancy without cross-linking.

STAT3 has been shown earlier to regulate allergic response in asthma. Epithelial STAT3 was identified as a critical regulator of allergen-induced allergic inflammation and AHR in a murine model of asthma (387), IL-17A-induced STAT3 activation mediated the CCL11/eotaxin-1 production in HASM (325), and PDGF-induced STAT3 and small GTPase Rac1 mediate the proliferation in HASM cells (373). Moreover, polymorphisms in STAT3 gene are associated with decreased lung function in asthma patients (388). Besides PDGF, IgE is earlier known to phosphorylate STAT3 in mast cells to induce transcription of pro-survival genes (374). We observed a clear phosphorylation of STAT3 in response to IgE, the functional role of which was indeed confirmed by lentivirus-shRNA-mediated STAT3 inhibition that completely abrogated the IgE-induced HASM cell proliferation. Interestingly, although both PDGF and IgE activated STAT3, we did not observe any synergy between both in modulating HASM cell proliferation (data not shown).

Although FcεRI-induced signaling pathways are well characterized in inflammatory cells, there is limited information on this area in HASM cells. MAPK family is fundamental in regulating multiple cell functions such as cytokine expression, proliferation and apoptosis. IgE-induced signaling pathways involved Erk1/2, p38, JNK MAPK, Akt, and GSK-3α/β which was confirmed by the abrogation of IgE-induced proliferation following the use of MAPK inhibitors. Although Erk1/2 and p38 MAPK were shown to mediate IgE-induced proinflammatory gene expression in HASM recently (278), Akt and GSK-3α/β were observed first time to be activated

in response to IgE in HASM. The role of Akt (also called protein kinase B) is well defined in HASM mitogenic signaling (389). Interestingly, GSK-3 represses several pro-mitogenic signaling pathways in its unphosphorylated form, and activation of this kinase is shown to play a critical role in HASM cell proliferation (390). It is well known that MAPK (at least ERK1/2) can modulate the STAT3 activation in HASM (325, 391) and HEK293 cell line (392). Taken together, IgE-induced activation of ERK1/2, p38, JNK MAPK and STAT3 in our study suggest a complex network of regulatory cross-talks of MAPK, Akt, GSK-3α/β, and STAT3 pathways in mediating IgE-FcεRI signaling in HASM cells. Further studies are underway to demarcate these cross-regulatory interactions in HASM cell proliferation.

Our study also has some limitations. For instance, the IgE dose we have used (10 μg/ml) is outside the clinical spectrum and may not necessarily support these conclusions *in vivo*, wherein mean IgE levels are considered to be 25-150 ng/ml in serum. However, these levels may rise up to about 35μg/ml in patients with hyper-IgE syndrome or atopy (11, 185). Omalizumab/Xolair^{TM}, the clinically approved anti-IgE antibody blocks the interaction of IgE with FcεRI and has shown clinical benefits in controlling allergic inflammation, significant improvement in asthma symptoms, reduced frequency of asthma exacerbations, and significantly lowered the use of inhaled corticosteroids (393, 394). However, it remains yet to be investigated whether omalizumab therapy can improve airway remodeling, particularly in the context of current findings.

In conclusion, IgE sensitization induces HASM cell proliferation through the activation of multiple signaling pathways. Blocking the IgE-FcεRI interaction, not only on inflammatory cells but also on the airway structural (HASM) cells, should therefore be considered as a novel tool to inhibit allergic sensitization-mediated airway remodeling in asthma. In addition, targeting

the FcεRI-mediated signaling components such as MAPK, GSK-3α/β, and STAT3 may also prove beneficial in controlling excessive ASM remodeling.

7.4.0. ACKNOWLEDGEMENTS

Authors wish to thank Dr. Ali Saleh (St. Boniface General Hospital Research Centre, Winnipeg), and Dr. Sam KP Kung lab for assistance with the lentiviral vector work. Part of this work has previously been presented in abstract form in Keystone Symposia 2011.

8.0. CHAPTER 8

GENERAL DISCUSSION

8.1.0. THE ROLE OF ASM IN CHRONIC AIRWAY INFLAMMATORY DISEASES

Collective evidence suggests that both asthma and COPD occur due to an abnormal immune response. Traditionally, chronic inflammation is believed to trigger and perpetuate a vicious circle of tissue 'injury and repair' culminating in tissue remodeling. The focus of the research for many years has been on lung-infiltrating immune cells and the array of locally released pro-inflammatory mediators called cytokines (395, 396). The role of Th1 and Th2 cells and associated cytokines was suggested by animal models and confirmed in human studies (15, 397). However, later studies did not support this hypothesis since the proposed Th2 cytokines (IL-4, IL-5, and IL-13) did not correlate with the disease severity in humans, eventually putting in question the role of T cells in these pathologies (397-400). Eventually, the focus started getting shifted towards the local tissue components (73). Although the role of T cells and Th cytokines (Th1, Th2, Th17, Treg, and other unknown subtypes) is certainly an active area of investigation, reviewed in (401, 402), structural components have emerged as key players in instigating the local remodeling and inflammation. In fact, the data from pediatric severe asthma showing early presence of structural alterations in bronchial tissue suggests that inflammation and remodeling may occur in parallel and also that the latter may arise independent of former (73, 76, 403). Interestingly, the rhesus monkey model also suggests that airway wall remodeling can occur long before any signs of inflammation; particularly the smooth muscle was found to play a special role in commencing the attraction and activation of infiltrating immune cells (148, 279, 375, 404).

Among other components, ASM cells have emerged as crucial players in regulating the

airway tissue homeostasis, and remodeling associated with chronic diseases. Asthma was indeed described first as a pathology of ASM since all asthma cases presented with increased ASM mass (139). An increased ASM mass caused by hypertrophy and hyperplasia is a cardinal feature of both asthma and COPD. Airway wall remodeling occurs in the upper airways in asthma and in the distant airways in COPD (93, 405). As Gounni AS notes, "ASM cells can contribute directly to the pathogenesis of asthma by expressing cell adhesion and costimulatory molecules and by secreting multiple proinflammatory cytokines and chemokines that may perpetuate airway inflammation and the development of airway remodeling" (97, 116, 406). Convincing evidence suggests that ASM cells might be compared to, and indeed classified as, 'inflammatory-like' cells by virtue of their capacity to produce and respond to multiple pro-inflammatory mediators (116, 117, 406).

8.2.0. IgE RECEPTORS EXPRESSION AND FUNCTION IN ASM

Extensive studies of bronchus tissue sensitization with atopic serum from 1990s provide a potential, yet debatable link between IgE and smooth muscle interaction. For instance, serum IgE levels (i) affected the pathogenesis of smooth muscle hyperreactivity, (ii) correlated with bronchial hyperresponsiveness which was shown to be transferable from asthmatic to non-asthmatic subjects with IgE-rich serum (166), (iii) IgE-rich serum from atopic subjects induced ASM hyperreactivity in isolated airway preparations (170), and (iv) IgE caused abnormal smooth muscle contractile function through binding to ASM and inducing subsequent membrane hyperpolarization (172). ***However, a molecular link between IgE and ASM cells was missing from these important studies*.**

IgE is known to exert its effector functions on inflammatory cells through binding to and

activation of low affinity receptors FcεRII/CD23 and Galactin3/ε-BP, and the high affinity FcεRI (11). However, FcεRI binds to IgE with more than 1000-fold higher affinity than FcεRII/CD23 (382) and therefore is a likely determinant of majority of IgE effects. Hakonarson and Grunstein (96) showed in late 1990s that ASM cells from rabbit and human origin express the FcεRII/CD23, and its activation by atopic serum was found to provoke pro-asthmatic-like changes in ASM responsiveness such as increased contractility and attenuated relaxation. The same group also provided early evidence that IgE sensitization of ASM through FcεRII/CD23 leads to autocrine release of IL-13 and IL-5, which may underlie the altered ASM responsiveness (96, 174, 272). In 2005, Gounni et al discovered the expression of a functional high affinity FcεRI in cultured primary human ASM cells, and FcεRI immunoreactivity was observed in ASM bundles of bronchial tissue from allergic asthmatic subjects. FcεRI activation in ASM cells led to marked transient increase in intracellular Ca2+ concentration, an effect that is likely a critical determinant of AHR through the regulation of ASM contraction/relaxation (97, 98). ***However, the molecular mechanisms explaining IgE effects on ASM cells function remain elusive. Some of the central questions include how IgE-FcεRI network affects airway inflammation, AHR, and remodeling in allergic asthma. Therefore, understanding the ASM phenotype and function in context of FcεRI activation was the major thrust for this thesis, essentially to fully appreciate the role ASM plays in allergic airway inflammatory disease.***

8.3.0. REGULATION OF FcεRI EXPRESSION IN ASM CELLS

Extensive studies in inflammatory cells suggest that the amount of FcεRI present on cell surface determines the effector functions of these cells and thus the intensity of allergic reaction. Early studies showed the expression of transcripts of α, β, and γ subunits of FcεRI in human

ASM cells. The surface expression of IgE-binding subunit FcεRI-α was also shown by flow cytometry, besides the detection of its protein expression by immunoprecipitation (IP)-coupled-Western blotting (WB), immunocytochemistry, and immunohistochemistry. Even though the FcεRI-α chain immunoreactivity was shown in ASM bundles of allergic asthma patients (98), the successful detection of FcεRI expression in ASM cells has been a matter of debate; while some groups could not detect either FcεRII/CD23 or FcεRI in ASM (276). However, we must agree that the abundance of FcεRI in ASM cells is low compared with immune cells such as mast cells; however it can potentially be augmented by proinflammatory (TNF-α, IL-1β) and Th2 (IL-4) cytokines as we reported recently. This upregulation has functional consequences such as the enhanced expression of CC- and CXC-chemokines (321). A microarray-based study, preceding our report (98), also suggests an IL-13-inducible FcεRI-α mRNA expression in ASM cells (277). Roth and Tamm have reported the WB detection of both FcεRI and FcεRII/CD23 (278). This report besides confirming both the Fcepsilon receptors expression in ASM, demonstrates the IgE-induced synthetic function that is inhibited using the therapeutic anti-IgE Omalizumab antibody (278, 397). While human serum has been proposed to augment the synthetic functions and mitogenesis of human mesenchymal cells (407, 408), the use of fetal bovine serum (FBS) in culture of ASM cells has been proposed to partly underlie the inconsistencies in detection of FcεRs in some of the studies (276). The discrepancies observed in detection of FcεRI may be explained through multiple factors that need to be considered in assessing this critical receptor, some of which were discussed by us recently *(N. S. Redhu, L. Shan, and A. S. Gounni, Am. J. Respir. Cell Mol. Biol., 2012, in press)*. Studies that failed to detect FcεRI expression in ASM have concluded on the basis of two main approaches, immunohistochemistry and flow cytometry. Both of these techniques utilize the antibodies to FcεRI-α chain. It has been known

for a long time that autoantibodies to IgE and FcεRI-α chain are produced and bound to FcεRI on cell surface in many autoimmune and allergic diseases including chronic urticaria, allergic rhinitis, autoimmune arthritis, and allergic and non-allergic asthma (409-412). At least one third of anti-FcεRIα autoantibodies are competitive with IgE (413, 414). Although their role in pathogenesis of allergic diseases is a matter of debate, autoantibodies to FcεRI-α chain or IgE may mask the detection of FcεRI or IgE bound to FcεRI (414-416).

Since FcεRI is abundantly expressed in mast cells and basophils and can be detected fairly easily; the use of similar techniques and efforts in detecting FcεRI in mesenchymal cells may be disappointing. Assessing the surface expression of FcεRI is challenging in adherent (ASM) cells which are generally trypsinized prior to detection of FcεRI surface expression. Trypsin has been shown to degrade the cell surface receptors (417, 418), and therefore jeopardize the detection of less abundant FcεRI receptor on ASM cells by flow cytometry per se. Moreover, considering the low abundance of FcεRI on ASM cells (98), analysis by flow cytometry may not be appropriate in its detection. Instead, more rigorous approaches using high amount of cellular protein such as Western blotting combined with or without immunoprecipitation may prove beneficial in detecting FcεRI in ASM (98, 278, 321). An interesting parallel can be drawn from a recent study by Wang J et al (419) in which authors for the first time detected the FcεRI-α chain expression in human vascular (aortic) smooth muscle (VSM) and endothelial cells by immunoblotting. Results of this study fully support the arguments presented above since authors failed to detect the FcεRI-α chain expression by immunoblot analysis in VSM and endothelial cells. However, a weak band at baseline and a strong upregulation with interferon-γ (IFN-γ) was detected in VSM and endothelial cells only when the cell lysate input in immunoblot analysis was increased. Furthermore, a modest degree of immunoreactivity for FcεRI-α chain was also

detected in α-smooth muscle actin region of human vascular tissue in this study. This could be an important study in opening a whole new area of investigation in which IgE can potentially affect the cells of non-inflammatory phenotype and modulate the biological function of vascular tissue beyond classical allergic response (419). In addition, thorough investigation of the function of IgE-FcεRI network in vascular tissue is warranted in context of pulmonary vascular biology.

IgE was found to be the principal factor governing surface FcεRI-α expression in inflammatory cells and amplification of subsequent functions such as mast cell degranulation, and antigen presentation by APCs (252-256). Although IgE induced a slight increase in FcεRI-α transcript in ASM cells, we did not observe any change in its protein expression (data not shown). Another group also confirmed our observation and did not observe the regulation of FcεRI-α chain by its ligand IgE (278), altogether suggesting that different regulatory mechanisms may exist in ASM cells. However, various other factors control the regulation of FcεRI-α expression such as other (β and γ) subunits of FcεRI, cytokines, and growth factors present in local milieu (64, 248). As presented in *Chapter 4*, we found that proinflammatory cytokines TNF and IL-1β induced a remarkable increase in FcεRI-α transcripts whereas only TNF-induced effect translated into FcεRI protein. Th2 cytokine IL-4, on the other hand, induced a modest increase in transcription and protein level of FcεRI-α expression in ASM cells. In DCs, FcεRI-γ chain serves as a limiting factor in modulating the FcεRI-α surface expression. However, unlike DCs, we did not find any change in FcεRI-γ chain expression in response to different cytokine stimulations, though TNF induced a slight increase in transcript levels. At basal levels, we have observed the protein expression of both α- and γ-chains of FcεRI, however due to lack of good antibodies we have not been able to show the expression of β-chain protein. Nonetheless, we have initiated collaboration with CANGENE Corporation, (Winnipeg, MB, Canada) to custom-synthesize FcεRI-β chain

antibodies. In fact, preliminary data with serum from immunized mice showed promising results in detecting specific FcεRI-β chain expression in basophilic cell line KU812 (data not shown). Since the β-chain has been shown to enhance the expression and function of FcεRI-α chain in mast cells (197, 268), additional regulatory mechanisms of FcεRI-α chain expression in ASM may also exist which require further investigation and are of utmost priority to us. Not only TNF and IL-4 enhanced the protein expression of FcεRI-α chain, this regulation had a functional outcome on IgE-induced synthetic function in ASM cells (321). Our findings indeed partly corroborate a recent study on distal promoter of human FcεRI gene wherein IL-4 was found to enhance the intracellular expression of FcεRI-α chain (260).

Classically, TNF-α and IL-1β are prototype pro-inflammatory cytokines, which have been shown to play a central role in lung inflammation both in animal models and allergic patients (334, 335, 337). IL-1β is known to alter airway function by inducing cellular infiltrate, mucus hyperplasia, fibrosis, and airway wall thickening (420). It induces IL-8/CXCL8 release which has been shown to modulate ASM contraction and migration mediating AHR and remodeling in asthmatic subjects (421). TNF has been proposed to exert deleterious effects directly on airway smooth muscle, including its most recently explored roles in (i) activation of transient receptor potential channel 3 (TRPC3) leading to abnormal store-operated calcium influx, and (ii) upregulation of CD38 which regulates intracellular calcium and plays a role in AHR (341, 342). Th-2 cytokines are also known to play a critical role in pathogenesis of allergic disorders, and have been investigated extensively in strategies targeting respiratory diseases (338, 401). In particular, IL-4, in combination with IL-13 is known to induce Th-2 humoral immune responses leading to IgE production by B-cells (11). Altogether, the data presented in *Chapter 4* demonstrated that the FcεRI-α expression in human ASM is modulated by

proinflammatory and Th2 cytokines. Considering that these cytokines are detected in high levels in local airway milieu in allergic asthma patients (35, 120, 122, 334), our results suggest that targeting these mediators may offer novel means of managing allergic airway inflammation.

8.4.0. CYTOKINE AND FcεR-INDUCED SYNTHETIC FUNCTION OF ASM

Immunoglobulins were initially thought to activate only inflammatory cells which subsequently activate the ASM via release of cytokines and growth factors. However, in contrast to being just passive recipients of inflammatory cell input, ASM has recently been demonstrated to behave as potential 'inflammatory-like' cells (272, 279, 422). Indeed, IgE sensitization of ASM through FcεRII/CD23 was shown to cause autocrine release of IL-13 and IL-5, which may underlie the altered ASM responsiveness (96, 174, 272). Later, FcεRI activation following IgE/anti-IgE cross-linking induced IL-4, IL-5, IL-13, and CCL11/eotaxin-1 release in human ASM cells. FcεRI cross linking by IgE/anti-IgE also induced a rapid and transient increase in intracellular Ca^{2+}, a likely determinant of ASM contractility and subsequent AHR (**Figure 8.4.1**). Particularly, a blocking antibody for FcεRI-α chain abrogated the release of these mediators, suggesting that these effects were FcεRI-mediated (97, 98). As shown in *Chapter* 4, mimicking of asthmatic airway milieu with proinflammatory and Th2 cytokines enhanced the expression of FcεRI-α chain, and TNF presensitization of human ASM amplified the IgE-induced release of chemokines such as CCL11/eotaxin-1, CCL5/RANTES, CXCL8/IL-8, and CXCL10/IP-10 (321). Mechanistic analysis further showed that the expression of these chemokines was a result of promoter activation by IgE sensitization. Lentiviral-shRNA-transduced approach showed that the transcriptional activation of these chemokine promoters was abrogated in Syk-silenced ASM cells. Since Syk is crucial for FcεRI signal transduction in inflammatory cells (227), our results

argued for an FcεRI-specific effect of IgE in ASM cells (321). Another study showed that the IgE/anti-IgE treatment of human ASM cells leads to modest levels of matrix metalloprotease 1 (MMP-1) production which may contribute to airway inflammatory and remodeling responses (383). Roth and Tamm (2010) recently showed that the ASM cells from asthma patients showed de novo synthesis, and released higher levels of pro-asthmatic mediators such as IL-4, IL-6, IL-8/CXCL8, and TNF compared with the ASM cells from COPD patients or from healthy controls (278). An abstract study utilizing PCR gene array also showed the expression of chemokines such as IL-8/CXCL8, CXCL6, CXCL1, and CXCL2 in response to IgE in human ASM cells (423). Collective data thus far suggests that IgE can induce a plethora of cytokine and chemokine mediators in ASM **(Figure 8.4.1).** Moreover, these mediators are also known to be produced by ASM cells *in vivo* (116), which may augment the recruitment of multiple inflammatory cells such as eosinophils, basophils, neutrophils, monocytes, DCs, and Th2 lymphocytes. The accumulation of these inflammatory cells may release granular enzymes, proteases, and other mediators which may in fact contribute to the development of airway inflammation, AHR, and local tissue injury.

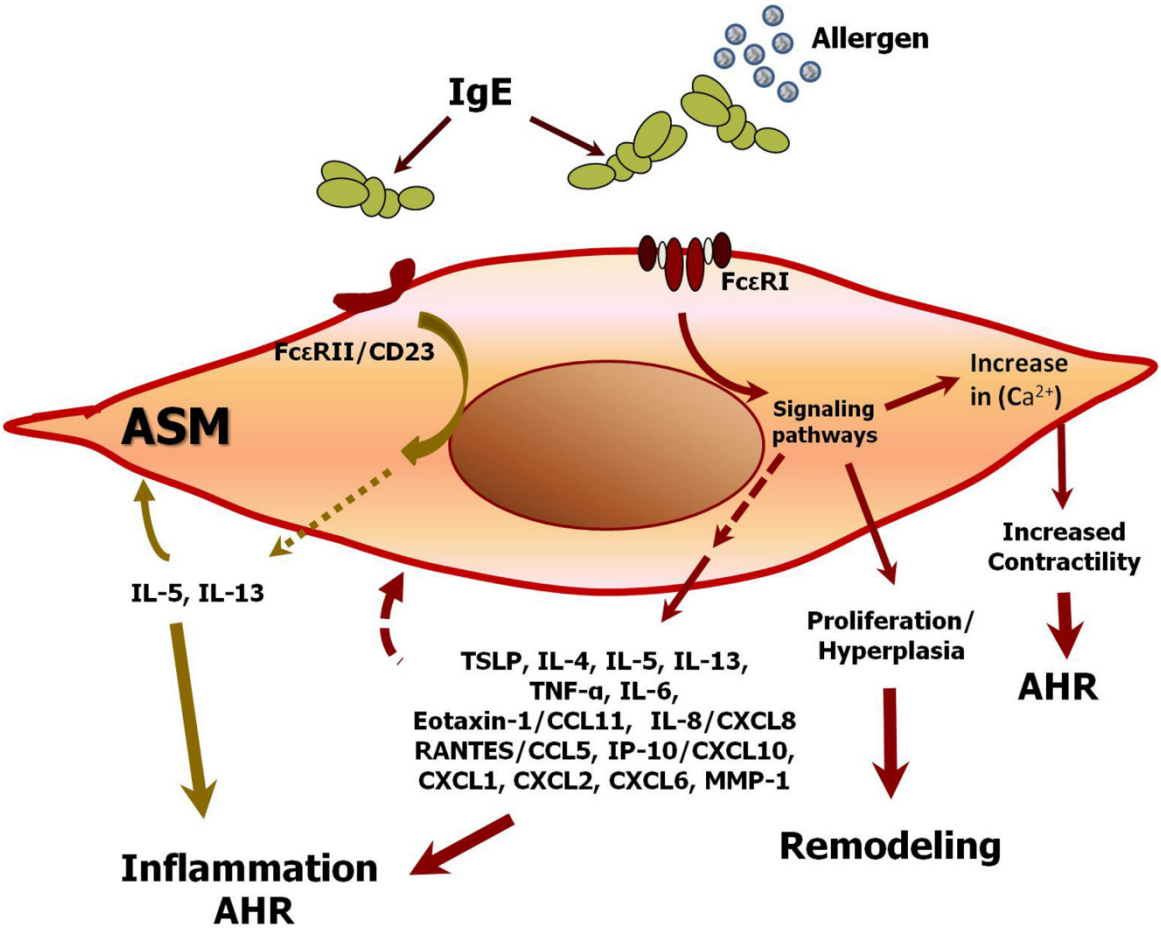

Figure 8.4.1. FcεR activation-induced effector functions on ASM. Both FcεRI and FcεRII/CD23 activation on ASM by IgE (allergen-dependent or -independent) leads to proinflammatory mediator (98, 321, 326) **(271, 278, 383, 423)** and intracellular Ca^{2+} release (98), which may eventually contribute to airway inflammation, and AHR. Data from *Chapter 7* and animal models also suggest that IgE can directly induce ASM cell proliferation (hyperplasia) which may account for the ASM remodeling observed in allergic asthma.

Data from past decade has explored the synthetic response of ASM in response to cytokines such as TNF, IL-1β, IL-4, and IL-13 (114, 119, 424, 425). One of the recently discovered mediators that are pivotal in shaping allergic immune responses is TSLP. TSLP-stimulated mDCs can prime naïve CD4+ T cells in an antigen-specific manner, resulting in T cells showing characteristic features of Th2-differentiated inflammatory cells (i.e. production of IL-4, IL-5, IL-13 and TNF), with the exception of IL-10 production (302). Interestingly, in the absence of IL-12, TSLP induces the expression of OX40L, the ligand for cell survival factor OX40 on DCs; and OX40-OX40L interactions were found to be critical for the ability of the mDCs to drive Th2 cell differentiation (307). Given the emerging role of TSLP in allergic inflammatory diseases (302, 307, 308) and the 'inflammatory-like' nature of ASM (116, 117, 406), it was enticing to assess whether ASM could contribute to airway inflammatory response via production of, and/or responsiveness to TSLP. Indeed, a constitutive, TNF or IL-1β-inducible, and *in vivo* expression of TSLP was demonstrated in ASM of COPD patients (292). Ying et al (291) also showed an elevated mRNA expression of TSLP, and chemokines TARC/CCL17, MDC/CCL22, and IP-10/CXCL10 in bronchial mucosa, with corresponding elevation of these proteins in the BAL fluid of COPD patients. As demonstrated in *Chapter* 5, we further demonstrated that the NF-κB, and AP-1-like transcription factors are critical in TNF-induced TSLP release in ASM cells **(Figure 8.4.2)** (426). Airway epithelial cells also express IL-1-induced TSLP via NF-κB-mediated pathway (290). However, in contrast to airway epithelial cells (290), stimulation of ASM with LPS (TLR 4 agonist) and cytosine phosphate guanine (CpG) (TLR 9 agonist) did not induce TSLP release, even though ASM cells express TLR 2, 3, 4 and 9 (427, 428); suggesting perhaps different triggers and signaling mechanisms in ASM compared to the epithelial cells. Moreover, supernatants of IgE/anti-IgE-activated mast cells

induced the TSLP release in ASM cells (288). Notably, neither additional Th1 (IFN-γ) nor Th2 (IL-4, IL-9) cytokines induced the TSLP expression in ASM (288, 292). Except TNF-α, other mast cell mediators (e.g. tryptase, histamine, leukotriene C_4, prostaglandin E_2, and prostaglandin D_2) could not induce TSLP release in ASM cells (288). Interestingly, the culture supernatants of IL-1 and TNF-stimulated ASM cells triggered the release of IL-5 and IL-13 by mast cells, the response that was completely inhibited by anti-TSLP antibody, suggesting a TSLP-dependent interaction (288).

Chapter 6 of this project was aimed at investigating the TSLP expression in ASM in response to IgE sensitization. Notably, TSLP was recently found to be necessary and sufficient to drive Th2 cytokine-mediated airway inflammation in murine models of asthma. Lung-specific expression of a TSLP transgene induced allergic airway inflammation characterized by a massive infiltration of inflammatory cells, goblet cell hyperplasia, and subepithelial fibrosis, with increased serum IgE levels (305). On the contrary, mice lacking the TSLPR failed to develop asthma in response to inhaled antigen (ovalbumin plus alum), probably due to the inability of CD4+ T cells to respond to TSLP, as reconstitution with TSLPR-positive T cells restores the aspects of the inflammatory disease (306). The findings from these animal models are closely dictated in human asthmatic subjects where higher concentrations of TSLP have been detected in the lungs, correlating with Th2 attracting chemokines and disease severity (291, 293). Interestingly, IgE also induced the de novo TSLP transcription and protein release in ASM cells through the activation of NF-κB and AP-1 transcription factors (326). Interestingly, Syk played a crucial role in IgE-induced TSLP expression since Syk inhibition by pharmacologic or genetic approached attenuated the TSLP promoter activity (*Chapter 6*). In support of our observations, human ASM tissue from asthma patients was recently shown to express TSLP *in vivo*, the

expression was increased in mild-moderate disease but not in severe asthma (429). Therefore, IgE-induced TSLP expression in ASM clearly points towards a vicious cycle of allergic airway inflammation mediated by TSLP (326).

Other triggers of TSLP release in ASM cells include IL-4, β2-adrenergic receptor (AR) agonists (296), and cigarette smoke extract (CSE) (310). The mechanisms of TSLP induction primarily involve the activation of p38, Erk MAP kinases, and NF-κB and AP-1 transcription factors, as we summarized lately in (430) **(Figure 8.4.2)**. Of particular interest, it was recently reported that the cytokine (IL-4 plus TNF)-induced TSLP production by lung mesenchymal (ASM and fibroblasts) and epithelial cells was enhanced by β_2-agonists of either long-acting (salmeterol, formoterol) or short-acting (salbutamol) nature (431). The effects of β_2-agonists on TSLP release in ASM were found to directly involve cAMP-PKA pathway. Moreover, corticosteroid (fluticasone) was found to significantly inhibit the cytokine- or β_2-agonist-induced TSLP production by all lung tissue cell types. Collective data from these studies provides a better rationale for combination therapy with β_2-agonists and corticosteroids to effectively inhibit the TSLP-mediated lung inflammation (296, 430) **(Figure 8.4.2)**.

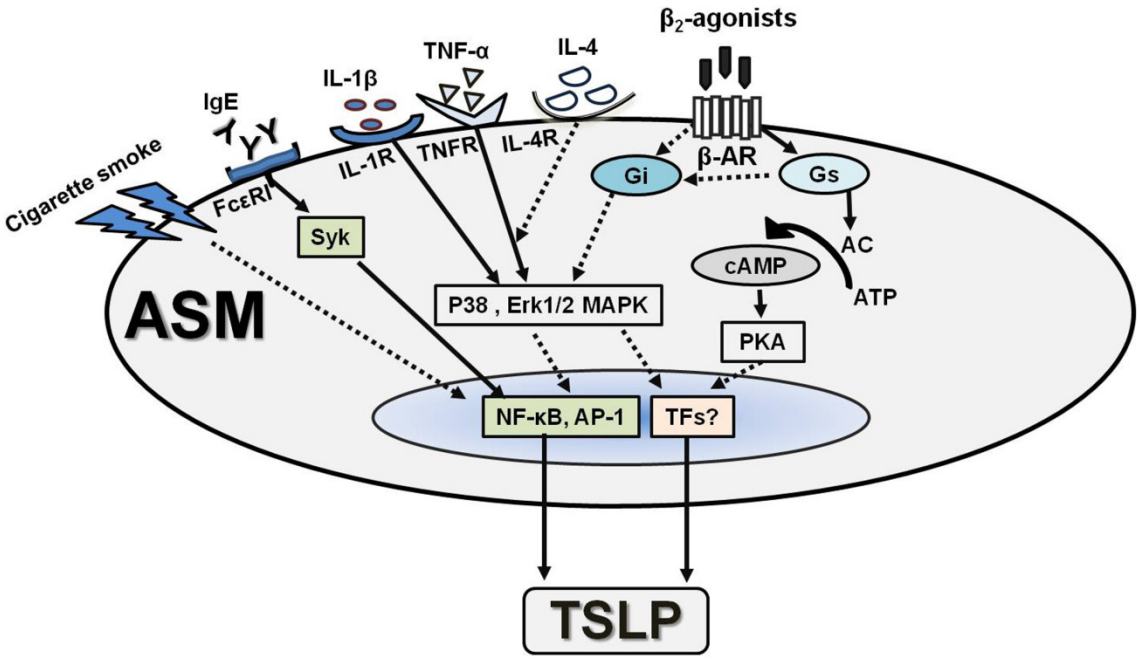

Figure 8.4.2. Triggers and mechanisms of TSLP expression in ASM. Proinflammatory (IL-1β, TNF-α) cytokines induce TSLP in ASM via MAPK, NF-κB and AP-1; while IgE utilizes NF-κB and AP-1 pathways. Th2 cytokine IL-4 synergizes with TNF-α, cigarette smoke extract induces TSLP, whilst β2-agonists were recently shown to augment the cytokine-induced TSLP expression in ASM cells. Although mechanisms remain obscure, β_2-agonists may involve the G-protein coupled receptor (GPCR)'s Gi subunit stimulation that induces Erk activation and eventually synergize with cytokine-induced Erk MAPK pathways. This might also involve unknown transcription factors that are activated through cAMP-PKA pathway. Solid line arrows indicate known pathway(s) and dotted line arrows represent plausible pathway(s). β-AR, β-adrenergic receptor; Gs, stimulatory GPCR subunit; Gi, inhibitory GPCR subunit; AC, adenylate cyclase; PKA, protein kinase A; and TFs, transcription factors (430).

8.5.0. TSLP/TSLPR-MEDIATED AUTOCRINE/PARACRINE INFLAMMATORY NETWORK IN ASM

In context of the global hypothesis of ASM's role as a critical airway immunomodulatory cell (116, 117, 406), numerous studies have implicated the proinflammatory and Th-2 cytokines/chemokines and their receptors in ASM cell activation. For instance, ASM cells are a rich source of IL-8/CXCL8 (323) and express the IL-8/CXCL8 receptors (CXCR1, and CXCR2), activation of which leads to increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$), cell contraction and cell migration (421). Similarly, ASM cells produce and respond to eotaxin-1/CCL11 (432), and IL-6 (433). Therefore, the ASM-produced TSLP may either be involved in regulation of local airway immune response e.g. by targeting mast cells that are in close vicinity of ASM (47, 289), eosinophils (434) or dendritic cells (302).

During the course of this project, we also reported the expression of a dimeric TSLP receptor (TSLPR, consisting of IL-7Rα chain and a common γ receptor like TSLPR chain) in ASM cells *in vitro* and in smooth muscle bundles of mild allergic asthma patients. TSLPR activation on ASM by recombinant human TSLP induced a significant release of both eosinophil- (CCL11/eotaxin-1) and neutrophil-attracting (CXCL8/IL-8) chemokines, and proinflammatory cytokine IL-6 (324). Mechanistically, TSLP transiently activates all three classes of MAPKs (p38, JNK, and Erk1/2), and induces STAT3 but not STAT5 phosphorylation in ASM cells **(Figure 8.5.0)**. Notably, TSLPR activation in bronchial epithelial cells involves both STAT3 and STAT5 phosphorylation to induce IL-13 production and cell proliferation (435). It is indeed surprising that TSLP does not induce STAT5 phosphorylation in ASM, particularly since STAT5 is considered to be a signature transcription factor in TSLPR-signaling (287, 435-438). However, lack of STAT5 phosphorylation does not preclude its role in inducing gene

transcription in TSLP-stimulated ASM cells. STATs can in fact mediate transcriptional activity without phosphorylation, as has been shown for STAT1 in some cell lines (439) and for STAT6 in IL-17A-induced eotaxin-1/CCL11expression in ASM cells (325). Therefore, further studies are required to understand the TSLPR-induced complex signaling network in ASM cells.

Another group (310) recently confirmed the TSLPR expression on ASM cells. Authors also reported an enhanced expression of both TSLP and TSLPR upon CSE exposure. Interestingly, TSLP stimulation of ASM cells led to enhanced $[Ca^{2+}]i$ responses to bronchoconstrictor agonists, a critical determinant of ASM contractility. Moreover, the use of TSLP and TSLPR blocking antibodies abrogated the CSE-induced $[Ca^{2+}]i$, suggesting TSLP to be a potential mediator of airway contractility, at least from an ASM cell perspective (310). Collectively, these studies suggest that ASM cells are a rich source and target of TSLP, and uncover a novel role of TSLP in modulating the function of ASM that may eventually contribute to airway inflammation and bronchoconstriction in a TSLP/TSLPR-dependent manner. This may also partly explain the attenuated bronchial hyperresponsiveness and inflammation in $TSLPR^{-/-}$ mice (306). Further investigation is required to establish whether ASM cell-derived TSLP activates ASM cells in an autocrine/paracrine manner, or is consumed readily and preferably by other cells of local milieu (e.g. mast cells, eosinophils, or epithelial cells) **(Figure 8.5.0).** Since TSLP induces proliferation in airway epithelial cells (435), a similar role in ASM cells is also plausible.

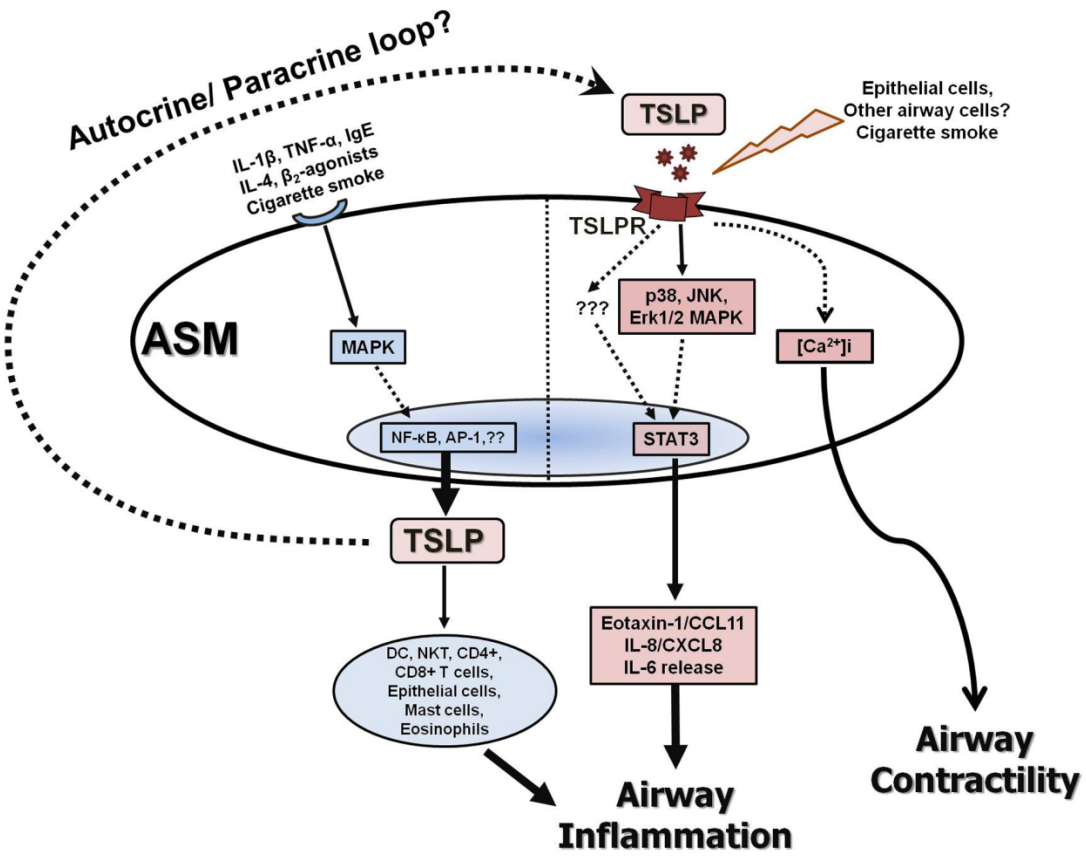

Figure 8.5.0. TSLP/TSLPR-mediated autocrine activation of ASM. Cytokines-, β2-agonists-, or cigarette smoke-induced TSLP from ASM may act on airway structural or lymphoid/myeloid cell types eventually contributing to airway inflammation. A functional TSLPR presence on ASM also suggest an autocrine loop of inflammation whereby ASM-produced TSLP activates the cell in autocrine/paracrine manner and induces inflammatory cytokine (IL-6) and chemokines (IL-8/CXCL8, eotaxin-1/CCL11) release in a MAPK (p38, JNK, Erk) and STAT3-dependent manner. ASM-produced TSLP may also enhance the $[Ca^{2+}]i$, eventually resulting in increased ASM contractility. Solid line arrows indicate known pathway(s) and dotted line arrows represent plausible pathway(s) (430).

8.6.0. ASM-MAST CELL INTERACTION: TSLP AS A NOVEL MEDIATOR?

Mast cell and ASM interaction has recently been demonstrated as a critical determinant of airway pathology. Regardless of the severity of the disease, activated mast cell accumulation has been found within the bronchial smooth muscle bundles in asthma (47). Co-culture studies have shown that this interaction may contribute to the development of ASM hypertrophy, hyperplasia, and hyperreactivity; potentially via the release of mast cell tryptase, IL-13, TNF, and TGF-β (136, 440). FcεRI-dependent activation of mast cells and ASM was also proposed to facilitate this interaction, potentially via the expression of CCR3 on mast cells in response to FcεRI activation-induced CCL11/eotaxin-1 release by ASM cells (97). Conversely, mast cell-derived TNF can induce CCR3 in ASM cells (432). From the perspective of current studies, TSLP represents a novel mediator of this cross-talk. The TSLP produced by ASM cells was shown to potentiate mast cell release of IL-5 and IL-13, whereas supernatants of IgE/anti-IgE-stimulated mast cells induced TSLP release in human ASM cells which was found to be predominantly mediated by mast cell released-TNF (288). Recently, another study confirmed the expression of both TSLP and TSLPR in mast cells and ASM cells. However authors could not find any TSLP/TSLPR-mediated functional relationship between both cell types (429). However, in light of the findings that IgE can induce TSLP expression in both cell types and a functional TSLPR is reported on both cell types (288, 430, 441), further studies are required to assess whether a TSLP/TSLPR-mediated functional cross-talk exists between the two.

8.7.0. IgE-MEDIATED MODULATION OF ASM GROWTH

Airway remodeling is the cardinal feature in most allergic asthma patients. Despite a good control of inflammation, most asthma medications fail to reduce airway remodeling (442).

ASM hyperplasia and hypertrophy are thought to be the leading causes of ASM remodeling, besides attenuated apoptosis and fibrocytes/myocytes migration mechanisms (443). Particularly, asthmatic ASM proliferate faster, at least due to the lack of C/EBPα expression (108). BAL fluid from asthmatic subjects exerted mitogenic effects on ASM cells (152), while it is known that BAL from asthmatics contain high levels of IgE in segmental allergen challenge (444). Since IgE was recently shown to exert pro-survival effects in inflammatory cells such as mast cells, monocytes, and asthmatic neutrophils (231, 239, 241, 242), we assessed whether IgE can modulate ASM growth directly. Indeed, as shown in *Chapter* 7, IgE caused a significant increase in DNA synthesis and cell number (**Figure 8.4.1**). IgE-induced mitogenic signaling was found to be predominantly through Erk1/2, p38, JNK MAPK, Akt, and GSK-3α/β. Interestingly, IgE induced STAT3 activation in ASM which was found to be crucial in IgE-induced DNA synthesis. Although the previously well-established mitogen of ASM (PDGF-BB) also signals through STAT3 (373), IgE effect on ASM growth were found to be independent of synergy with PDGF-BB. It is unclear whether IgE can modulate human ASM growth/proliferation *in vivo*, however further investigation is warranted to assess this possibility. At least in a murine model of chronic asthma, anti-IgE treatment decreased the thickness of ASM layer and peribronchial fibrosis compared with the untreated mice; implicating that (i) IgE could perhaps be one of the factors inducing ASM remodeling *in vivo,* and (ii) targeting IgE/FcεR network on ASM with anti-IgE represents a novel approach in reducing airway remodeling (384). In this line, anti-IgE asthma therapy, Omalizumab/Xolair® has been proposed to provide some benefits in overall airway tissue remodeling (442). However, detailed studies are lacking to reach any conclusion.

8.8.0. IgE-INDUCED SIGNALING AND PROPOSED MODEL OF FcεRI ACTIVATION

IN ASM

IgE induces multiple signaling pathways in inflammatory cells such as mast cells and basophils. These signaling mechanisms differ in IgE 'sensitization' versus 'cross-linking' models. However, some of the signaling molecules activated in response to IgE are common in both models (239, 240). In our efforts to understand the function of FcεRI activation in human ASM cells, we investigated some of the putative signaling mechanisms in parallel. IgE-activated ASM cells showed Syk requirement to induce synthetic (release of CC/CXC chemokines and TSLP, *Chapters 4, 6*) and mitogenic function (*Chapter 7*). Although various tyrosine kinases have been implicated in FcεRI-mediated signaling, only the lack of Syk results in complete inhibition of cell degranulation and cytokine release (227, 445). Syk-deficient cells are defective in most of the signaling events that occur downstream of FcεRI activation, and Syk-/- mast cells fail to activate NFAT or NF-κB transcription factors (227). In fact, novel Syk inhibitors are in clinical development for the treatment of airway diseases (446), with preliminary evidence of success in controlling the allergen-driven symptoms in human subjects (447). Our genetic ablation and biochemical data not only validates these observations and establish an upstream signaling target in ASM, it also provides an evidence of FcεRI-specificity of IgE-induced ASM activation since Syk serves as a signature pathway of FcεRI activation (53, 97, 226, 248) (**Figure 8.8.0**).

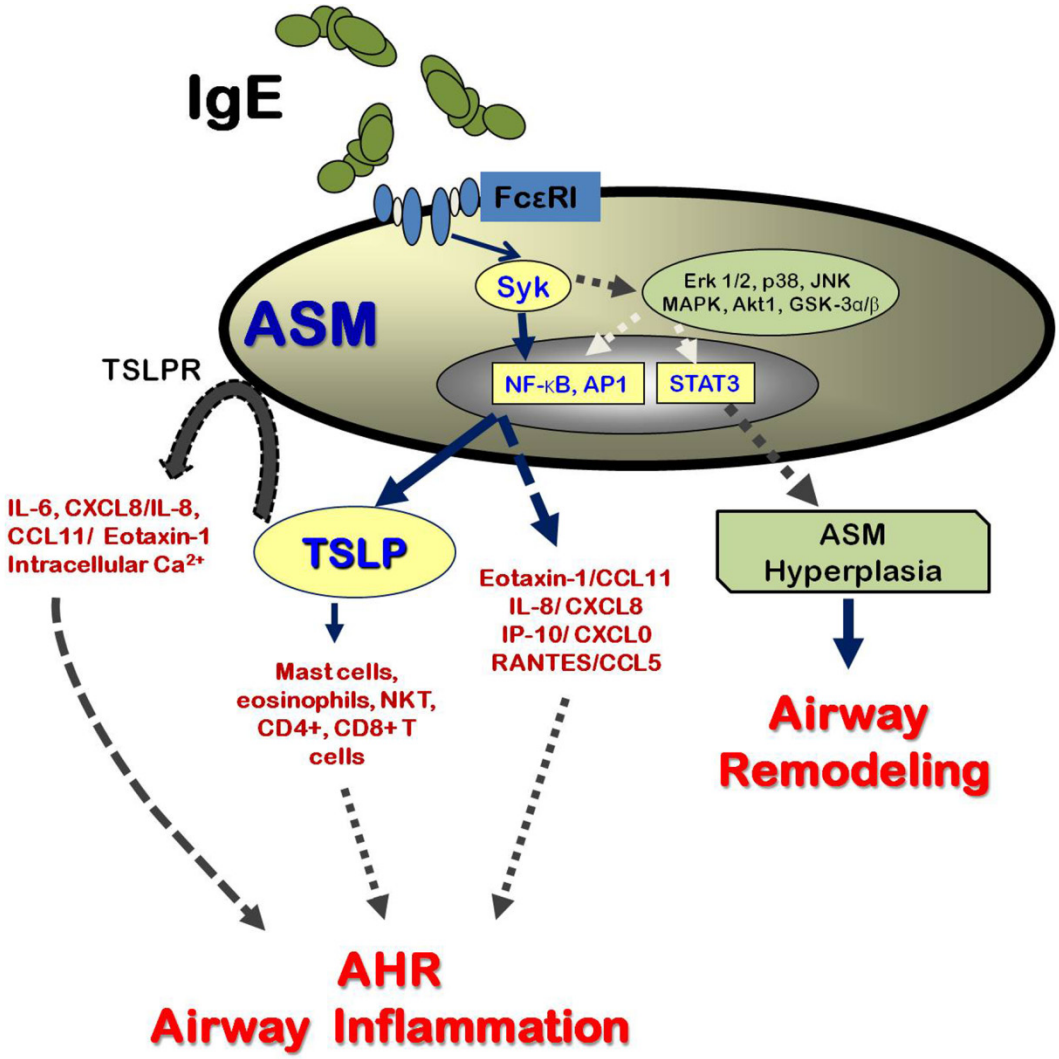

Figure 8.8.0. Proposed model of IgE sensitization-induced signaling events in ASM. FcεRI activation by IgE leads to Syk activation which can activate Erk1/2 and other MAPKs culminating in NF-κB, AP-1, and/or STAT3 activation. Eventually, the NF-κB and AP-1 transcription factors may translocate to the nucleus and induce proinflammatory mediator expression such as CC and CXC chemokines (*Chapter 4*), and TSLP (*Chapter 6*). Various MAPKs and STAT3 may also cooperate to regulate cell proliferation (*Chapter 7*).

IgE sensitization also induced phosphorylation of MAPK such as Erk1/2, p38, JNK MAPK, and Akt and GSK-3α/β. Other groups have also shown an IgE-induced activation of Erk1/2 and p38 MAPK in ASM synthetic response (278). We also have some evidence of various transcription factors activation in human ASM cells. Whereas IgE-induced TSLP expression required NF-κB and AP-1 activation (*Chapter 6*), the involvement of STAT3 was found to be crucial for IgE-induced mitogenic signaling in ASM cells (*Chapter 7*) **(Figure 8.8.0)**.

Although it is not completely known whether IgE-induced signaling in ASM involve FcεRI cross-linking due to IgE aggregate formation, preliminary evidence from *Chapter 7* suggests that the IgE cross-linking by anti-IgE antibodies from various sources does not augment the IgE-induced ASM proliferation. Cumulative data thus far suggest that IgE can activate ASM cells in both antigen (or anti-IgE)-independent (*Chapter 7*) (278, 321, 326) and antigen-dependent manner (97, 98). However, detailed studies are required to fine-tune the IgE-induced mediator release in ASM, particularly to understand whether it requires FcεRI cross-linking. A plausible signaling network induced by IgE binding to FcεRI is proposed in **Figure 8.8.0.**

9.0. CHAPTER 9

GENERAL CONCLUSIONS AND SIGNIFICANCE

Classically, IgE was thought only to activate the inflammatory cells such as mast cells, basophils, and APCs, which altogether plays an indispensable role in allergic reactions including asthma, atopic dermatitis, and allergic rhinitis. However, FcεRI transcripts have been detected in the cells of non-hematopoietic origin such as mouse neurons (448), mouse melatonin-secreting pinealocytes from pineal gland (449), mouse and human aortic smooth muscle cells, and epithelial cells in acute myocardial infarction (419), and human intestinal and airway epithelial cells (80, 83), thus suggesting an evolving role of IgE-FcεRI complex interaction beyond the 'classical' inflammatory cells. Interestingly, local IgE expression has been demonstrated *in vivo* in the airways of atopic subjects (12). Recent data from animal studies have provided insights about the transport of IgE and IgE-allergen complexes to sub-epithelial tissue where smooth muscle reside. For instance, a splice variant of the IgE receptor, expressed by epithelial cells, was shown to bind and transport allergens into sub-epithelial tissue (209). In fact, there is evidence for IgE-allergen transport through epithelial cells in large quantities without changing their structure (82). More recently, house dust mite allergens disrupted the cell-cell contacts and created gaps in epithelial cell barrier, by involving the activity of protease activated receptor (PAR)-2, which may facilitate allergens to infiltrate into sub-epithelial tissue (450). Altogether, data from these studies suggest a rational *in vivo* interaction of ASM with IgE/ IgE-allergen complexes.

Our project evaluated the factors governing the FcεRI expression in ASM cells and demonstrated a significant effect of TNF and IL-4 cytokines on this process. IgE sensitization was found to induce the expression of multiple proinflammatory mediators such as eotaxin-

1/CCL11, RANTES/CCL5, IL-8/CXCL8, IP-10/CXCL10. These mediators have been accepted widely to exhibit dramatic effects on airway inflammatory pathology including the Th2 cell differentiation, initiation and perpetuation of airway inflammation by promoting inflammatory cell recruitment, induction of AHR by direct or indirect mechanisms, and tissue remodeling due to structural alterations resulting from inflammatory cell activation (116, 312, 451). IgE-induced TSLP may in fact promote a Th2-dominated immune response within airways, suggesting an active contribution of ASM in this process. IgE was found to regulate ASM growth by inducing *de novo* DNA synthesis and increase in cell number, a novel observation which could have dramatic consequences on airway remodeling observed in allergic asthma. Collectively, IgE-dependent FcεRI activation in human ASM cells may contribute to the airway inflammation, AHR, and tissue remodeling. Our data recommends the use of IgE, FcεRI, Syk, TSLP, or TSLPR neutralizing approaches in controlling the allergic asthma.

9.1.0. CLINICAL RELEVANCE OF RESEARCH

Aberrant IgE production is probably the strongest contributing factor in childhood and adult asthma pathology. Owing to its central role in allergic asthma (393), IgE has long been perceived as an attractive regimen for asthma therapy. However, only recently a recombinant, humanized monoclonal anti-IgE antibody, omalizumab/ Xolair® (Genentech/Novartis, South San Francisco, CA, and Tanox, Inc., Houston, TX) has been approved by the Food and Drugs Administration (FDA) for the treatment of allergic asthma (452). Omalizumab targets the Cε3 region on the Fc fragment of IgE which binds the α-chain of the high affinity Fc receptor (FcεRI), and low affinity FcεRII/CD23, thereby blocking the binding of IgE with its receptors (453). Omalizumab treatment has been shown to be well tolerated and clinically effective in the

management of moderate-to-severe allergic asthma (452, 454). Anti-IgE therapy acts by reducing serum IgE levels, FcεRI expression on mast cells, basophils, and antigen-presenting cells, and by attenuating tissue mast cell function (393, 442). Omalizumab also reduces submucosal IgE+ and FcεRI+ cells, and eosinophil numbers in airway tissue and induced sputum in mild-to-moderate asthma patients (455). Majority of patients maintained a good level of asthma control after up to 3 years of omalizumab withdrawal, suggesting that the anti-IgE therapy may indeed prove as disease modifying in asthma (456). Although there are contentions regarding the safety of omalizumab due to potential risk of anaphylaxis, neoplasms due to long-term immunosuppression, and parasitic infections (442, 457); detailed studies are underway to establish the safety and efficacy of omalizumab therapy in allergic asthma.

From ASM perspective, omalizumab treatment abrogated the IgE-induced mediators of asthma relevance such as IL-4, IL-6, CXCL8/IL-8, and TNF from human ASM cells, the effect was surprisingly independent of FcεRI or FcεRII/CD23 downregulation (278). Although omalizumab inhibits both early- and late-phase asthmatic response to inhaled allergens (458), and decreases nonspecific AHR *in vitro* (459), it had little effects on AHR to methacholine in asthma patients (455, 458); while its effects on airway remodeling remain completely unknown. At least in a murine model of chronic asthma, anti-IgE treatment decreased the thickness of ASM layer and peribronchial fibrosis compared with the untreated mice (384). Nonetheless, although a relationship between chronic inflammation and structural remodeling in asthma remains debated, it would be worthwhile to test whether anti-IgE therapy can improve airway remodeling in allergic asthma.

It has been known from large epidemiological studies such as *Tucson Children's Respiratory Study* (460-462), that early childhood sensitization with food antigens or any single

inhalant along with a clinical profile of recurrent wheezing is one of the most sensitive and highly specific risk factor that would predict future asthma. Although the mechanism and impact of early sensitization on ASM function is considered a critical determinant of asthma pathophysiology, very little is known in this regard. Our data showing the IgE-induced release of multiple proinflammatory mediators and a pro-mitogenic effect addresses at least some of the mechanisms involved in sensitization-induced ASM function.

9.2.0. LIMITATIONS AND ALTERNATE VIEW OF OUR FINDINGS

Although the data presented in this thesis essentially underlines the critical role if IgE/FcεRI pathway in allergic sensitization of ASM, there are some limitations of the study which may suggest an alternate explanation of some of these findings. For instance, although primary ASM cells were used in this study, the outcome observed may not reflect an in vivo physiological condition. Cells undergo extreme stress starting from their isolation in tissue form from airways, followed by culture and passaging up to 5-10 generations. In fact, it has been suggested that isolating pure smooth muscle from large unfixed pieces of tissue is difficult and may carry contamination of fibroblasts (463). The latter, in presence of serum and multiple passages may become myofibroblasts by expressing α-smooth muscle actin, calponin, desmin, and various other putative ASM markers. The phenotype switching of ASM from 'contractile' to 'synthetic-proliferative' in presence of serum is nearly interchangeable with myofibroblasts (463, 464). Therefore, while the data from our project reflects an essential consideration of IgE/ FcεRI pathway in airway structural cell biology, the existing debate about their true identity as 'ASM', 'fibroblast', or 'myofibroblasts' cautions any strong conclusions about their cell-specificity.

A biphasic change in FcεRI-α transcription following proinflammatory stimuli suggest

that the factors produced early (within 2-6 hrs) may act on ASM in an autocrine/paracrine manner to modulate subsequent synthetic response of the cells. This also applies to the interpretation of our data about TSLP/TSLPR pathway. Although the TSLP produced by TNF or IgE (322, 326, 430) may act on the cells in vicinity (such as mast cells, CD4+ T, and CD8+ T cells), a more likely possibility could be an autocrine/paracrine activation of ASM by its own TSLP which can lead to IL-6, IL-8/CXCL8, and eotaxin-1/CCL11 production (324). This response may also represent what we observed in *Chapter 4* where IgE was shown to induce higher levels of IL-8/CXCL8, eotaxin-1/CCL11, IP-10/CXCL10, and RANTES/CCL5 following TNF presensitization (321).

Moreover, IgE-induced proliferation in ASM *(Chapter 7)* may also have an alternate explanation. We have shown that ASM can produce TSLP in response to IgE (326), and also possess a functional TSLPR (324). Interestingly, TSLP seem to induce proliferation in ASM cells *(Shan L, Redhu NS, and Gounni AS, manuscript under preparation)*, besides a well reported mitogenic function in epithelial cells (435). This could in fact be interpreted as if IgE first induces TSLP which then acts on ASM in an autocrine/paracrine manner to induce subsequent proliferation. A constitutive expression of TSLP in ASM (292, 322) may also suggest that it works as a feed forward mechanism to regulate ASM growth following the stress it undergoes during tissue isolation procedures. These are certainly some of the possibilities worth exploring in near future. Collectively, although our data may have alternate explanations, it does not preclude an outstanding contribution of IgE/ FcεRI pathway in modulation of ASM phenotype and function.

9.3.0. FUTURE DIRECTIONS

Since the detection of FcεRI on ASM cells, modest efforts have been made to understand the regulation and function of this important receptor. Considering a critical role of ASM in allergic asthma pathophysiology, much more is needed to understand IgE-FcεRI interaction. While substantial efforts have been invested in examining the FcεRI activation on inflammatory cells, only limited reports are available in context of its function on ASM cells. To understand the composition, dynamics, and effector functions of FcεRI activation in ASM, some of the key areas that need to be addressed are:

- Although FcεRI expression in ASM bundles of allergic asthma patients was shown earlier, there is a stern need to evaluate the association between FcεRI expression in ASM tissue and allergic asthma severity in large population
- Uncover the differential signaling events between 'sensitization' and 'cross-linking' modes of FcεRI activation, and to understand whether FcεRI signaling network in ASM is similar to mast cells
- Elucidate the IgE-induced expression of novel proinflammatory mediators by utilizing multiplex platforms to understand the global synthetic potential of this interaction on ASM.
- Evaluate in detail the role of individual Fc receptors (FcεRI, FcεRII/CD23) activation and their contribution to ASM function in airway inflammation, AHR, and remodeling in allergic asthma
- Determine the *in vivo* outcome of FcεRI activation on ASM cells and other structural cells such as epithelial cells and fibroblasts by utilizing FcεRI-transgenic animal models. Development of such a transgenic mouse model is underway.

It is a matter of great debate whether FcεRI activation on ASM can directly modulate the immune response within the airways. This project has provided some leads in unraveling the

IgE-induced expression of pro-allergic TSLP in ASM. Notably, TSLP can induce the maturation of dendritic cells (DCs) which in effect can induce naïve CD4+ T cells to differentiate into Th2 cells *in vitro* and *in vivo*. DCs do this potentially via upregulation of OX40L on their surface in absence of IL-12, which triggers the inflammatory Th2 differentiation following an OX40L-OX40 interaction between DC and T cells (284, 305, 306). In addition, ASM itself can express enhanced OX40L in inflammatory milieu (131), and has been shown to possess the molecular machinery for antigen presentation (MHC I and II, co-stimulatory molecules, and cellular adhesion molecules) (127-129). In light of these observations, the role of ASM in antigen presentation to T cells through the modulation of DC function or through TSLP-OX40L-OX40 interaction between T cell and ASM itself is enticing to explore. Therefore, understanding the role of ASM-produced TSLP in this Th2 differentiation process may establish a direct role of ASM in airway immune responses.

10.0. CHAPTER 10

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