Role of IgE in modulating the expression and function of smMLCK in human airway smooth muscle cells

By

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FIGURE LEGENDS

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Abstract

Aberrant phenotypes of airway smooth muscle cells are central to the pathophysiology of asthma. The hypercontractile nature of these cells and hypertrophy are the key reasons for the excessive narrowing of the airways observed in allergic asthma. Although previous studies have indicated a role of enhanced content of smMLCK in modulating the contractile reactivity, as well as an indication of hypertrophy of HASM cells in asthmatic conditions, the effect of IgE on the expression of smMLCK in HASM cells is not fully understood. In this study, we demonstrate that IgE augments the expression of smMLCK at the mRNA and protein level. Inhibition of IgE binding with anti-FcεRI blocking antibody, Syk silencing, pharmacological inhibitors to MAPK (ERK1/2, p38, and JNK) and PI3K significantly diminished the IgE-mediated smMLCK expression in HASM cells. Finally, we found that IgE, similar to metacholine induces the contraction of HASM cells grown on collagen gel matrix. Our data suggest that IgE stimulates the phosphorylation of ERK, P38, STAT3 and induces the dephosphorylation of smMLCK to phosphorylate myosin regulatory light chain in HASM cells. Taken together, our data suggest a modulatory role of IgE in regulating the contractile machinery and hypertrophic phenotype of HASM cells.

Review of literature

1.1. Asthma:

According to the World Health Organization, asthma is now a serious public health problem with over 300 million sufferers worldwide. Further, it is estimated that the prevalence of asthma globally increases every decade by 50% (1). Approximately 500 Canadians die from asthma each year (www.asthma.ca). Asthma is a disease of the lungs, clinically recognized by excess mucus production, difficulty in breathing due to airway narrowing and airway inflammation. It is a complex representation of an inflammatory response to specific allergens, which otherwise are harmless. According to a report by Braman (1), collateral increase in atopic sensitization and other allergic conditions such as eczema and rhinitis are associated with an increase in asthma pervasiveness. Allergic sensitization may occur as early as *in-utero*. Genetic or hereditary predisposition, environmental factors and gene-environmental interactions also contribute to the development of asthma in susceptible subjects.

Genetic factors:

Susceptibility to asthma is partly associated with genetic predisposition due to which it is seen to run strongly in families (2, 3). Determination of the genetic regions co-inherited with the disease by positional cloning and linkage studies identifies the genes or gene complexes related with genetic predisposition. Vercelli et al. (4) (Figure 1) carefully segregated asthma susceptibility genes into four major categories: “genes associated with innate immunity and immunoregulation; genes associated with Th2-cell differentiation and effector functions; genes associated with epithelial biology and mucosal immunity;

and genes associated with lung function, airway remodelling and disease severity". The first category refers to polymorphism in genes encoding PRR or surface receptors which have marked impact on the development of allergic inflammation. Given the significance of IL-4, IL-5, IL-9, IL-13 in the maturation of Th2 response, they constitute the second category (4). The next category comprised of the genes associated with the functions of epithelial cells which serve as an interface between innate and adaptive immunity. These include ADAM33, PHF11, DPP10, GRPA and SPINK5 (5-9). The last group of candidate genes affects lung physiology and phenotype. This group is indicated to be highly heterogeneous ranging from the adrenergic beta receptor 2 (ADRB2) (10) and extracellular matrix protein tenascin c (11) to proinflamatory molecules such as TNF (12), leukotriene C4 synthase (13) and TGFB1 (14). Moreover epigenetic factors also regulate the development and progression of disease. Epigenetics refers to the inheritance of characters which are not encoded by DNA. Such changes may arise before or during the occurrence of disease. For example, methylation of promoter and intronic regions of IL-4 gene modulate its expression (15). Similarly, hypermethylation of IFN-γ gene suppresses its production, thereby promoting a Th2 response (16). Although genetic factors play a central role in determining the outcome of the disease, influence of the environmental factors on genetics can further modulate the phenotype.

Environmental factors:

Asthma is more prominently seen in western countries of the world, particularly in the societies which are less exposed to infections or allergens (17). Such reports support 'the Hygiene

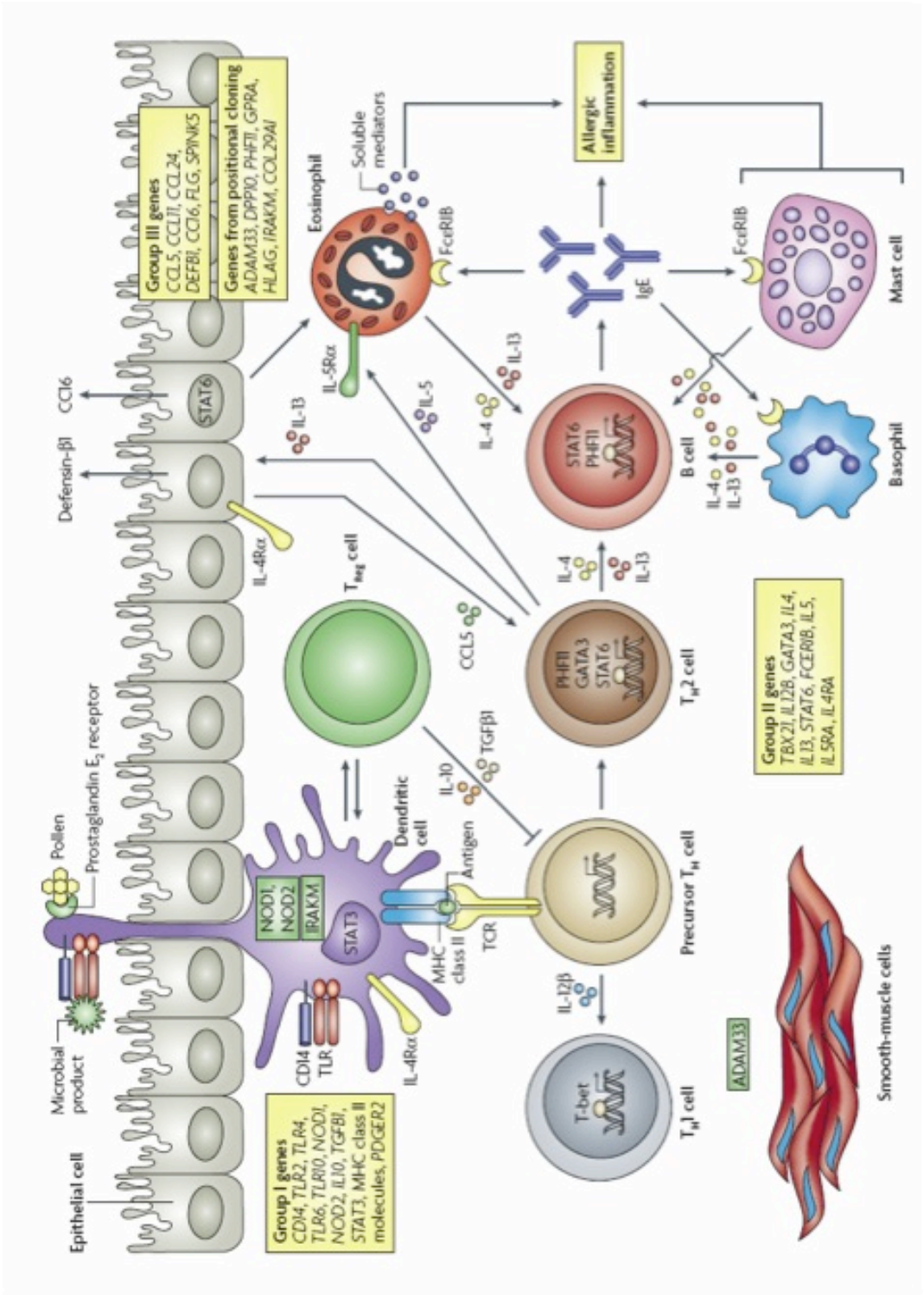


Figure 1. Schematic representation of genes expressed by various cells involved in asthma. The figure also shows the regulation of such genes in asthmatic milieu. ((4) used with permission)

hypothesis' according to which an environment rich in microbes provides protection against diseases (18). These also indicate the role of environmental factors in the perpetuation of allergic asthma. Environmental factors may vary from respiratory viruses, tobacco smoke, air pollutants, toxins, allergens in air, diet to variable lifestyle (19).

1.1.1. Mechanisms of allergic asthma:

Allergic asthma is type I allergic hypersensitivity consisting of an early phase allergic reaction (EAR) and a late phase allergic reaction (LAR). EAR occurs within seconds to minutes after re-exposure to allergen while LAR develops within a few to many hours later. Diagrammatic representation of different phases of asthma is shown in figure 2.

Sensitization: Upon first time exposure to allergen, antigen presenting cells, such as DCs, take up the allergen/antigen and migrate to the draining lymph nodes where they process and present antigenic epitopes to naïve CD4 T cells. These T cells are induced to differentiate into Th2 cells which secrete cytokines, such as IL-4 and IL-13. IL-4 and IL-13 induce heavy chain isotype class switching in allergen-specific B cells, resulting in subsequent secretion of IgE antibodies. Allergen specific circulating IgE binds to its receptors on the surface of mast cells and basophils and results in their activation.

The early phase allergic response: Upon re-exposure to the same allergen, allergen interacts with IgE which is already loaded on the receptor, resulting in aggregation of the receptor and subsequent activation of mast cells and basophils. These activated cells then release preformed mediators, such as histamine. They also secrete newly

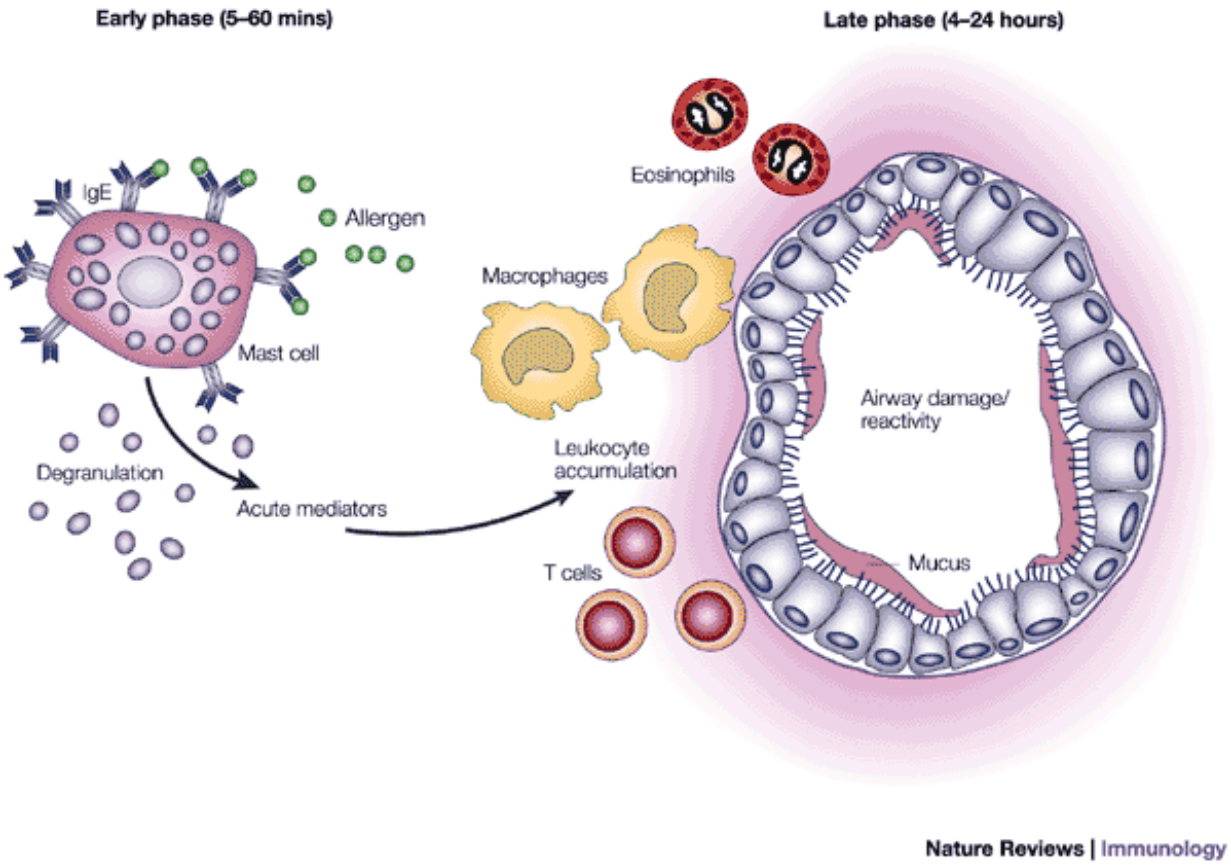


Figure 2. Diagrammatic representation of different phases of asthma and the cells involved in each phase. ((20) used with permission)

synthesized lipid mediators (e.g. leukotrienes) and inflammatory cytokines (e.g. TNF) (21). The crucial role of mast cells and basophils in EAR is discussed further in succeeding sections.

The late phase allergic response: Development of LAR takes place hours after allergen re-exposure. Inflammatory molecules secreted during EAR initiate LAR. This phase is characterized by recruitment and activation of leukocytes, including DCs, lymphocytes and eosinophils and their migration to the affected tissue. More precisely, LAR is predominated by Th2 mediated responses. Th2 cytokines such as IL-4, IL-5 and IL-13 play a central role in LAR (22). IL-4 promote IgE production by B cells, IL-13 induces augmented production of mucus by epithelial cells (23) and IL-5 activates eosinophils (24).

1.2. IgE and asthma

Asthmatic manifestations are correlated with total serum IgE levels (25). In a cohort comprising of asthmatic children, increased IgE levels were associated with the diagnosis of asthma and associated bronchial hyperresponsiveness (26). Further, non-allergic asthmatics who have elevated IgE titre are also identified at risk of asthma (27). Given the relationship between IgE abundance and likelihood of asthma development, analysis of its titer is commonly used as a diagnostic as well as prognostic tool.

Like antibodies of other classes, IgE consists of two identical heavy chains and two identical light chains. Uniqueness of IgE lies in the heavy chain. Heavy ε chain has an additional domain in comparision to the heavy γ chain of IgG (Figure 3), and this extra domain is Cε2 that is positioned equivalent to flexible hinge region of IgG. Cε2, Cε3 and Cε4 heavy chain domains constitute its crystallisable fragment, **Fc fragment,** while heavy chain Cε1 and V_H domains

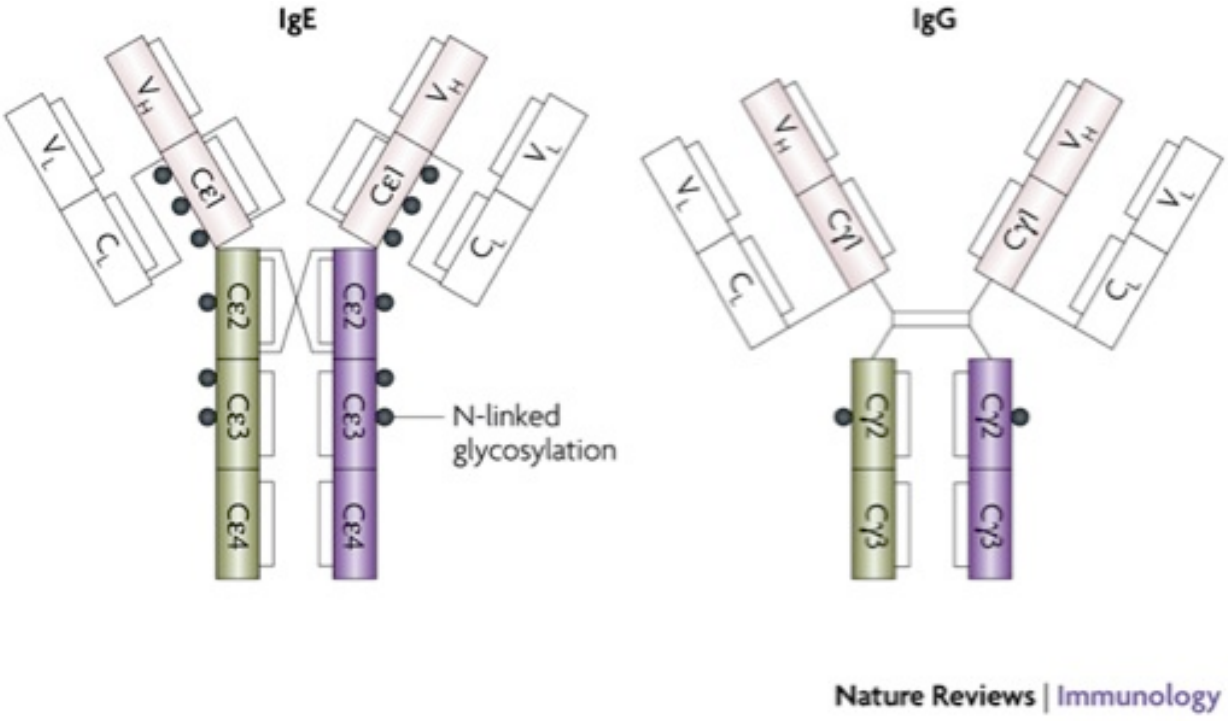


Figure 3. Comparison of the structure of IgE and IgG ((28) used with permission).

along with domains on light chains constitute the antigen binding fragment, **Fab region** of IgE. The Fab region specifically interacts with allergen, while Fc region interacts with IgE receptors (28, 29). Classical paradigm indicates that upon exposure to allergen, allergen-bound IgE interacts with its receptors and induces receptor aggregation, which leads to the activation of biochemical events downstream to receptor and subsequent activation of inflammatory cells, such as mast cells. Activated mast cells, eosinophils and basophils then degranulate and release inflammatory molecules which further cause inflammation and contribute to bronchial hyperresponsiveness (BHR) (30),(reviewed in (28)). Anti-IgE treatment blocks eosinophillic inflammation and hyperresponsiveness (31). However it does not implicate that asthma pathophysiology is entirely dependent on IgE. Acute manifestations, particularly the early phase response seems to be driven by a combination of IgE and other inflammatory cells. Late phase responses and chronic effects are determined by Th2 specific T cell response to allergens. Besides, murine studies have shown that bronchial inflammation and hyperresponsiveness can also be induced even without IgE prevelance (32). Eosinophilia was observed to be absent in IgE-sensitized athymic BALB/c nude mice. Full-fledged asthmatic responses were regained upon administration of T cells or IL-5 (33, 34). Collectively, the current reports propose IgE and its collaboration with T cells as a mechanistic paradigm resulting in the development of asthma.

1.2.1. Production of IgE:

IgE production by B cells is regulated by molecular genetic rearrangement at an Ig heavy chain locus, IgH. B cells which were producing IgM undergo genetic recombination and are induced to produce IgE in response to cytokines such as IL-4 and IL-13 or cell-cell interaction with

CD4+ T cells (35, 36). Briefly, the process of IgH class switching involves a recombination/deletion event, referred as class-switch recombination (CSR), in which the Cμ gene is replaced with a downstream Cε gene (37, 38). IL-4 and IL-13 bind to IL-4 receptor and activate JAK-1, and JAK-3 and JAK-2, respectively. Activated JAKs then phosphorylate and activate STAT6, which binds to specific sequences in the Iε encoding gene and trigger transcription at the Cε locus. The role of NF-κβ, PU-1 and BSAP (Pax 5) has also been identified in the expression of IgE. NF- κβ and PU-1 cooperate with STAT6 in activating the promoter at Cε locus (36).

Interaction with activated T helper cells also promotes the production of IgE by B cells. Activated T cells express CD40L on their surface through which allows interaction with CD40 of B cells and induction of class switching to IgE. This interaction activates NF- κβ through TRAF-2, 5 and 6. Activated NF- κβ and STAT6 both translocate to the nucleus and activate Iε promoter. CD40L-CD40 interaction also activates JAK-3 to induce the class-switching pathway (reviewed in (39)).

Such B cells retain antigenic specificity while changing the isotype of the antibodies as antigenic specificity is determined by variable region which is geneitically arranged early in B cell development through V(D)J recombination (40). Details of the entire process are shown in figure 4.

1.2.2. IgE receptors and their regulation

There are three receptors for IgE which are expressed on the surface of a variety of cells: FcεRI, the high affinity receptor, FcεRII (CD23), the low affinity receptor and C-epsilon binding protein, galectin 3.

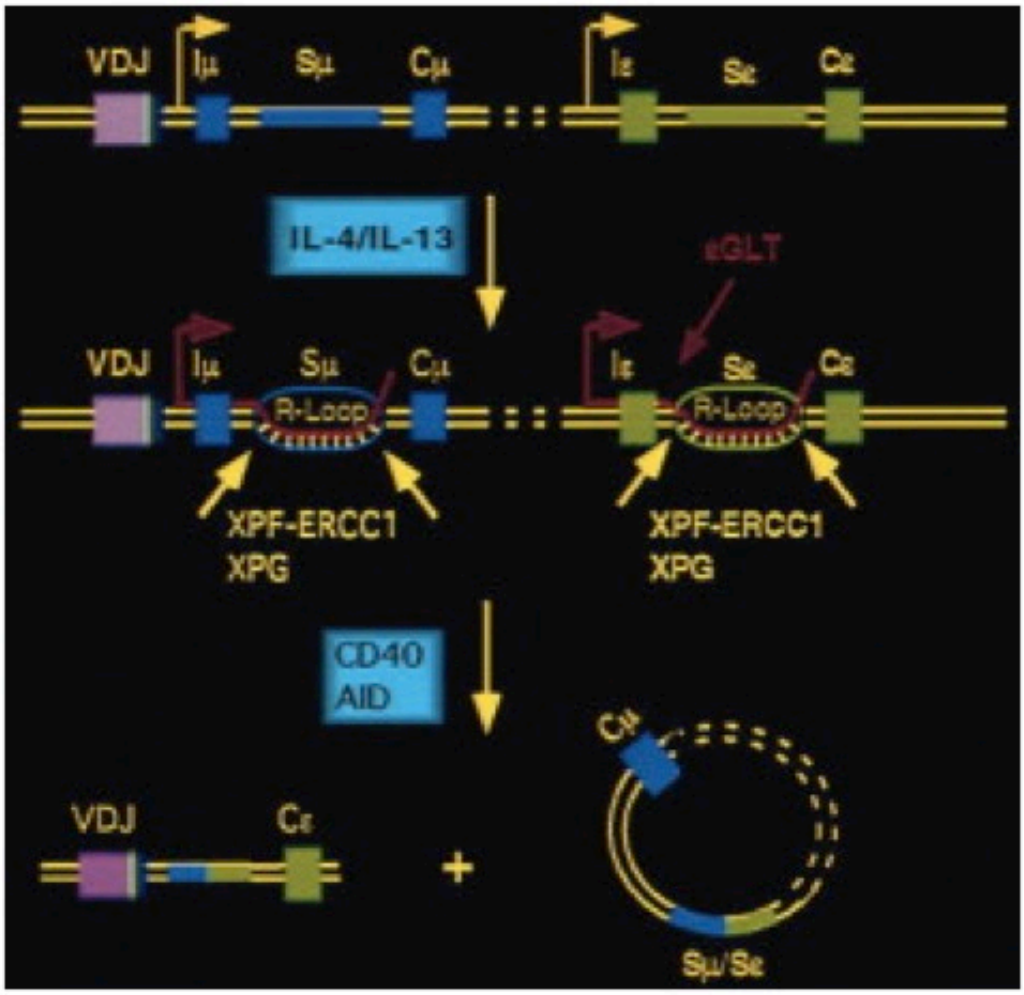


Figure 3a. Mechanisms of germline transcript-mediated class-switch recombination which results in the expression of IgE.
"Briefly, In naive resting B cells, the VDJ sequences encoding the immunoglobulin heavy chain variable region are positioned in the 5' end of the IgH locus, near the Cμ exons, which encode the IgM heavy chain constant region domains. After stimulation by IL-4 (or IL-13), RNA is transcribed at the germline ϵ heavy chain locus originating at the Iϵ promoter to produce ϵ-germline transcripts (ϵ-germline transcript is indicated in red). Sϵ RNA remains hybridized to one of the strands of Sϵ DNA, forming an R-loop structure, which is a substrate for endogenous nucleases, which introduce double-stranded DNA breaks. Analogous processes result in the introduction of DNA breaks at the Sμ locus. Joining of the DNA breaks upstream of the Cϵ cluster of exons and downstream of the VDJ exons then generates a complete IgE heavy chain gene containing VDJ sequences encoding the antigen-binding site and Cϵ exons encoding the constant region domains. The intervening DNA forms an episomal excision circle, which is eventually lost during cell divisio". ((39) used with permission)

1.2.2.1. FcεRI receptor:

Structure of FcεRI: Classically, FcεRI is composed of four subunits as $\alpha\beta\gamma_2$ in mast cells and basophils (Figure 4). However, $\alpha\gamma_2$ is found on eosinophils, langerhans cells, monocytes and monocyte derived DCs. The alpha chain constitutes the extracellular region and is the subunit which interacts with the Fc region of IgE. The beta chain stablizes the receptor and amplifies the signal, which is then transduced by disulphide-bond linked gamma chain-homodimer.

Alpha chain: In human, the gene for the alpha chain is mapped on chromosome 1q23. The alpha chain is a Type I transmembrane protein which belongs to the immunoglobulin superfamily. It consists of an extracellular N-terminal region (amino acids 1-176) and a cytoplasmic tail (amino acids after 177). The N-terminus contains two Ig like domains, referred to as D1 and D2, each of 86 amino acids. At the interface of α 1 and α 2, a hydrophobic patch is formed which contains the binding site for Fc region of IgE (41). FcεRI α chain binds with IgE in 1:1 stoichiometry with an affinity of Kd< 10^{-9} to 10^{-10} M (42). The alpha chain is a highly glycosylated subunit with seven N-glycosylation sites in human (41) and six in mice (43).

Beta chain: The gene for beta chain is located on chromosome 11q13. The molecular weight of this subunit is 30 kDa. It contains four transmembrane domains with a cytoplasmic C- and N-terminus. At the C-terminal, the beta chain contains an immunoreceptor tyrosine based activation motif (ITAM) which amplifies the signal following alpha chain engagement (44, 45). It also regulates the expression of FcεRI which is discussed later in this section.

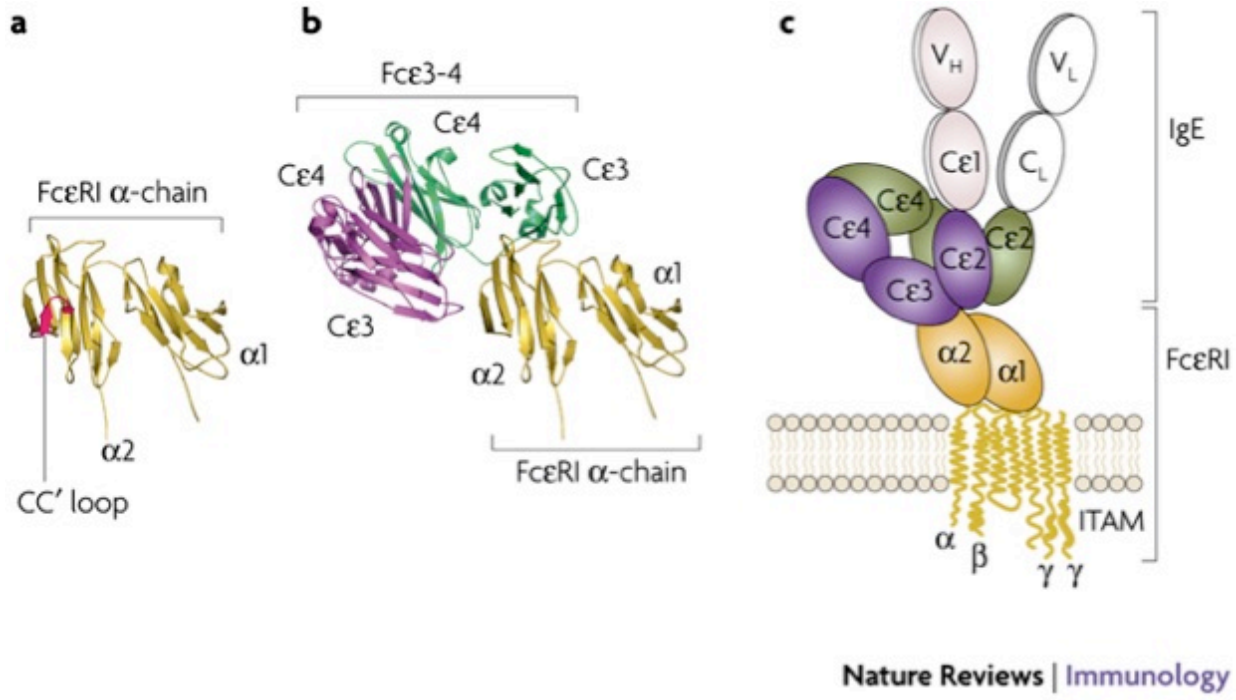


Figure 4: a. Ribbon structure of alpha chain of FcεRI showing α 1 and α 2 domains, b. Ribbon structure of alpha chain of FcεRI and IgE showing their interaction, c. Cartoon showing the intraction of IgE molecule with FcεRI ((28) used with permission)

Gamma chain: The γ chain of FcεRI is a 10 kDa protein, comprised of a short extracellular domain, a transmembrane domain and a cytoplasmic tail. The γ-chains form disulfide linked homo-dimers. Each γ-chain contains an ITAM in their cytoplasmic tail through which they mediate the signaling downstream to FcεRI α chain (45, 46). The γ subunit is shared by other Fc receptors such as FCγRIII, FcγRI, FcαR and TCR (47).

Regulation of FcεRI expression: Regulation of FcεRI is dependent on many factors which includes FcεRI beta chain and local IgE abundance. The FcεRI beta chain plays a critical role in the assembly of FcεRI complex, validated by the fact that association of a splice variant of FcεRI beta chain, FcεRI-β_T with the alpha chain leads to the proteosomal degradation of associated partners (48). Normally, association of the beta chain with the alpha chain renders their translocation from ER to Golgi bodies for glycosylation and subsequent migration to the cell surface, attributed to the presence of a ER retention sequence on alpha chain. FcεRI-β_T is the truncated version of beta chain and it masks the ER sequence, hindering the post-translational modifications of the alpha chain, resulting in degradation (49). Further, SNPs in alpha and beta chains also regulate the receptor expression. SNP in the promoter region of the alpha chain introduces an additional GATA-1 site, thereby augmenting the promoter activity (50). A variant of beta chain: E237G, was found to be associated with enhanced expression of FcεRI complex on basophils. However the mechanism by which such a polymorphism in the beta chain gene affects the receptor complex expression is not known. (51). Two more SNPs in beta chain promoter were observed to increase the promoter activity. In this report, SNPs in the beta chain were also suggested to be correlated with atopy (51).

Expression of FcεRI on the surface is also dependent on its engagement by IgE. It is the aggregation of receptor complex that prevents the internalization of the receptor and following degradation (52). Free receptors on the cell surface are constantly internalized and degraded.

Signaling downstream to FcεRI: Upon allergen exposure, allergen bound IgE binds to the alpha chain of FcεRI and induces the receptor aggregation. It leads to the activation of Lyn which subsequently phosphorylates ITAMs in β and γ chains. The tyrosine phosphorylated ITAM then binds to SH2 domain of protein tyrosine kinases mainly Lyn, Fyn and Syk. Lyn and Syk kinase associate with FceRI beta and gamma chains, respectively. Syk then activates linker for activation of T cells (LAT) and SLP 76 which thereby activates other signaling complexes which includes Bruton's tyrosine kinase (BTK) which activates phospholipase C gamma (PLCγ). This induces the production of diacylglycerol (DAG) and inositol-1,4,5-triphosphate (InsP3)-induced Ca^{2+} mobilization. Tyrosine phosphorylation and activation of other enzymes and adaptors such as Vav, Shc, Grb2 and Sos, stimulate small GTPases such as Rac, Ras and Rho , which is followed by the activation of ERK, JNKand p38 MAP kinases (53). Following the activation of these signaling events, degranulation of cells such as mast cells, eosinophils and basophils is induced which then release granule stored inflammatory molecules and leads to synthesis and secretion of eicosaniods, cytokines and chemokines. Also stimulation with monomeric IgE is also reported to stimulate these functions. Although, *James et al*. suggested a mechanism by which unknown multivalent ligands may crosslink monomeric IgE and promote FcεRI aggregation, it is controversial whether such a phenomenon occurs *in-vivo*.

1.2.2.2. FcεRII receptor:

Structure of FcεRII: FcεRII or CD23 is the low affinity receptor of IgE. CD23 gene is mapped on chromosome 19p13. Unlike other immunoglobulin receptors, it is a type II transmembrane protein, comprised of a calcium dependent lectin domain in the C-terminal end and intracellular N-terminal as shown in figure 5. The structure of the CD23 is comprised of three lectin domain 'heads' which are connected with the membrane by a stalk made up of a triple alpha helical coiled coil.

Human CD23 exists in two isoforms: CD23a and CD23b, attributed to difference in the first seven (CD23a) or six (CD23b) amino acid residues in N-terminal region (54, 55). CD23a is expressed on antigen-activated B cells prior to differentiation to antibody secreting plasma cells, while expression of CD23b is observed on the surface of several cell types such as T cells, B cells, macrophages, eosinophils and other hematopoetic cells. Due to the effect of MMP ADAM10, human and murine cells also release soluble fragments of CD23 of different sizes (56). The functional activity of these soluble forms depends upon their oligomeric state. Fragments containing stalk form trimer either by themselves or upon binding with IgE, whereas smaller fragments without stalk region remains in their monomeric state (57). The IgE binding region of CD23 lies in the lectin head domain (58), however independent of carbohydrate requirement (59).

Functions of FcεRII: CD23 on B cells interact with allergen bound IgE and present the antigen to T cells (60). The process is known as facilitated antigen presentation (FAP). The role of CD23 in allergic diseases has caught further attention due to its effects on the regulation of IgE production. Membrane bound CD23 interacts with soluble IgE and signals downregulation

of B cell production of IgE. Moreover, anti-CD23 antibody, lumiliximab, is shown to curtail the production of IgE as well as inflammatory responses generated in response to allergens (61). However when soluble CD23 binds with membrane bound IgE and CD21 (another ligand of CD23), it forms a stable trimer and upregulates B cell proliferation and IgE production. Polymorphism in the CD23 gene which results in mutation in the stalk region of murine CD23, destabilizes the stalk and thus decreases its surface expression, upregulateing B cells IgE production (62).

The role of CD23 independent of IgE is observed in chronic idiopathic urticaria where an autoantibody against CD23 induces histamine release from basophils. This function is attributed to the release of MBP from eosinophils upon CD23 engagement. The MBP thus released, induces the release of histamine from basophils, irrespective of the presence of allergen (63). IgE independent CD23-mediated cytotoxicity of monocytes is also identified. CD23 fragments interact with $\alpha_M\beta_2$-integrin and $\alpha_X\beta_2$-integrin on monocytes and induce the production of TNF and other inflammatory cytokines such as IL-1 and IL-6, potentially perpetuating non-specific inflammation (64).

1.2.2.3. Galectin 3

Galectin-3, originally known as ε-binding protein, is also recognized to have IgE binding capacity. More interestingly, it can also interact with FcεRI and both these interactions are mediated through β-galactose containing oligosacceride chains (65). Structurally, it comprises a carbohydrate-recognition domain and a N-terminal domain. It is through the N-terminal domain that galactin-3 forms pentameric structures upon interaction with oligosaccerides (66).

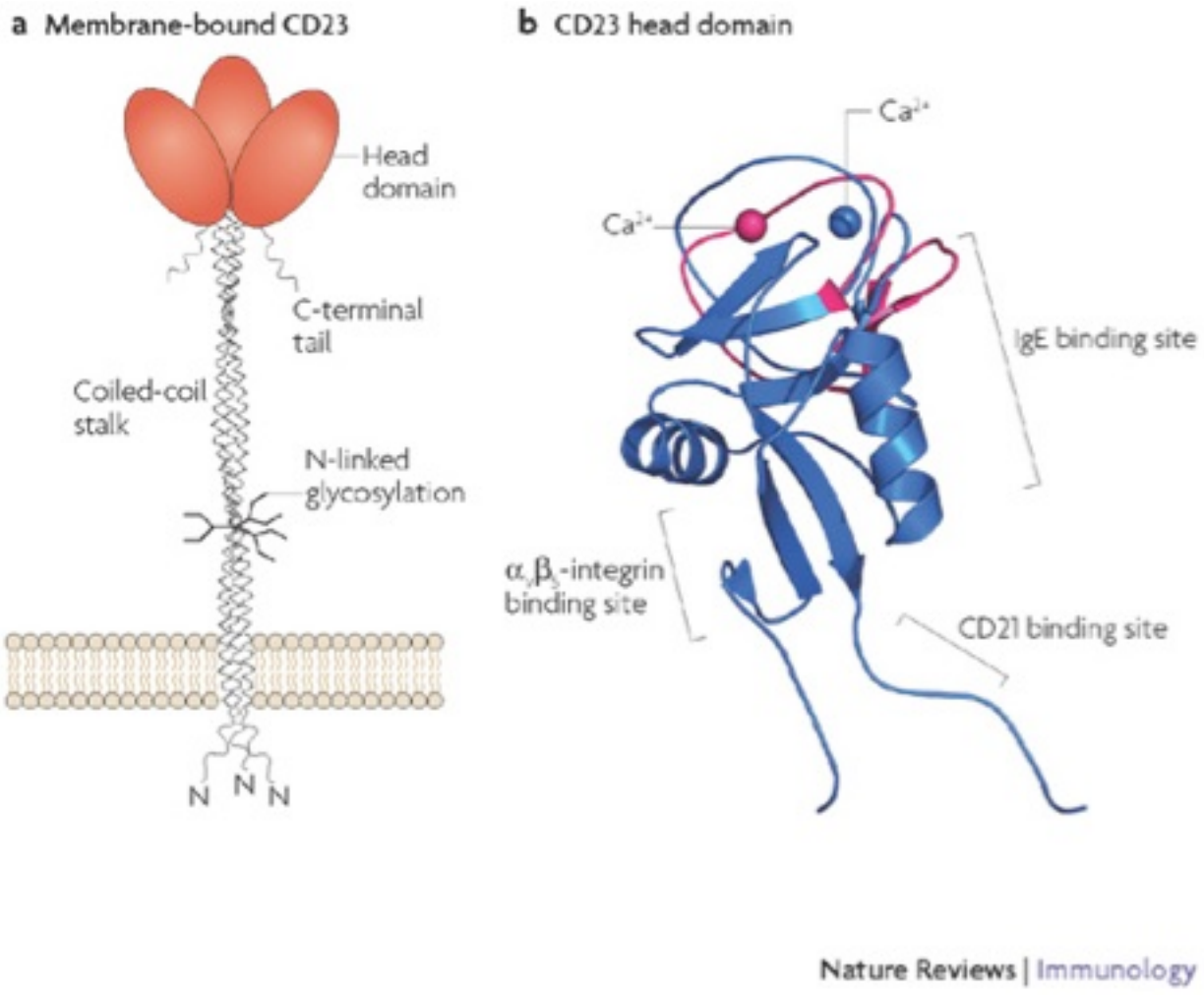


*Figure 5. **a**. Cartoon showing the structure of membrane bound CD23, **b**. Ribbon structure of CD23 head domain showing the binding sites for IgE, CD21 and $\alpha_2\beta_2$ integrins. ((28) used with permission)*

1.3. Cells involved in the pathology of asthma

Various inflammatory and structural cell types (Figure 6) which play a critical role in the pathogenesis of asthma are detailed below:

1.3.1. Mast cells:

Mast cells are found on the airway surface, in submucosa and deep in the airway wall (67). These mast cells are mostly the mucosal type, designated as MC_T and contain tryptase in their secretary granules. Other kinds of mast cells, designated as MC_{TC}, are tissue type which contain tryptase and chymase. Mast cells are the primary responders to the antigens entering into the airways. Their activation is mediated by FcεRI receptors, which bind with antigen-bound IgE. Once mast cells are activated, they degranulate and release preformed and newly synthesized mediators, such as tryptase, heparin and histamine, and PGD_2 and LTC_4, respectively (68, 69). The products released by mast cells are potent bronchoconstrictors. Their effect on vascular permeability has also been shown. The role of mast cells in the elaboration of proinflamatory cytokines has also been studied. Mouse mast cells produce IL-4, IL-5 and IL-6 in response to IgE. Human mast cells are also found to produce IL-4, IL-5, IL-6 and TNF in asthmatic condition. In chronic asthma, the number of MC_{TC} is found to be increased in smooth muscle layer where products released from mast cells contribute to ASM remodeling (70, 71). IL-8 and IL-10 produced by ASM mediate the infiltration of mast cells and also prime them to manifest an enhanced elaboration of mediators. Mast cells in turn, secrete IL-19 which contributes to the migration and hyperplasia of airway smooth muscle (ASM) cells.

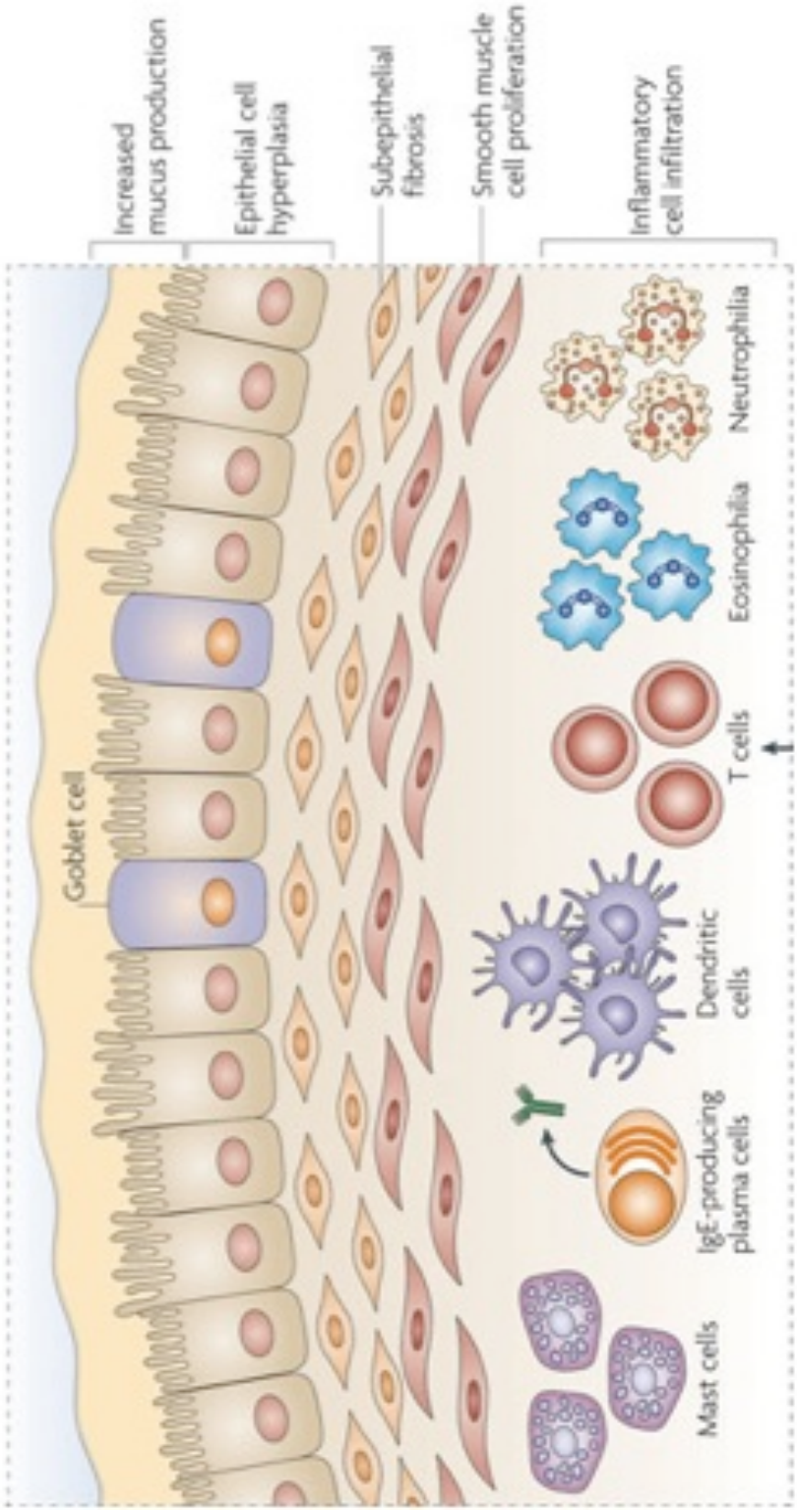


Figure 6: Diagrammatic representation of asthmatic bronchial tissue showing the cell types involved in asthma ((72) used with permission)

1.3.2. Eosinophils:

Lung tissue is one of the major sites where eosinophils reside. They originate from CD34+ progenitor cells in the bone marrow followed by release in peripheral blood (73). Eosinophils contribute to the late phase of inflammation in asthmatic manifestations. Their numbers increase with the severity of the disease (74). Unlike mast cells, activated eosinophils degranulate and release cytotoxic and proinflamatory mediators. The products released from them, such as reactive oxygen species, major basic protein, eosinophils cationic protein, eosinophils peroxidase, eosinophil derived neurotoxin, cytokines and chemokines contribute to the mucosal damage (75). Eosinophil priming, activation and survival are regulated by Th2 cytokines, particularly IL-3, IL-5 and GM-CSF. Other cytokines that help in the differentiation, proliferation and recruitment of eosinophils are TNF, eotaxin-1/CCL-11, MIP1-a/CCL-3, RANTES/CCL-5, MCP-3/CCL-7 and CCL22 (76, 77).

1.3.3. Basophils:

Like eosinophils, basophils also originate from CD34+ progenitor cells in the bone marrow and are released into the circulatory system. Basophils are present in the airways and sputum of asthmatics and express high levels of FcεRI (78-80). Interaction between IgE and FcεRI activate basophils to perpetuate inflammation by releasing mediators. Importance of basophils in allergic manifestations was further strengthened by a study which showed the induction of allergic inflammation in FcεRI deficient mice upon transfer of FcεRI expressing basophils. However, inflammation thus induced was a delayed response, thereby suggesting the role of basophils in LAR, unlike mast cells. They are a rich source of IL-4 and IL-13 (81). In LAR, they play a central role due the elaboration of chemokines such as CCL-3, CCL-5CCL-7, CCL-

8, CCL-11, CCL-13, CCL-24 and CCL-26 which are chemoattractants not only for basophils but also promote infiltration of other pro-inflammatory cells into asthmatic lungs (82, 83).

1.3.4. Mononuclear cells:

1.3.4.1. Macrophages: Macrophages are the most prominent cells inhabiting airway mucosa (84) and form an innate immune system barrier against foreign bodies. When macrophages encounter allergens, they mediate a proinflamatory response, which is intended to clear the potential foreign danger. However, macrophages enhanced inflammatory response against an otherwise harmless agent cause tissue injury. Anti-inflammatory molecules elaborated by macrophages are TGF−β, IL-10 and PGE_2 and Th1 cytokines such as IFN-γ, IL-12, IL-18 and NO (85, 86). Further, macrophages clear the cellular debris and prevent the lung injury potentially caused by necrotic inflammatory cells. On the other hand, they produce molecules which promote inflammation. Macrophages express IgE low affinity receptor, CD23 (87, 88) and produce leukotriene B_4, LTC_4, PGD_2, superoxide anion and lysosomal enzyme in response to IgE (89-91). Expression of CD23 increases with the severity of diseases (88).

1.3.4.2. Dendritic cells: The number of dendritic cells (DCs) in lungs increases during the course of asthma. Perpetual presence of allergen primed activated DCs sustains T-cells mediated inflammation (92). On repeated allergen exposure, DCs stimulate memory T cells residing in the bronchial tissue and contribute to LAR (93). Human DCs can produce CCL-2, CCL-3, CCL-4, CCL-17, CCL-22 and CXCL-8 (94). Expression of FcεRI presents an additional mechanism by which DCs present IgE bound allergen to T cells. IgE crosslinking in

plasmacytoid DCs (pDCs) reduce IFN–γ production upon CpG stimulation by reducing the expression of TLR-9. On the contrary, if TLR9 on pDCs are first stimulated by CpG, it downregulate the expression of FcεRI and sustain IFN-γ production. Altogether, this suggests that pDCs regulates the balance between Th1 and Th2 cytokines on occasions when allergic reaction is accompanied with microbial infection, attributed to the temporal sequence of allergen challenge and microbial exposure.

1.3.5. Epithelial cells

Epithelial cells form a physical barrier which regulates the accessibility of allergens to the bronchial tissue. However their barrier function is found to be paralysed in asthmatic state due to the shedding of columner cells, disruption of tight junction and increase in epithelial permeability (95, 96). Moreover, they produce chemokines such as MIP3/CCL-7 which attract Th2-inducing DCs. Other cytokines elaborated by ECs that drive inflammation are TSLP, GM-CSF, IL-1b, IL-33 and IL-25 (97). During airway inflammation, number of mucous producing goblet cells also increase (98). Excessive mucous accumulation in the airways decreases the intraluminal space in asthmatic subjects as compared to healthy subjects.

1.3.6. Airway smooth muscle cells

1.3.6.1. Origin of airway smooth muscle cells (ASMC): The precise paradigm of the origin of muscle cells is not entirely known yet. During the embryonic development of the lungs, airway smooth muscle (ASM) cells originate from mesenchymal and neural crest cells and establish a smooth muscle lining in a cranial-to-caudal pattern (99). ASM surrounds the airways

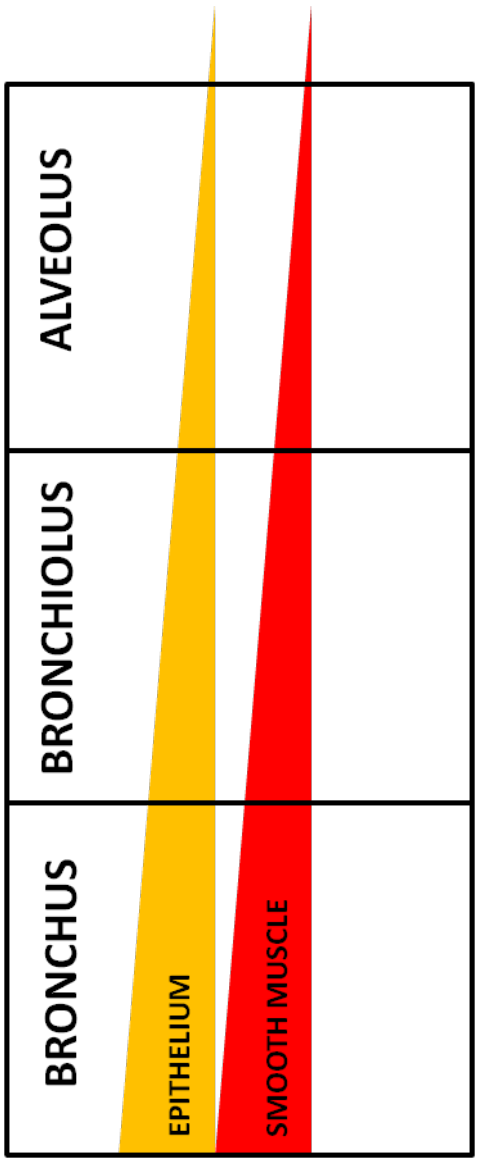


Figure 7. Diagrammatic representation of the organization of ASM in alveolar tree.

circumferentially, however, their density decreases as they reach the terminals of the alveolar tree. Detailed structure of ASM organization in the lungs is shown in figure 7.

Differentiation of ASM cells is catalyzed by various transcription factors such as Sp1, myocyte enhancer factor 2, activator protein 2, serum response factor (SRF) and GATA-5 (100, 101). Morphogenic factors elaborated by airway epithelium also support the differentiation and organization of ASMC. For example laminin-1 and laminin-2 regulate ASMC differentiation by promoting attachment and spreading of primitive mesenchymal cells on the basement membranes (102). Mechanical tension provided by abutting parenchyma in the form of hydrostatic or sheer pressure facilitates the elongation and spreading of the differentiating ASM cells. Mechanical forces from outside, as well as those generated due to stretching of cells, function by activating an SRF dependent pathway (102, 103). Upon activation, SRF translocates from cytoplasm to the nucleus and upregulates the expression of smooth muscle related genes (103). Once the lungs are completely developed, the ASM cells population is renewed by their constant proliferation. However, the role of circulating mesenchymal stem cells in the replenishment of ASM population is uncertain. Circulating fibrocytes are also proposed as potential progenitors of ASM cells, which differ from mesenchymal stromal cells (MSC) as they express hematopoetic markers and differentiate into ASM cells under specific conditions (103).

1.3.6.2. Airway smooth muscle cells in asthma: Airway smooth muscle cells are the major effector cells of airway narrowing observed in asthmatic lungs. Physiologically, ASM contracts in response to agonists and narrows of airways. However, in asthma, such narrowing is abnormally exaggerated even in response to mild stimuli. ASM are affected by a plethora of

proinflamatory cytokines and in turn elaborate inflammatory cytokines and promote airway inflammation. Another mechanism by which ASM contributes to asthmatic manifestations is by participating in airway remodeling through hyperplasia and hypertrophy. Different roles of ASM in asthma (Figure 8) are discussed in detail in the following sections:

1.3.6.2.1. Role of ASM cells in airway remodeling:

An increase in smooth muscle mass is one of the major cause of airway remodeling which manifests profound physiological consequences. To dissect the precise mechanism of increase in smooth muscle thickness, *Ebina et al.* (104) employed a combination of dissector method and a serial sectioning technique to examine the airways of patients suffering from fatal asthma. According to their observation, asthma is categorized into two groups: Type I and Type II. In Type I, smooth muscle mass was found increased only in the central bronchi whereas in Type II, smooth muscle thickness was found to be increased throughout the airway tree. Type I pathology was suggested to be due to hyperplasia while Type II due to hypertrophy. Although there were reports that showed asthmatic patients and animal models manifesting either ASM hypertrophy or hyperplasia, it is rarely reported that both of these phenomenon co-exist (105, 106). Collectively, it is suggested that hyperplasia and hypertrophy both play role in asthma pathology, however interplay between them may vary with asthma phenotype, severity and duration. According to a review by *Hershenson et al* (107), the possibility of overlay of biochemical mechanism controlling ASM hyperplasia and hypertrophy was proposed. When mammalian cells undergo proliferation, mitosis occurs where cells transverse cell cycle, however in hypertrophy, cells undergo cell enlargement before mitosis but do not divide.

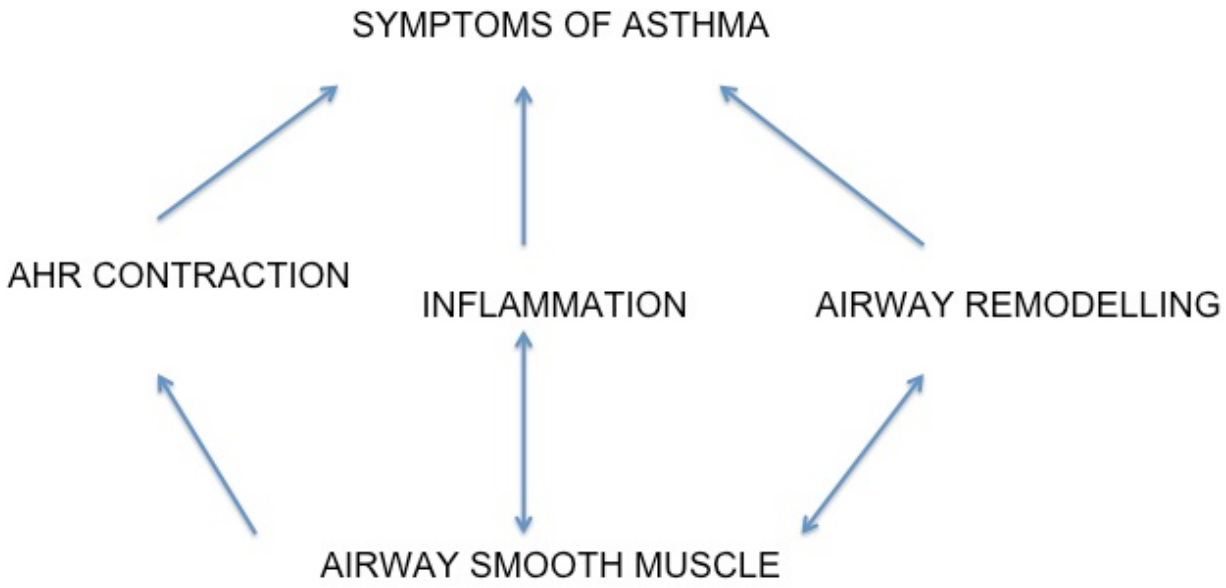


Figure 8. Diagrammatic representation showing the role of ASM in manifesting AHR, inflammation and airway remodeling

Proliferation of asthmatic ASM cells is faster than non-asthmatic individuals. Mitogens that cause hyperplasia range from growth factors such as PDGF, EGF and TGF-β (109-112) to inflammatory substances such as histamine, endothelin, tryptase and thrombin (113-119). Mitogenesis of ASM is regulated by the activation of PI3K through Rac1 which induces the expression of cyclin D1 via cAMP response element binding protein (CREB)/activating transcription factor (ATF-2) binding site (107). ERK activation in ASM also promotes ASM prolifearation through cyclin D1, however independent of PI3K pathway. Hyper-proliferative rate of ASM in asthma subjects is found to be resistant to corticosteroids. This effect was suggested due to dysfunctional interaction between C/EBPa and the glucocorticoid receptor (107).

Another substantiating theory that may play a role in increasing the number of ASM cells surrounding the airway lumen is the migration of ASM cells from ASM bundles (120). Migration of ASM cells is seen in response to growth factors, chemokines and cytokines which are produced in asthma in a highly elaborated manner (70, 121, 122). Also asthma associated extracellular matrix (ECM) alterations also affect ASM cell migration *in-vitro* (121, 123). Nevertheless, studies to investigate this phenomenon *in-vivo* will shed more light on the actuality of this concept. ECM, in general, helps in normal maintenance of ASM which range from differentiation of ASM cells to their proliferation. It also regulates the contractility of smooth muscle cells. However in asthmatic airways, alteration in ECM is observed which is suggested to contribute to airway wall thickening. In culture conditions, ASM cells from asthmatics produce more perlecan and collagen I as compared to non-asthmatic counterparts (124). This could also be a mechanism by which ASM cells produce increased amounts of connective tissue growth factor (CTGF) in response to a profibrotic stimulus (125). ECM from

asthmatic patients amplifies the proliferation of ASM cells, irrespective of asthmatic phenotype, which indicates the likelihood of aberrant ASM phenotype due to self-produced molecules (124).

1.3.6.2.2. Role of ASM cells in airway inflammation:

The concept of secretory role of ASM was conceived after it was found that ASM promotes the number of mast cells in asthmatic condition (126). These intra-ASM mast cells, in turn, release proinflamatory mediators such as histamine, prostaglandin D2 and leukotriene C4 which further exaggerate the inflammation in the tissue. ASM cells were then suggested to elaborate substances which might attract mast cells or other inflammatory cells. Later it was shown that ASM cells secrete chemokines such as CCL-11 (127), CXCL-10 (128) and CX3CL-1 (129). Elaboration of eotaxin/CCL-11 by ASM cells was observed in culture in-vitro condition in response to proinflamatory cytokines such as IL-1β, TNF-α, IL-4, IL-9, IL-13 and TGF-β . Moreover, strong mRNA and protein expression of eotaxin/CCL-11 were observed in ASM and bronchial epithelium of asthmatic airways which is otherwise undetectable in healthy airways (130).

Similarly, induction of ASM cells to produce other chemokines was also demonstrated upon stimulation with cytokines found in inflamed asthmatic airways was also detected. Human ASM cells secrete RANTES/CCL-5 when stimulated with TNF-α, IL-1β and platelet activating factor. Th2 cytokines, such as IL-4 and IL-13 seems to enhance this effect. However, no change was observed in the abundance of RANTES in asthmatic ASM bundles (131). Similarly, ASM cells also produce TARC/CCL-17 and IL-8/CXCL8 but only in cultured condition but their production by intact airways has yet not been demonstrated (132).

Besides promoting the infilitration of proinflammatory cells by producing chemokines, ASM cells directly perpetuate inflammation by elaborating proinflammatory cytokines. These produce IL-1β in response to self-elaborated IL-5 (133). Asthmatic serum induces the production of IL-10 from ASM cells, inducing IL-5 release. IL-5 primarily recruits eosinophils to the pulmonary tissue. IL-1β on the other hand, coordinates inflammation by producing other molecules of the IL-1 axis such as IL-1α, IL-1 β converting enzyme, IL-1 receptor accessory protein, IL-1 receptor, IL-1 receptor like 1 and IL-18 receptor 1. Furthermore, ASM derived IL-1β perpetuates airway pathophysiology by affecting ASM contractile responses (134). IL-1β stimulated cultured ASM cells elaborate GM-CSF (135) and IL-11(136) expression. Effect of TNF and TGF- β is reported to stimulate the production of GM-CSF and IL-11, respectively (135, 136). They also prime ASM cells to produce IL-6 and leukemia inhibitory factor (136). Other molecules which are elaborated by ASM cells and contribute to airway inflammation are COX2, IFN-β (137) and growth factors, such as TGF-β, PDGF and VEGF (138).

ASM cells express a variety of cell surface markers which are used to interact with inflammatory cells. ICAM-1 and VCAM-1 along with CD44 is required for adhesion of T cells to ASM cells. Such interaction activates both cells via cell adhesion molecules and co-stimulatory molecules (139). ASM cells also express OX40 ligand which upon engagement results in the production of IL-6 (140). Surface expression of TLRs, such as TLR-2, TLR-3 and TLR-4, is also found on ASM cells. Activation of these TLRs stimulates the production of IL-8 and eotaxin from ASM cells (141). Altogether it is clear that ASMC employ multiple mechanisms to perpetuate inflammation.

1.3.6.2.3. Role of ASM cells in airway hyperreactivity:

Asthmatic airways exhibit exaggerated narrowing in response to even milder doses of agonists as compared to healthy individuals. Constriction of airways due to bronchial provocation with agents, such as methacholine, is a normal phenomenon, however in asthma, this effect is profoundly increased and is attributed to increased contractility of ASM cells (107) (142). It is possible that hyperresponsiveness in asthma occurs due to increased agonist sensitivity of ASM cells and their greater maximal force generation by them (143). Such a functional outcome may also be due to increased ASM mass which collectively enables smooth muscle bundles to generate greater force in response to provocative agents and thus amplifies airway narrowing.

Moreover, in asthmatic condition, ASM manifest an impaired relaxation. Such an abnormal behavior was observed *in-vitro(144)* as well as *in-vivo (145)* in response to β-adrenergic agonists. β-adrenergic agonist binds to its receptor (β2AR) which interacts with Gα s subunit of G protein and induces the relaxation of contracted ASM cells through the activation of K+ channels. β-adrenergic agonists also stimulate adenylate cyclase to generate cAMP which allows relaxation through the activation of protein kinase A (PKA). However, long term exposure to b-adrenergic agonists terminates signaling pathways activated downstream to β2AR due to its phosphorylation by PKA and specific G protein kinases. The phosphorylated receptor is internalized and limits further activity of β-adrenergic agonists. In the context of asthma, it was found that increased cytokine expression favor the desensitization of β2AR, which paralyses the relaxation (107). In particular, IL-1β stimulates the release of Prostaglandin E2 from ASM cells through a COX-2 dependent mechanism, activating the β2AR signaling pathway at an extensive level (146-149). Such an exhaustive activation of β2AR signaling in

turn impairs the relaxation process. Similar out come was observed in response to TNF and IL-13, but not to IL-4 (150, 151).

1.4. IgE-ASM axis:

Airway smooth muscle cells express IgE high and low affinity receptors which might explain the ability of IgE rich atopic serum to induce hyperreactivity of ASM cells in the isolated airway preparations (152, 153). CD23, a low affinity receptor for IgE is expressed on ASM cell surfaces and the expression is upregulated upon exposure to IgE rich atopic asthmatic serum (154, 155) and IL-4 and GM-CSF either alone or in combination (156). Interaction of CD23 with IgE immune complexes is shown to cause enhanced contractility or attenuated relaxation (154, 155). Subsequently, expression of FcεRI on the surface of cultured ASM cells was also reported. Its expression on ASM cells was also quantified in asthmatic tissue biopsy (157). IgE mediates the release of a variety of proinflamatory factors from ASM cells such as IL-4, IL-5, IL-13 and eotaxin-1/CCL-11. The release of these cytokines is inhibited by the treatment with anti-FcεRI alpha chain antibody (157). IgE was also shown to cause aberrant contractile functions in these cells. Exposure to allergen-specific IgE enhances contraction of bronchial rings obtained from non-atopic individuals (153). Given that ASM cells express receptors for IgE, it is possible that airway smooth muscle cells comprising these bronchial rings are mediating the contractile function in response to IgE. In ASM cells, the contractile apparatus is tightly regulated by the activity of smooth muscle myosin light chain kinase (smMLCK). Given that ASM cells in an asthmatic state manifest a hypercontractile phenotype, it was postulated that molecules elaborated in asthma might enhance the content of smMLCK to cause hypercontractility. Ammit et al. showed an increase in smMLCK abundance in human airways which were passively sensitized with atopic serum (158). Increased mRNA expression of

smMLCK was also observed in endobronchial biopsies obtained from asthmatic subjects (143). Similar conclusions were derived from canine studies (159, 160). Since smMLCK is acontraction regulatory protein, increase in its content is also associated with the predominance of a contractile phenotype. As discussed earlier, ASM cells manifesting hypertrophy show increased abundance of contractile proteins such as alpha-smooth muscle actin, SM22 and smMLCK. It is not impossible that factors in atopic serum, which cause hyperresponsiveness due to hypercontractile phenotype and/or hypertrophy, do so by upregulating the protein content of smMLCK.

1.5. Smooth muscle myosin light chain kinase (smMLCK):

Vertebrates contain two genes for MLCK (figure 9): the skeletal MLCK and the smooth muscle MLCK (161). The smooth muscle MLCK gene encodes three cell specific transcripts as a result of different promoters (162-164). The three transcripts are short form MLCK (110-130kDa), long form MLCK (220kDa) and telokin (20kDa). Although short form of MLCK is ubiquitously expressed, its greatest expression is observed in smooth muscle tissues, and is therefore also known as smooth muscle MLCK (smMLCK), which contains a catalytic core and regulatory segment. It contains three Ig modules, one Fn module, PEVK repeat rich region and an actin binding sequence at the N terminal. Although the function of the Ig and Fn (fibronectin) module, and PEVK (proline rich sequence with unknown secondary and tertiary structure) region is not clear in MLCK structure, actin binding region is necessary for mediating contraction by smMLCK (165, 166). The actin binding region is present in amino acids 2-63 of the short form of kinase which contains three repeat motifs (DFRXXL). Each of these repeats may potentially bind to single actin monomer in an actin filament (167-169).

Another transcript of the gene is the long form of MLCK, which contains all the region present in smMLCK. It holds an additional Ig module and two additional actin binding motifs at its N terminus (170). The long form of kinase is also referred to as non-muscle (nmMLCK), embryonic and endothelial MLCK, pertaining to its expression in these cells. It is also expressed in smooth muscle cells in culture or in embryonic stage, however adult smooth muscle cells do not express it (162, 171, 172). Non-contractile functions of MLCK are attributed to nmMLCK.

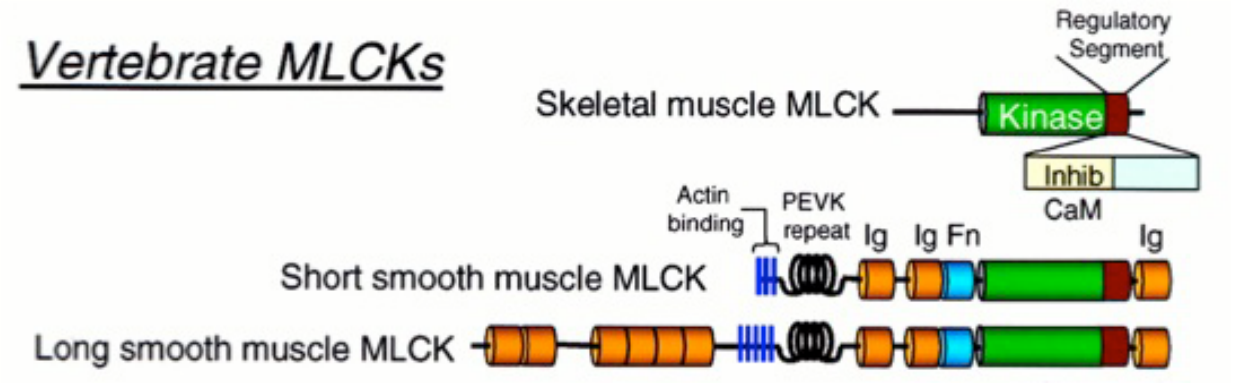


Figure 9: Pictorial representation of structure of long and short forms of MLCK. ((173) used with permission)

The third transcript is telokin, which contains C terminal Ig module (164). Telokin participates in calcium desentization of smooth muscle force by cyclic nucleotides (174).

1.5.1. Phosphorylation of MLCK

Traditionally, the active state of MLCK is attributed to the unphosphorylation of MLCK. Certain kinases phosphorylate MLCK at serine 815 and serine 828 in the regulatory region (175-177) and play a regulatory role in its kinase activity. Such kinases are protein Kinase A (177), protein kinase C (178, 179), Ca^{2+}/calmodulin-dependent protein kinases II (CAMK II) (180, 181) and p21-activated kinase (PAK). Phosphorylation of MLCK at Serine 815 paralyzes the kinase activity of MLCK, while Ser 828 phosphorylation inhibits the actin activated ATPase activity of phosphorylated myosin and increases the ATPase activity of unphosphorylated myosin (175, 176). Phosphorylation at serine 1760 severely affects the activation of MLCK. Serine 1760 is located in the pocket which is used for interaction with calmodulin. Phosphorylation of this site hinders the binding of the calcium-calmodulin complex with MLCK (182). MLCK also contain several phosphorylation sites for proline-directed protein kinase (181, 183). Collectively, phosphorylation of specific sites of MLCK plays an important role in determining its activation and function.

1.5.2. Regulation of contraction by smMLCK

Agonist stimulation induces intracellular mobilization of calcium which then complexes with calmodulin. Thereby, in the presence of Ca^{2+}, C-terminal domain of calmodulin binds to the N-terminal of calmodulin binding sequence in MLCK. This interaction activates MLCK (both skeletal and smooth muscle MLCK) which then phosphorylate myosin regulatory light chain at serine 19. The phosphorylation of RLC leads to the activation of myosin ATPase allowing

interaction with actin, subsequently resulting in the induction of contraction (shown in figure 10) (reviewed in (173)).

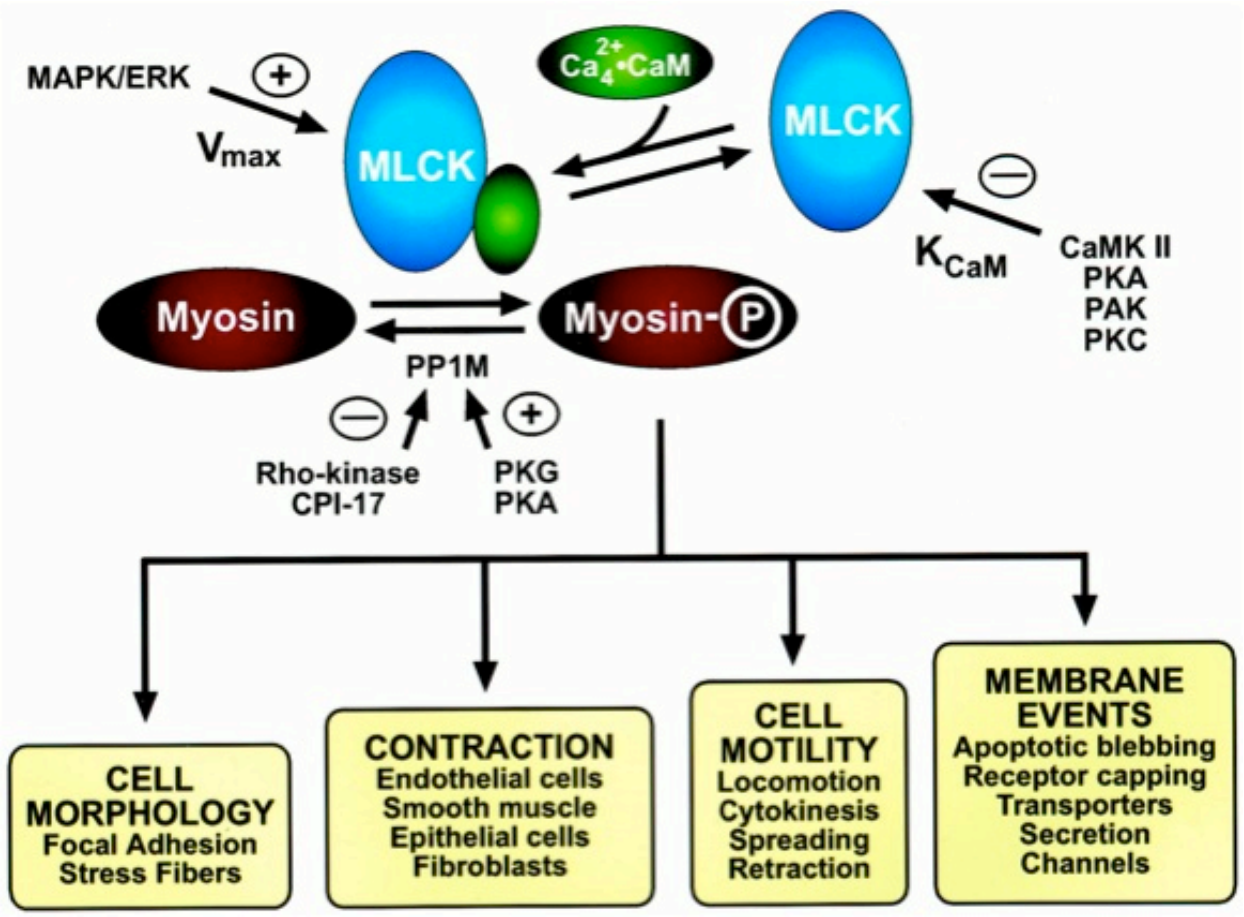


Figure 10. Diagrammatic representation of role of MLCK in regulating cellular morphology, contraction, cellular motility and other membrane events. ((173) used with permission). Calcium –calmodulin complex interacts with MLCK which phosphorylates myosin light chain. The role of other kinases including ERK, PKA, PKC as discussed in (173) is also shown here. Activation of this pathway results in cell contraction, migration, movement of membrane to allow blebbing in apoptotic cells, phagocytosis, secretion and also allows cells to communicate and transport molecules between cells. This schema applies to both long and short form of MLCK.

1.6. Overall aim and concept of the study

Asthma is a chronic inflammatory disease of the airways, clinically characterized by airway obstruction, inflammation and hyperresponsiveness. The inflammatory components of this disease include an increased infiltration of activated T lymphocytes, mast cells, eosinophils and neutrophils within the airway lumen and bronchial submucosa (184, 185). Besides inflammatory cells, structural cells including airway smooth muscle cells also play an important role in the development of asthma. Airway smooth muscle cells are the primary effector cells that control the bronchomotor tone within the airways (107).

Exaggerated bronchoconstriction due to excessive contraction and aberrant relaxation of airway smooth muscles is one of the critical contributor to airway hyperresponsiveness observed in asthma (107). Enhanced contractility is possibly attributed to increased velocity combined with extent of shortening of HASM cells (143). Contractility of airway smooth muscle cells is primarily controlled by smooth muscle Myosin Light Chain Kinase (smMLCK) activity (186). This step involves the activation of the calcium-calmodulin complex, which activates smMLCK and subsequently phosphorylates 20kDa myosin regulatory light chain, triggering contraction (187). smMLCK content has been shown to be increased in sensitized atopic humans (158), and ragweed-sensitized canine airway smooth muscle (159). Furthermore, enhanced smMLCK content associated with enhanced contractility was observed in passively sensitized bronchus with serum from asthma subjects (158) and human airway smooth muscle (HASM) cells from asthmatic subjects (143).

Hypertrophy of airway smooth muscle cells is also known to aggravate the lung functions of asthmatic individuals (107). Benayoun et al. (105) demonstrated that HASM cells obtained from severe asthmatic subjects had larger lumen diameter as compared to their healthy or mild

asthmatic control. They and others (reviewed in (107)) provided convincing evidence of hypertrophy in asthmatic airways. In the same study, increase in smMLCK was also observed (105). Increase in contractile proteins including sm22 and smMLCK, is proposed as a mechanism of hypertrophy of HASM cells (107).

Collectively, these reports suggest that certain components of serum that are associated with asthma can potentially regulate the expression of smMLCK, a mechanism through which they can modulate the role of HASM cells in asthma.

It is well recognized that most asthma in children and adult subjects is associated with atopy, characterized by an increased serum IgE against the most common allergens. Indeed, two-thirds of asthmatics are allergic and more than 50% of patients with severe asthma have allergy (188). Furthermore, emerging evidence suggests that IgE (26, 189) and IgE induced proinflammatory cytokines (reviewed in (190)) are closely correlated with functional, morphological and phenotypical defects manifested by HASM cells in asthma. However, it is not completely understood whether a direct link exists between IgE and smMLCK and aberrant phenotype of HASM cells. We have previously shown that HASM cells express the high affinity IgE receptor (FcεRI). FcεRI cross-linking on HASM cells induced Th2 (IL-4, -5, and -13) cytokines and CCL11/eotaxin-1 release and led to transient intracellular calcium (Ca^{2+}) mobilization. Recently, we demonstrated that FcεRI expression in HASM cells is under the regulatory control by proinflammatory (TNF and IL-1β) and Th-2 (IL-4) cytokines. This up-regulation results in enhanced expression and release of CC and CXC chemokines upon IgE stimulation (191). Taken together, our data suggest a critical role of IgE in mediating abnormal inflammatory function of HASM cells, but the role of this pathway in modulating contractile functions is not known.

In this study, we aimed to elucidate the role of IgE in regulating smMLCK expression and whether this effect is mediated through FcεRI. We also examined the signaling pathway which is activated upon IgE stimulation in HASM cells and the role of this pathway in HASM cell contraction *in vitro*. We found that IgE enhanced the smMLCK expression in HASM cells. This effect is mediated through FcεRI since inhibition of IgE binding with anti-FcεRI antibody 15-1 directed against the IgE binding site significantly diminished the IgE-mediated smMLCK expression in HASM cells. Similarly, IgE mediated smMLCK overexpression depends upon Spleen Tyrosine Kinase (Syk), MAPK (ERK1/2, p38, and JNK) and PI3K as showed by shRNA mediated Syk silencing and pharmacological inhibitors, respectively. IgE stimulation was shown to induce phosphorylation of ERK, P38, STAT3 and myosin RLC and dephosphorylation of smMLCK. Finally, IgE incubation leads to decrease in HASM-cultured collagen gel area, suggesting an IgE-induced contractile response in HASM cells.

Chapter 2. Materials and Methods

2.1. Ethics statement

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

2.2. Reagents

Recombinant human IgE was obtained from Antibody shop (BioPorto Diagnostics A/S, Denmark). Fetal bovine serum (FBS) and sodium pyruvate were purchased from HyClone (Logan, UT, USA). 100X L-glutamine, DMEM, Ham's F-12, trypsin-EDTA, and antibiotics (penicillin, streptomycin) were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada).

2.3. Preparation of human airway smooth muscle (HASM) cells

Human telomerase reverse transcriptase (hTERT)-immortalized bronchial smooth muscle cells were used which were prepared as we described previously (192). Briefly, these cells were obtained from macroscopically healthy segments of second to fourth generation lobar or main bronchus of patients undergoing surgery for lung adenocarcinoma as we described previously (192, 193). To extend the life span of these cells, primary low-passage cultures were infected with a retrovirus vector encoding the (hTERT) gene. A plasmid (pGRN145) containing hTERT cDNA expression vector was a gift from Geron (Menlo Park, CA). The hTERT expression cassette was cloned into pLXIN (figure 11) (Clontech cat# 631501), and replication-

incompetent Moloney murine leukemia virus retrovirus was generated in HEK293 retroviral packaging cells. Primary and first-passage cultures of human airway smooth muscle cells were infected with the hTERT retrovirus and selected with 100 mg/ml G418 for 1 wk. The expression of hTERT was verified in immortalized cells by RT-PCR using telomerase-specific primers. Immortalized cells were passaged (4:1 dilution) up to 50 times with no evidence of senescence (192, 194). Furthermore, these hTERT HASM cells are shown to retain smooth muscle-specific actin, SM22, and calponin protein expression and mobilize intracellular Ca^{2+} in response to acetylcholine, a physiologically relevant contractile agonist as described previously (192). We also examined the calponin levels in these cells as shown in Figure 13. The predominance of contractile phenotype of these cells upon serum starvation was analysed by cell cycle analysis (PI staining). As shown in Figure 13a, serum starvation induces the arrest of cells in G1/M phase which is representative of a non-proliferating population or contractile HASM cells. Briefly, HASM cells were harvested and fixed overnight in 70% cold ethanol. Next day, cells were washed twice with PBS and stained with PI in 300ul PI solution (20ug/ml in PBS) (Sigma) for 30 min at room temperature. 20ul of 10mg/ml RNase stock solution was added per tube. Stained cells were then aquired on FACS Canto II (Becton Dickinson).

2.4. Cell stimulation

Sub-confluent HASM cells were serum deprived for 48 hrs in Ham's F-12 medium containing 5 µg/ml human recombinant insulin, 5 µg/ml human transferrin, 5 ng/ml selenium, and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were then stimulated with IgE (5µg/ml) in fresh FBS-free medium for the specified time. In a selected experiments, cells were pre-treated with MAPK inhibitors: ERK1/2 inhibitor U-0126 (10µM), p38 inhibitor SB-203580

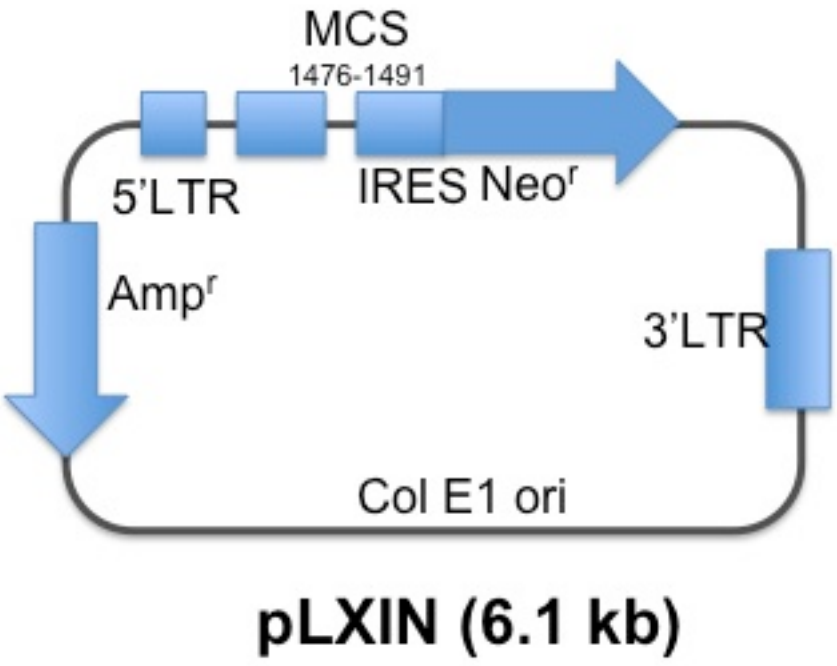


Figure 11. Diagrammatic representation of vector pLXIN various sites present in the vector. hTERT was cloned in multiple cloning site (MCS) in this vector.

(10μM), JNK inhibitor SP600125 (40nM) or phosphatidylinositol-3-kinase (PI3K) inhibitor wortmannin (100nM) for 45 minutes prior to stimulation with IgE. For signaling experiments, HASM cells were serum deprived for 48 hrs followed by stimulation with IgE 5ug/ml. Lysate was collected at different time points until 30 minutes. Westerm blotting was done on the protein lysate to determine the phosphorylation levels of MAPKs, Akt, STAT3, myosin and smMLCK.

2.5. RNA isolation and Real-time RT-PCR analysis

Serum-deprived confluent HASM cell cultures were stimulated, harvested, and total cellular RNA was extracted from them using TRIzol® method (Invitrogen Canada Inc., Burlington, ON). 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). Primers for human housekeeping gene, glyceraldhyde-3-phosphate dehydrogenase (GAPDH) are forward primer 5′-AGCAATGCCTCCTGCACCACCAAC-3′ and reverse primer 5′-CCGGAGGGGCCATCCACAGTCT-3′. Primers for smMLCK are forward Primer: 5' GACTGCAAGATTGAAGGATAC 3' and Reverse Primer: 5' GTTTCCACAATGAGCTCTGC 3'. Real-time quantitative PCR was carried out using 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. Briefly, the amplification of smMLCK gene in stimulated cells

was calculated first as the copy number ratio of smMLCK to GAPDH, and then expressed as normalized values of fold increase over the value obtained with unstimulated (control) cells.

2.6. Western blot

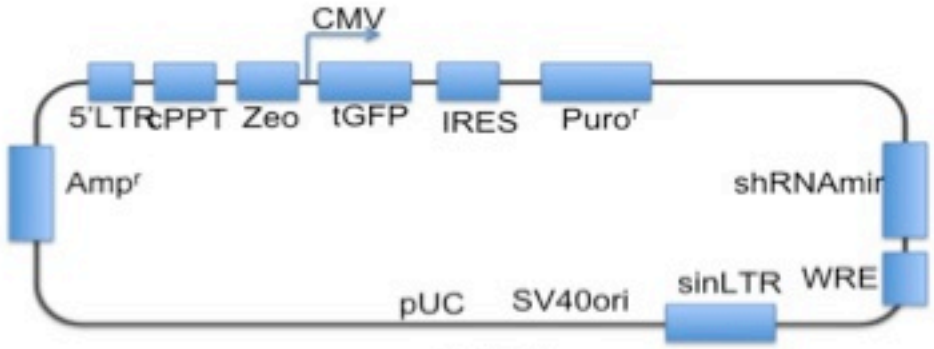
For western blots, HASM cells were lysed for 2 min on ice in M-PER lysis buffer (Thermo Scinitific) supplemented with a cocktail of protease inhibitors (Sigma-Aldrich) and centrifuged for 20 min to collect protein lysate. For immunoblotting, 10 μg of lysate from each sample was separated on 6% SDS polyacrylamide gel and electro-transferred onto PVDF membrane (Amersham Pharmacia, ON). The membrane was blocked at room temprature for 2 hrs with 5% skim milk, incubated with mouse anti-MLCK (K36 clone) polyclonal Ab (Sigma-Aldrich) (1:2000), or mouse anti-calponin antibody (Sigma-Aldrich) (1:5000) at room temperature for 2 h, followed by secondary antibody HRP-goat anti-mouse IgG (1:5000) prepared in 1% skim milk. All the blots were developed by enhanced chemiluminescence as recommended by the supplier (Amersham Pharmacia, ON). Beta-(β-) actin was used as internal control. The intensity of smMLCK, calponin and β-actin bands was determined by using AlphaEase FC software version 3.1.2 relative and presented as arbitrary value which is the densitometric ratio of target protein (smMLCK and calponin) to loading control (beta actin).

2.7. Lyn and Syk silencing in HASM cells by lentiviral shRNA transduction

Lyn and Syk kinases were silenced by transducing HASM cells with pseudotyped lentiviral vector (clone Id: V2LHS_134140; V2LHS_153702) (sequence of shRNA and vector map is shown in figure 12) expressing specific Syk and Lyn shRNA, respectively, were obtained from

Open-Biosystems (Huntsville, AL). A control shRNA unrelated to Lyn and Syk sequence (scramble shRNA) was used as a transduction control. 293T cells used for virus production and titration were cultured in Dulbecco's medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin/glutamate (PSG) (Gibco, Grand Island, NY) as described in (191). Briefly, $17.5X10^6$ 293T cells were seeded in T175 flasks and transfected with a mixture of expression vector, packaging vector (8.2 δ vpr) and envelope vector (VSVG) in the presence of $CaCl_2$. Three days after transduction, supernatant from 293 T cells was collected and centrifuged at 1500 rpm for 5 min at RT. The supernatant was then filtered which contains 1X virus. For silencing the protein expression of these kinases, HASM cells were transduced with virus at a multiplicity of infection (MOI) of 10 in the presence of polybrene (8 μg/ml). In brief, 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 FACS using the turbo-green fluorescent protein (tGFP) (Figure 17a and 18a). Viability of the transduced cells was >98% as assessed by trypan blue dye after completion of the experiment.

a

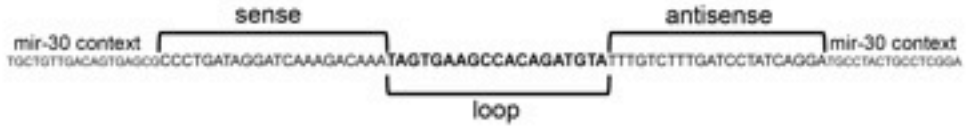


pGIPZ

b

Vector Element	Utility
CMV Promoter	RNA Polymerase II promoter
cPPT	Central Polypurine tract helps translocation into the nucleus of non-dividing cells
WRE	Enhances the stability and translation of transcripts
turbo GFP	Marker to track shRNAmir expression
Puro^r	Mammalian selectable marker
AMP^r	Ampicillin bacterial selectable marker
5'LTR	5' long terminal repeat
pUC ori	High copy replication and maintenance in *e.coli*
SIN-LTR	3' Self inactivating long terminal repeat
RRE	Rev response element
ZEO^r	Bacterial selectable marker

c **sh RNA_lyn**



d **shRNA_syk**

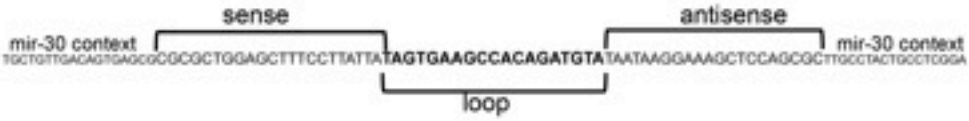


Figure 12. ***(a)*** *Diagrammatic representation of vector pGIPZ showing the site of shRNA.* ***(b)*** *Shows the role of various sites present in the vector.* ***(c), (d)*** *shows the sequence of Lyn and Syk shRNA, respectively. Their sequence contains a sense, loop and an antisense sequence. The positin of these sequences can be seen in (a).*

2.8. Contraction assay: Collagen type I gel deformation

The anchored collagen type I gel deformation method was performed to study the effects of monomeric IgE on the contractile responses in a 3D environment, as described by Takayama and Mizumachi (195). Briefly, collagen type I gel was prepared by gently mixing 7 ml of cold collagen I solution (3 mg/ml) (Purified Bovine Collagen solution from Advanced BioMatrix Inc., Arizona) and 2 ml of fivefold-concentrated cold DMEM-F-12 (Invitrogen). pH was adjusted to 7.4 using NaOH. The final volume was adjusted to 10 ml. Six hundred microliter aliquots of the collagen type I gel cocktail were added to 24-well dishes and allowed to solidify overnight. $1x10^5$ cells were seeded overtop the gel in each well. Cells were incubated in complete media and allowed to adhere and grow for 24 hrs, then serum deprived for another 48 hrs. HASM cells were then stimulated with IgE (5μg/ml) for 48 hrs, followed by detachment of the gel from the surrounding walls of the wells. Methacholine (MCh) was used as positive control for contraction. Wells were digitally photographed at different time points. Gel surface area on a 2-D digital image was quantified to measure contraction using Image J software (NIH, Bethesda, USA). The data is presented as % contraction which is calculated by subtracting area of collagen gel at a particular time to gel area at t=0, divided by 100.

2.9. Statistical analysis

All the data were obtained from experiments performed three or more times. Statistical analysis was performed by doing *Mann-Whitney U* test and one-way ANOVA with 95% confidence

level using GraphPad Prism Software Version 3.02 for Windows (GraphPad Software Software, San Diego, CA, USA). P values <0.05 were considered statistically significant.

Chapter 3. Results

3.1. Effect of IgE on smMLCK expression

3.1.1. IgE augments smMLCK mRNA and protein content in HASM cells

In order to assess the role of IgE on smMLCK expression in HASM cells, immortalized human bronchial smooth muscle cell line was used, as we previously described (192). These cells showed normal proliferative rate in the presence of serum (Figure 13A) while upon serum starvation, they manifest a contractile phenotype by undergoing less proliferation (less number of cells in G2-M phase of cell cycle) and enhanced calponin expression (Figure 13B) (192). IgE (5μg/ml) stimulation of HASM cells enhanced the smMLCK mRNA level significantly at 6, 24 and 48 hrs ($n>3$, $P<0.05$) (Figure 13C). The greatest detectable increase in smMLCK mRNA level was detected at 24 hrs timepoint. Furthermore, western blotting experiments show that IgE also augments smMLCK protein level in HASM cells at 48 and 96 hrs (Figure 13D). Collectively, our results indicate that IgE could play a substantial role in enhancing smMLCK content in HASM cells.

3.1.2. IgE enhances smMLCK content through FcεRI

We then investigated the involvement of FcεRI receptor in the regulation of smMLCK expression in HASM cells using FcεRI blocking monoclonal Ab (mAb) (15-1) directed against the IgE binding site (196). Serum deprived HASM cells were pretreated with 10μg/ml of mAb 15-1 or mouse IgG1 isotype control (MOPC1) for 1 hr and then stimulated with IgE 5μg/ml for 48 hrs. As shown in Figure 14, in contrast to isotype control mAb (MOPC21), pre-treatment with mAb15-1 significantly diminished IgE enhanced expression of smMLCK ($n=3$, $P<0.05$). Taken together, these results suggest that IgE stimulates the expression of smMLCK in HASM cells mainly via FcεRI.

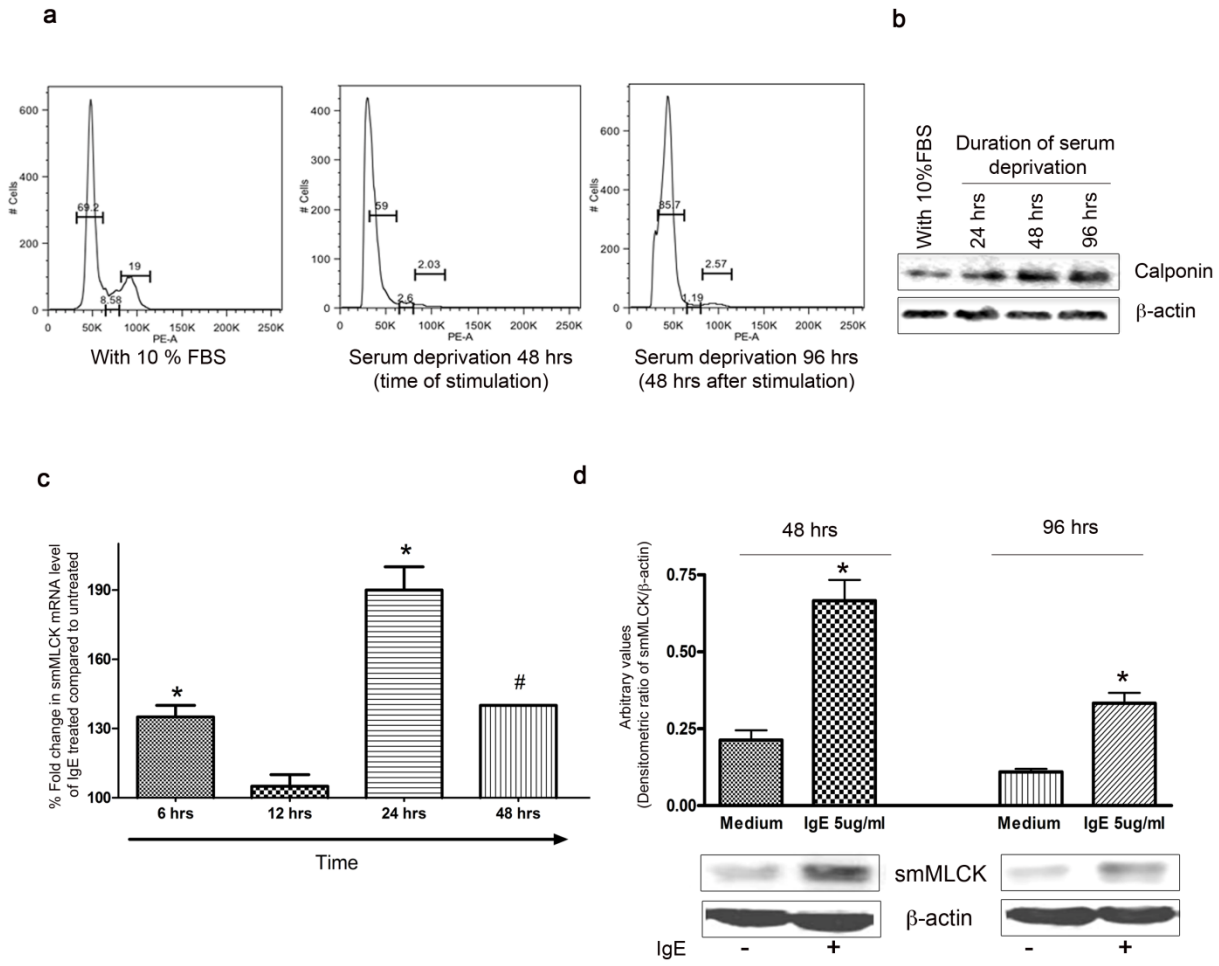


Figure 13. Characterization of HASM cell preparations and smMLCK mRNA and protein analysis. (a) Cell cycle analysis of HASM cells showing a decrease in proliferating population upon serum deprivation and (b) a representative immunoblot showing calponin protein level demonstrating increase in contractile phenotype. (c) HASM cells were serum-deprived for 48 hrs and then stimulated with IgE 5μg/ml. Shown is smMLCK mRNA level compared to medium of respective time point. (d) HASM cells were stimulated with IgE 5μg/ml for 48 and 96 hrs and smMLCK protein expression was analysed by western blotting. The intensity of smMLCK band was normalized with that of β-actin. The shown blot is representative of more than three different experiments. Densitometric analysis of smMLCK protein to β -actin is also shown above the immunoblot. Mann-Whitney U test was performed to test the difference between the samples P<0.05 (*), P<0.01 (#).

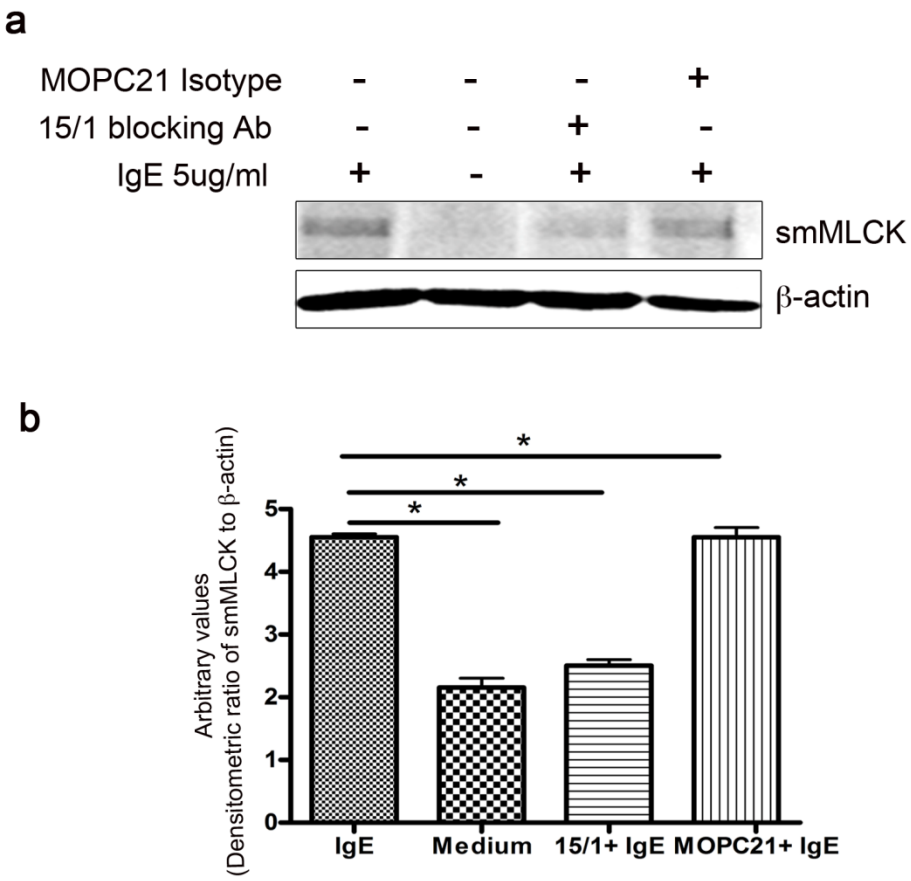


Figure 14. Neutralizing anti-FcεRI mAb (15-1) abrogates IgE-induced smMLCK upregulation in HASM cells. Cells were pretreated anti-FcεRI mAb15–1 or isotype control (MOPC1) for 1 h before stimulation with IgE. (a) Western blot is representative of three different experiments showing smMLCK protein content in different treatment groups. (b) Densitometric analysis of smMLCK protein compared to β-actin (n=3). Mann-Whitney U test was performed to determine the mean difference of different IgE treated samples. P<0.05 (*).

3.1.3. TNF priming enhances monomeric IgE mediated smMLCK upregulation

Our lab had previously reported that TNF induces upregulation of IgE high affinity receptor FceRI alpha chain expression (191). The upregulatory effect of TNF was shown to augment IgE mediated release of eotaxin-1, IL-8, RANTES and IP-10 (191). Therefore we hypothesized that TNF priming which upregulates FcεRI α chain protein expression would increase the effect of IgE on smMLCK protein expression. HASM cells were serum deprived for 48 hrs and then primed with TNF 10ng/ml for 48 hrs followed by stimulation with monomeric IgE for 48 hrs. Lysate from TNF primed IgE stimulated HASM cells showed enhanced smMLCK protein content as compared to unstimulated TNF primed and unprimed HASM cells (figure 15) (n=3).

3.1.4. IgE mediates smMLCK upregulation through MAPKs (ERK1/2, P38, JNK) and PI3 Kinase

IgE mediates its effect via multiple signalling pathways in inflammatory cells that include MAPK pathways (197). IgE modulates the release of mediators through P38-dependent pathways in basophils. The prosurvival effect of IgE on mast cells is also attributed to the role of ERK, P38 and JNK (197). Similarly, activation of the PI3K dependent pathway is also implied in IgE signaling. IgE amplifies PI3K signaling through the FcεRI β chain to mediate the degranulation of mast cells (198). Further, IgE driven activation of akt which is a substrate of PI3K strenghthens the role of PI3K pathway in IgE signaling. To characterize the involvement of these signalling proteins in IgE-mediated smMLCK enhanced protein expression in HASM cells, cells were pretreated with pharmacological inhibitors of ERK1/2 (U0126), P38 (SB203580), JNK (SP600125) and PI3 Kinase (Wortmannin), and then stimulated with IgE for 48 hrs. Inhibition of MAPK (ERK1/2) or the PI3K pathway resulted in

a significant reduction of IgE-mediated smMLCK protein expression (n=3, P<0.05) (Figure 15). Pre-treatment with inhibitor of P38 and JNK also inhibited IgE-induced smMLCK upregulation (Figure 16), however the effect was less than the inhibition of ERK1/2 and PI3K. These results indicate that (MAPK) ERK1/2 and PI3K, and to lesser extent P38 and JNK are essential for IgE mediated enhanced expression of smMLCK protein in HASM cells.

3.1.5. shRNA mediated Syk silencing inhibits IgE mediated smMLCK protein expression in HASM cells

IgE mediates its action through the activation of FcεRI receptor followed by phosphorylation of Lyn and Syk kinase (53). As we have previously shown that IgE activates FcεRI and induces the release of cytokines from HASM cells through a syk dependent pathway (191). However role of Lyn in context with IgE signalling in HASM cells particularly has not been studied to date. Having identified that smMLCK protein expression is modulated by IgE through FcεRI, our succeeding aim was to understand the role of Lyn and Syk kinase in this process. We then sought to investigate the role of Lyn and Syk kinase in IgE-induced smMLCK expression. Lyn and Syk kinase silencing was induced in HASM cells by transducing HASM cells with a pseudotyped lentiviral vector expressing specific shRNA (191). Transduction of cells with specific Lyn or Syk shRNA resulted in a highly significant and reproducible decrease in their expression, as shown by western blotting in figure 16B and 17B, respectively. Scramble shRNA was used as a transduction control. In Syk silenced HASM cells, IgE stimulation fails to enhance smMLCK protein content as compared to scramble sequence transduced HASM cells (Figure 18C). However, Lyn silenced HASM cells responded to IgE in a similar fashion as

scramble transduced cells (Figure 17C). Collectively, our data suggest that IgE enhances the smMLCK expression via Syk-dependent but Lyn-independent pathway.

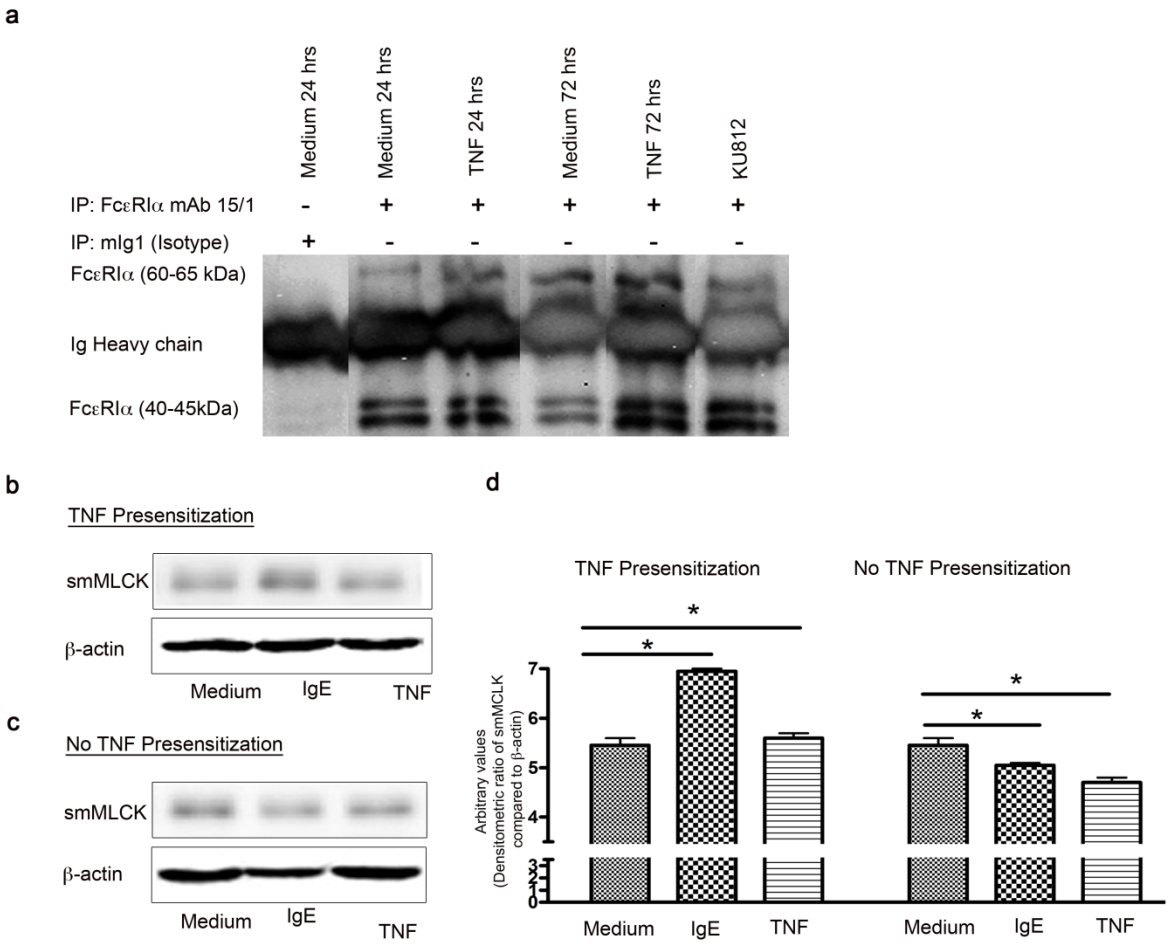


Figure 15. FcεRI-α protein expression is upregulated by TNF. Forty eight hour serum-deprived primary HASM cells were cultured in presence or absence of TNF-α. FcεRI-α protein from 24 h and 72 h culture cell lysates was Immunoprecipitated (IP) followed by Western blotting, as reported earlier (Redhu et al, PLoS One 2009). FcεRI-α protein was immunoprecipitated with either anti-FcεRIα mAb 15/1 or with isotype antibody mouse IgG1 (MOPC21) for negative control. The order of the gel lanes was adjusted from within a single blot with uniform resolution. ***(b)*** *HASM cells were TNF primed* for 48 hrs and stimulated with monomeric IgE (5ug/ml) for 48 hrs. **(c)** *HASM cells were cultured in medium for 48 hrs and then stimulated with IgE alone and TNF for 48 hrs.* ***(d)*** *Graphical representation of the densitometric ration of smMLCK to beta actin. The graph shows the protein level of smMLCK upon different treatments. Mann-Whitney U test was performed to determine the significance of data. P<0.05 (*)*

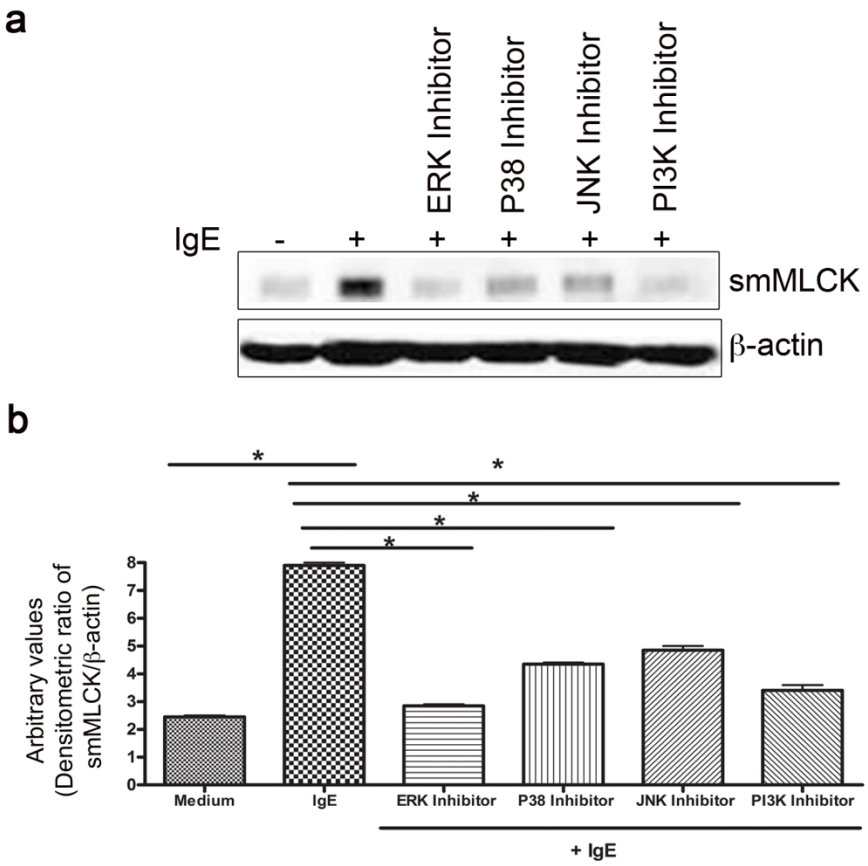


Figure 16. (a) Immunoblot showing smMLCK protein expression following stimulation with IgE (5µg/ml) and ERK1/2 Inhibitor: U-0126 (10mM), P38 Inhibitor: SB-203580(10mM), JNK Inhibitor: SP600125 (40nM) or PI3K Inhibitor wortmanin (100nM). (b) Graph shows densitometric analysis of smMLCK protein to β -actin (n=3). One-way ANOVA was performed to determine the significance of data. P<0.001 (*)

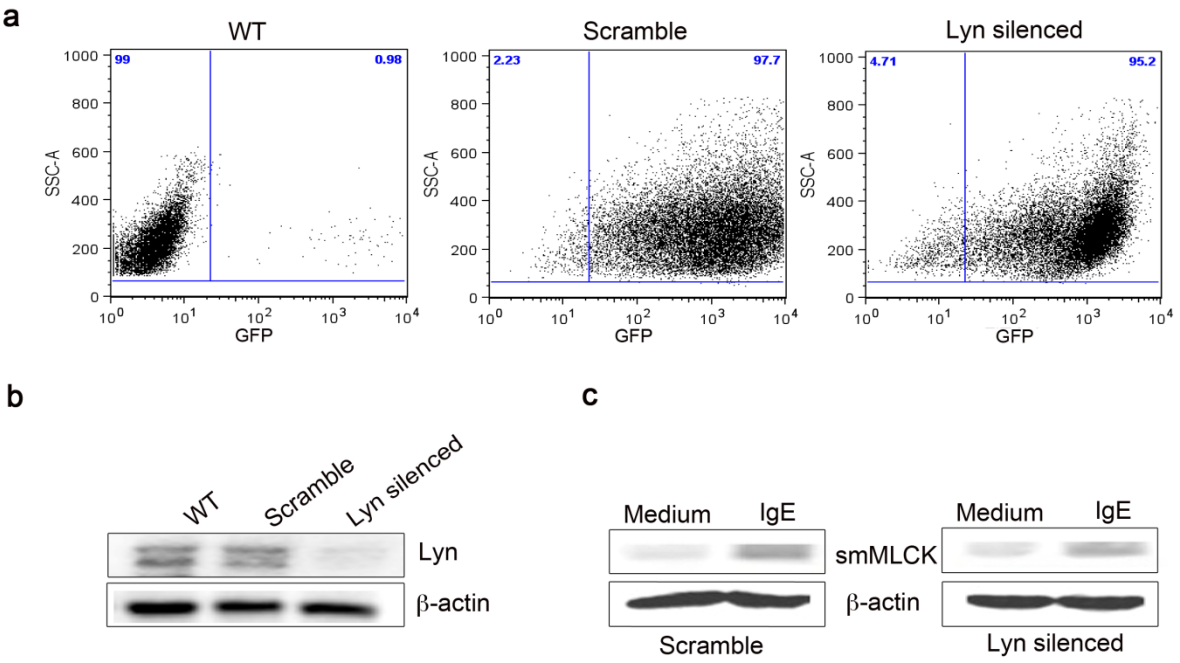


Figure 17. Lyn silencing was induced in HASM cells similar to Syk silencing (Figure 5). (a) Lentiviral transduction efficiency was found to be more than 90% for both scramble and Lyn specific shRNA as determined by measuring GFP content. (b) Lentivirus induced Lyn silencing in was shown by western blotting. (c) Protein extracts were prepared from Lyn silenced and scramble HASM cells, stimulated with IgE 5μg/ml for 48 hrs after serum deprivation and smMLCK protein content was assessed by western blotting.

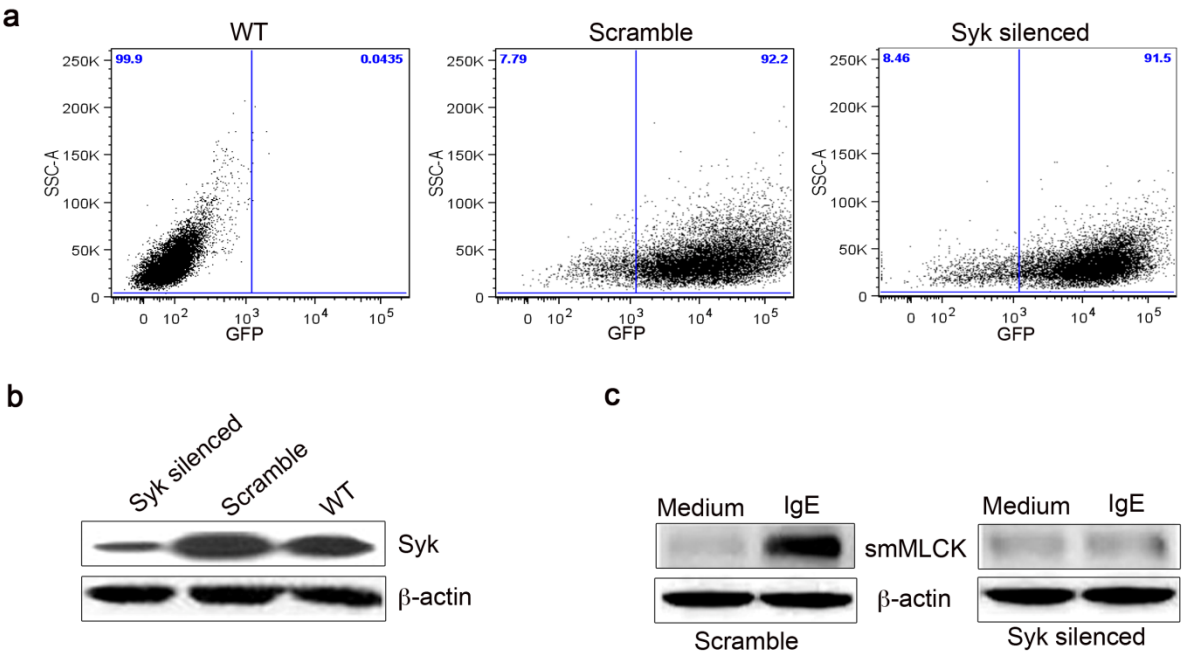


Figure 18. Syk silencing was induced in HASM cells by transduction with lentiviral vector expressing Syk specific shRNA. shRNA against unrelated scramble sequence was used as control. (a) Transduction efficiency was analysed by FACS using GFP as a marker. Lentiviral transduction efficiency was found to be more than 90% for both scramble and Syk specific shRNA. (b) Lentivirus induced Syk silencing was shown by western blotting. (c) Protein extracts were prepared from Syk silenced and scramble HASM cells which were stimulated with IgE 5μg/ml for 48 hrs and smMLCK protein content was assessed by western blotting

3.2. Effect of IgE on the contractile apparatus in HASM cells

3.2.1. IgE induces contraction in HASM cells

There are previous reports which indicated that IgE rich atopic serum induced contractile reponse (153). Therefore, we wanted to examine whether IgE has the ability to induce contraction.We examined the effect of IgE on the contraction of HASM cells using deformation of collagen type I gel matrices, a technique widely used as an *in-vitro* model of assessing contraction of cultured cells (195). Decrease in the area of collagen gel upon stimulation was measured at different time points. Analysis of the gel surface area showed that IgE (5μg/ml) induced greater contraction of the collagen type I gel as compared with the matrix contraction imparted by untreated cells (Figure 19). Methacholine (MCh), a well-known contractile agonist, also displayed contraction of collagen gel with its effect more prominent than IgE.

3.2.2. IgE stimulation induces dephosphorylation of smMLCK and phosphorylation of myosin RLC

Contraction in HASM cells is regulated by activated smMLCK which then phosphorylates myosin RLC, and induces contraction. Phosphorylation at serine 1760 inactivates smMLCK. Given that IgE can induce contraction in HASM cells, we therefore sought to investigate whether IgE can induce the dephosphorylation of smMLCK. By using the western blotting method, we found that smMLCK phosphorylation at serine 1760 decreased till 5 minutes after IgE stimulation (Figure 19). Thereafter we looked at the phosphorylation of myosin RLC which is a substrate of activated smMLCK. We observed that IgE stimulation induces the phosphorylation of myosin RLC (serine 20) within one minute after which the magnitude of phosphorylation decreased (Shown in figure 20).

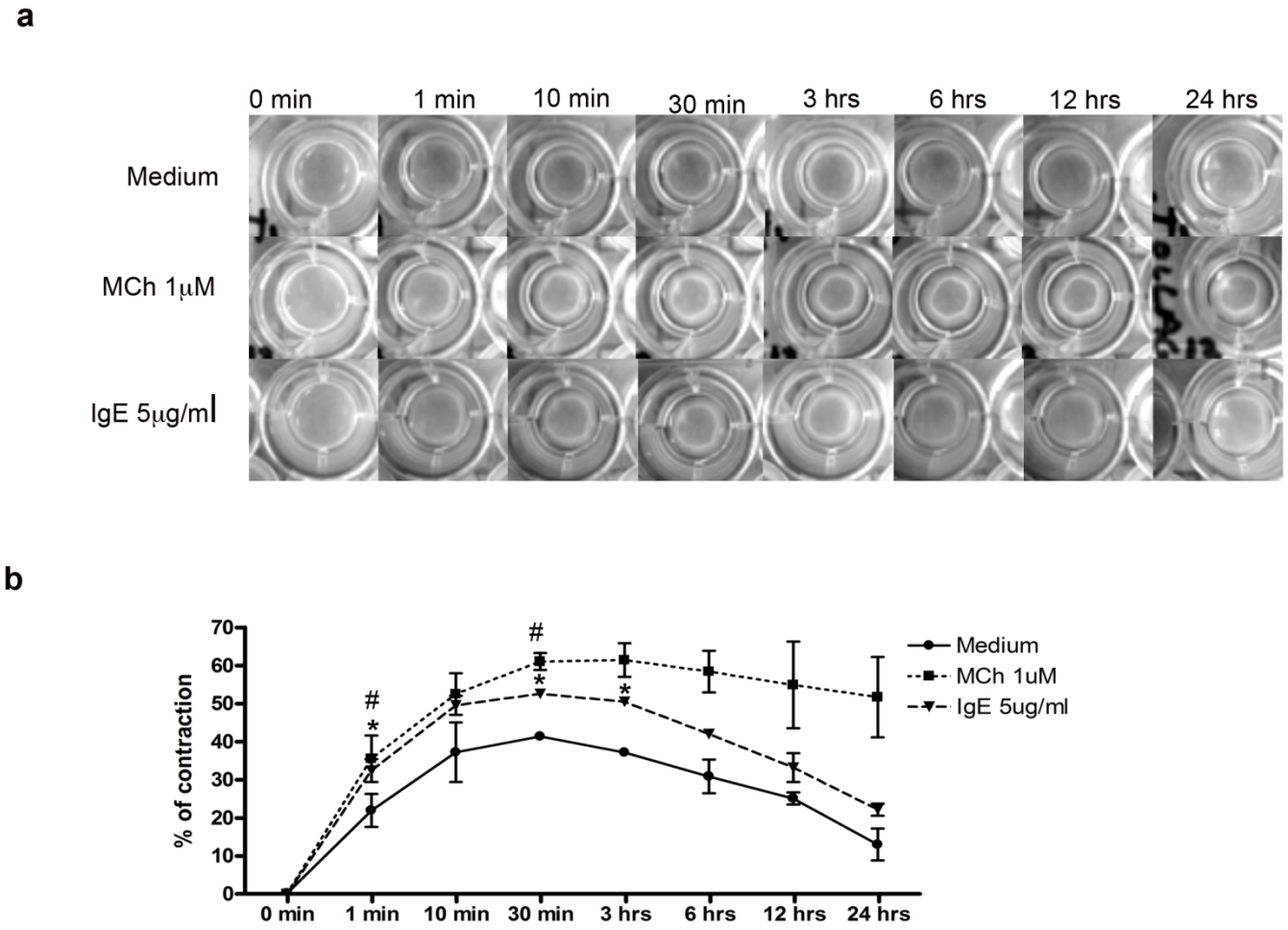


Figure 19. Contraction assay: HASM cells were seeded on collagen gel matrix and serum deprived for 48 hrs. The collagen gel was then detached from the walls of the well and stimulated with IgE (5ug/ml). Positive control group was stimulated with MCh (1μM) before detachment. (a) Decrease in the area of collagen gel was measured at different time points and (b) % of contraction was calculated. Mann-Whitney U test was used to determine the difference between contraction at 0 min and at different time points. P values <0.05 (IgE) (# MCh) were considered statistically significant.*

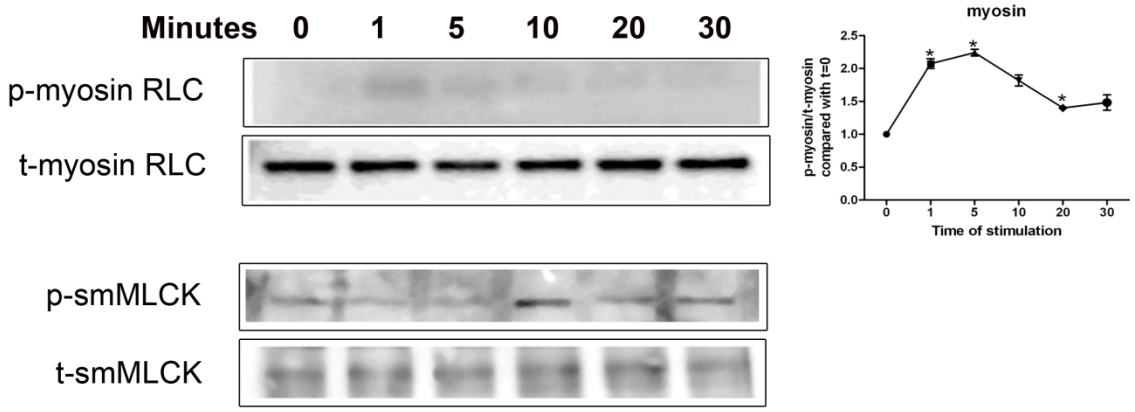


Figure 20. IgE stimulation induces the phosphorylation of myosin regulatory light chain and dephosphorylation of smMLCK. Serum starved HASM cells were stimulated with IgE (5μg/ml) and lysate was collected at different time points untill 30 minutes. Figure shows the western blots probed with antibodies recognizing phosphorylation at serine 20 on myosin and serine 1760 on smMLCK. These blots were then reprobed with total myosin and smMLCK ab, respectively. Mann-Whitney U test was performed to determine the significance of the effect shown at different time points compared to time =0 min. P<0.05(*)

3.2.3. IgE stimulation induces phosphorylation of ERK, P38 and STAT3

We also determined the phosphorylation of signaling proteins as discussed in section 3.1, downstream to IgE. By virtue of immunoblotting analysis, we observed that IgE stimulation induces the phosphorylation of ERK, P38 (Figure 21) and STAT3 (Figure 24). However, phosphorylation levels of JNK (Figure 21), Akt (Figure 23) and Lyn (Figure 22) upon IgE stimulation were found to be comparable to unstimulated HASM cells.

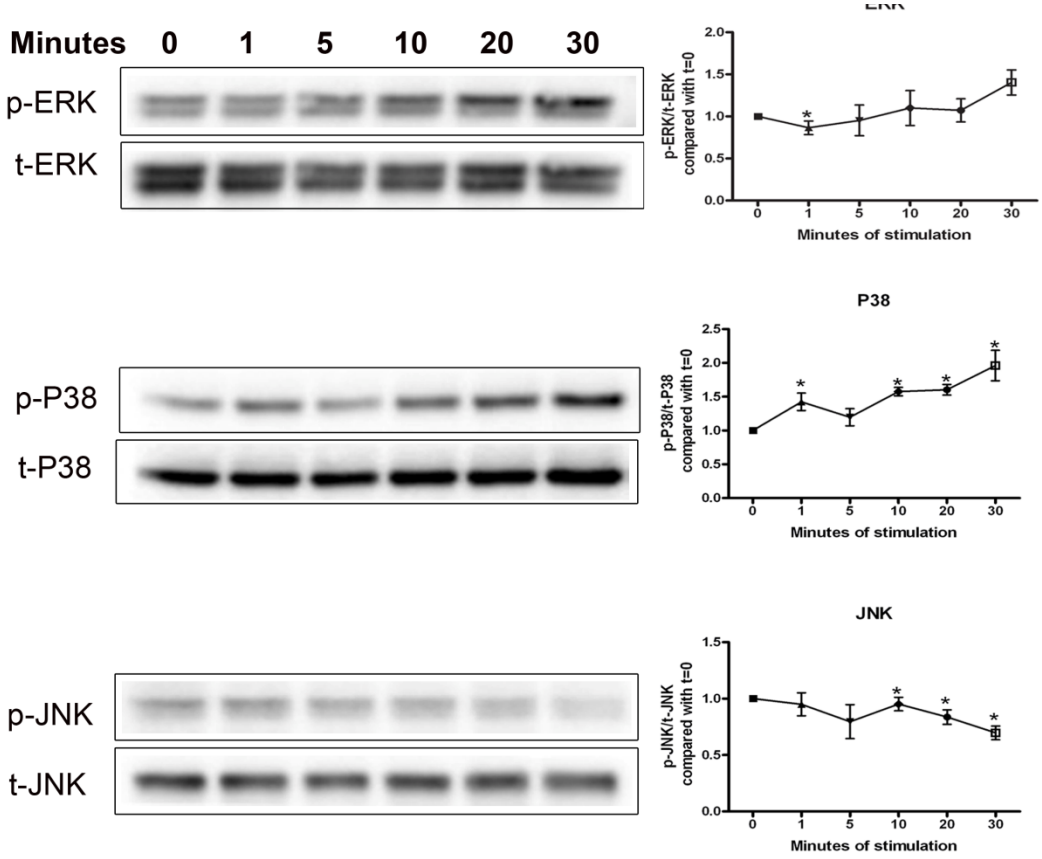


Figure 21. IgE stimulation induces the phosphorylation ERK and P38 but not JNK. Serum starved HASM cells were stimulated with IgE (5μg/ml) and lysate was collected at different time points until 30 minutes. Figure shows the western blots probed with antibodies recognizing phosphorylated ERK, P38 and JNK. These blots were then reprobed with total ERK, P38 and JNK ab, respectively. Mann-Whitney U test was performed to determine the significance of the effect shown at different time points compared to time =0 min. P<0.05(*)

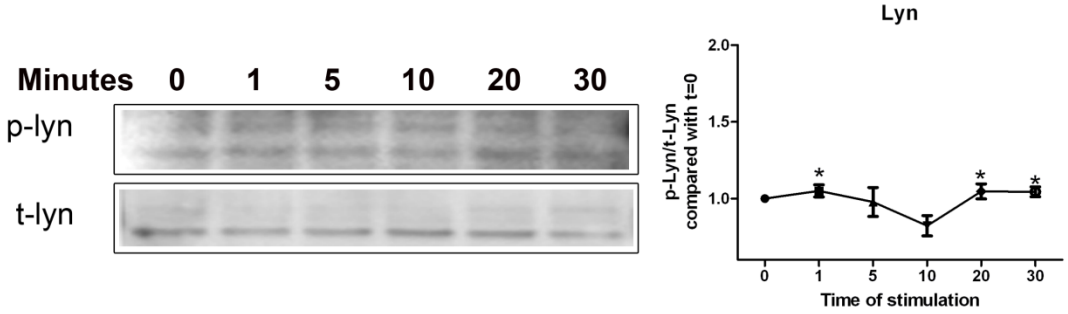


Figure 22. IgE stimulation induces the phosphorylation Lyn. Serum starved HASM cells were stimulated with IgE (5μg/ml) and lysate was collected at different time points until 30 minutes. Figure shows the western blots probed with antibodies recognizing phosphorylated Lyn. The blot was then reprobed with total Lyn ab. Mann-Whitney U test was performed to determine the significance of the effect shown at different time points compared to time =0 min. P<0.05(*)

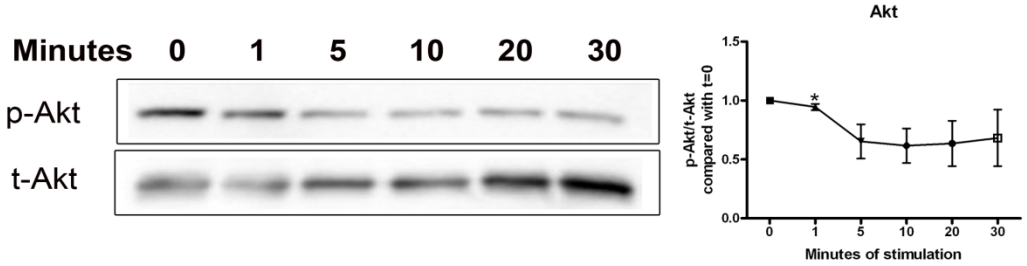


Figure 23. IgE stimulation induces the phosphorylation Akt. Serum starved HASM cells were stimulated with IgE (5μg/ml) and lysate was collected at different time points until 30 minutes. Figure shows the western blots probed with antibodies recognizing phosphorylated Akt. The blot was then reprobed with total Akt ab. Mann-Whitney U test was performed to determine the significance of the effect shown at different time points compared to time =0 min. P<0.05(*)

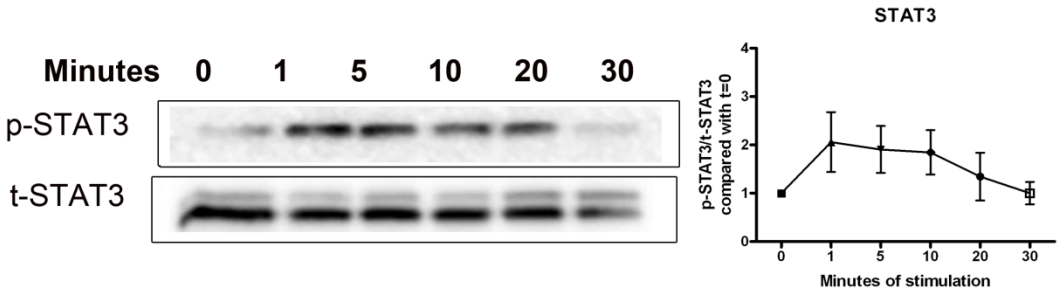


Figure 24. IgE stimulation induces the phosphorylation STAT3. Serum starved HASM cells were stimulated with IgE (5μg/ml) and lysate was collected at different time points until 30 minutes. Figure shows the western blots probed with antibodies recognizing phosphorylated STAT3. The blot was then reprobed with total STAT3 ab. Mann-Whitney U test was performed to determine the significance of the effect shown at different time points compared to time =0 min. P<0.05(*)

Chapter 4. Discussion

Previous studies have demonstrated that serum IgE level is associated with asthma and contractility of the asthmatic bronchial tissue (199). Incubation of HASM cells with atopic (rich in IgE) serum was shown to induce hypercontractility of isolated airway preparations (152, 153). In addition, IgE was shown to bind to smooth muscle membranes producing membrane hyperpolarization (200). Presence of both high (157, 201) and low affinity (155) Fc receptors of IgE on HASM cells suggests the likelihood of a direct role of IgE in modulating contractile and synthetic functions of these cells. However, the mechanism by which IgE modulates the contractile function of HASM cells is not fully understood.

In this study, we showed that IgE enhanced the expression of smMLCK (short form) in HASM cells, an effect that is found to be dependent on binding of IgE to its high affinity receptor, FcεRI. Here, the Ige driven effect was found to be dependent on MAPKs (ERK1/2, P38 and JNK) and PI3K to upregulate the protein expression of smMLCK.

We also observed that IgE could induce contraction of HASM cells grown on collagen gel matrix *in-vitro*. Interestingly, IgE stimulation is shown to phosphorylate ERK, P38 and STAT3. smMLCK dephosphorylation and myosin RLC phosphorylation is also observed upon IgE stimulation. Taken together, our data are the first showing a direct role of IgE on smMLCK protein expression and activity providing a plausible link between the effector controller of motor tone and IgE.

There has been considerable investigation on identifying the mechanism by which airway smooth muscle cells contribute to chronic asthma (107). HASM cells are the principal contractile component of the airways which are affected by the inflammatory cytokines secreted in peribronchial and perivascular milieu, thus contributing to airway remodeling (108). In severe chronic asthma, HASM cells also exhibit a hyper-responsive and hyper-contractile phenotype, an effect that is suggested to be due to altered smMLCK expression (108, 202).

MLCK exists in multiple tissues and developmental stage-specific isoforms. In HASM cells, smMLCK (130kDa) is the prominent isoform that controls the contractile apparatus to mediate contraction (186, 203). Several studies have demonstrated an enhanced expression of smMLCK in the asthmatic condition, suggesting its plausible role in the development of the hypercontractility observed in asthma (143, 202).

Although IgE binding to FcεRI in inflammatory cells was earlier thought to be a "passive sensitization" step requiring subsequent allergen/antigen cross-linking, recent data suggest some critical effector functions of IgE binding alone (called sensitization). IgE sensitization induced pro-survival effects in mast cells, monocytes and asthmatic neutrophils (197, 204, 205). Furthermore, we recently demonstrated that IgE sensitization of HASM cells induced the release of CC and CXC chemokines, Th2 cytokines (IL-4, -5, and -6) and the pro-allergic cytokine thymic stromal lymphopoietin (TSLP) (157, 191, 206). Roth and Tamm also observed similar effects and reported that the IgE-induced HASM synthetic function can be inhibited by therapeutic anti-IgE mAb Omalizumab/Xolair^{TM} (201, 207). Here we showed that IgE augments the expression of smMLCK in HASM cells. Moreover, this effect was found to

involve FcεRI as an FcεRI-α chain-specific blocking antibody mAb 15-1 (208) significantly reduced IgE-induced smMLCK expression in HASM cells. mAb 15-1 is anti-FcεRI alpha chain mouse antibody which specifically inhibits the binding of IgE to membrane bound FcεRI particularly (209, 210). Studies performed on mast cells suggested that 15-1 is neither anaphylactogenic by itself nor binds to Fcγ receptors, also giving insight to its inability to mobilize the cellular signaling by itself or through non-specific receptors (208). Previously, we demonstrated the inhibitory activity of mAb 15-1 on the cytokinergic functions of IgE in HASM cells (157). Based on our data, although we propose that the effect of IgE is predominantly mediated though FcεRI, we are not ruling out the plausible contribution of CD23. It would be interesting to examine whether 15-1 mediated inhibition of IgE binding to FcεRI also affects the expression of CD23 and whether the outcome of IgE is infact the result of activation of both FcεRI and CD23- dependent pathways. Besides extending the above-mentioned observations, our current study provides insight to the modulatory role of IgE on the proteins which contributes in the initiation of contraction.

The surface expression of FcεRI α chain in HASM cells is upregulated by proinflammatory cytokines, particularly TNF and IL-4 (191). As shown earlier by our lab, presensitization with TNF augmented IgE mediated release of cytokines from HASM cells (191). Here in this study we further substantiated this upregulatory mechanism and demonstrated that TNF presentization enhanced IgE mediated upregulation of smMLCK protein expression. Altogether it clearly indicated that IgE here mediated its effect through FcεRI as the mechanism which upregulates FcεRI surface expression and also enhances the IgE induced synthetic functions.

HASM cells are proposed to play a central role in airway narrowing due to two major physiological phenomenon: hypercontractility and increase in airway smooth muscle mass. Ebina *et al. (104)* observed an increase in the ASM area of asthmatic individuals. Although they did not determine the cause of greater ASM area in their studies, others demonstrated that it could be the result of an increase in size (hypertrophy) or number (hyperplasia) of smooth muscle cells. Benayoun *et al. (105)* then showed that HASM cells in airways of asthmatic subjects had larger lumen diameter but showed no evidence of hyperproliferation. Later, Woodruff *et al. (106)* also examined the airways of severe asthmatic subjects and provided evidence for hyperplasia rather than hypertrophy. Although the co-existence of both processes is not seen, altogether these events are suggested to root from the same pathway.

Benayoun's report also reported augmented levels of smMLCK in asthmatic hypertrophic HASM cells. They and others proposed that accumulation of structural proteins including contractile proteins, such as smMLCK, occurs during hyperplasia and hypertrophy as it is a mechanism through which cells undergo before mitosis. However, hyper-proliferating cells undergo mitosis and divide their contents while hypertrophic cells retain them, resulting in an increase in size. In our study, we found that IgE augments smMLCK mRNA and protein content in HASM cells, an effect that can be explained as being responsible for IgE-induced regulation of HASM cell functions, including hypercontractility and hypertrophy. Collectively, these results indicate the likelihood of a new paradigm by which IgE may regulate the role of HASM cells in causing airway narrowing.

MAPKs play a crucial role in modulating cellular functions, such as cell proliferation, survival, muscle contraction and cell migration (211-214). ERK1/2 is reported to be essential for MLCK expression and activity in vascular smooth muscle cells and breast cancer cells (213, 215-217). While in astrocytes, P38 but neither ERK1/2 nor JNK upregulates the expression of MLCK (108). In our study, we showed that ERK1/2 and to lesser extent P38 and JNK are essential for IgE induced smMLCK expression in HASM cells. We also examined the role of PI3K and observed that the inhibition of PI3K by wortmanin in IgE-stimulated HASM cells significantly decreased IgE-mediated smMLCK upregulation. Altogether, our study suggests the involvement of ERK, P38, JNK MAPKs and PI3K in mediating IgE-induced smMLCK protein abundance in HASM cells.

IgE-antigen complex aggregates and activates FcεRI, which induces Lyn kinase phosphorylation. Lyn is a protein tyrosine kinase of Src family which then phosphrylates ITAM on FcεRI and provides a docking site for the activation of Syk kinase. Thus, Lyn kinase facilitates the phosphorylation and activation of Syk kinase, resulting in phosphatidylinositol-3-phosphate (PIP3) production, activation of Phospholipase C and thus increased cytosolic Ca2+ levels (47). In our study, we wanted to investigate whether stimulation with IgE alone would also employ signaling pathways similar to IgE-antigen complex. Here, we found that silencing of Syk kinase abrogated IgE-mediated smMLCK protein expression in HASM cells. This result supports the pivotal role of Syk in both IgE-mediated FcεRI activation models: "cross linking model" versus "binding of IgE alone" (197, 218). Interestingly, our data in this study do not support the role of Lyn in IgE mediated signaling. We found that Lyn kinase silencing did not affect IgE induced smMLCK protein expression. Early studies have indicated that Lyn is a

positive regulator of FcεRI activation (47). However, according to electron microscopy and biochemical investigations, it was noticed that Lyn dissociates from FcεRI as soon as the latter is stimulated (219, 220). Furthermore, FcεRI, which is assoctiated with Lyn, was reported to be less competent in signaling (220). From here emerged the view that Lyn might be a negative regulator of the FcεRI activated pathway. Moreover, $Lyn^{-/-}$ mice display increased levels of serum IgE and expression of surface FcεRI on mast cells *in vivo* that account for the exacerbated allergic phenotype in comparison with WT littermates (221). Furthermore, FcεRI-mediated activation of $Lyn^{-/-}$ BMMCs (bone marrow derived mast cells) results in a greater increase in mRNAs encoding Th2 cytokines and chemokines compared to control cells (221-223). Collectively, the role of activated Lyn is not clear as it is suggested to regulate IgE-mediated functions in a positive as well as in a negative manner in mast cells (47, 53). Although anaphylaxis studies tried to partly dissect the exact mechanism and proposed that Lyn kinase is infact a negative regulator of IgE pathway. Earlier in an anaphylaxis study mast cells from Lyn kinase knock out mice were found resistant to degranulation which was the basis of conclusion that Lyn is required by the IgE activated signaling pathway for mast cell degranulation (224). Later, another anaphylaxis study reported a confounding observation that mast cells from lyn-/- mice showed greater degranulation (223). The discrepancy was later explained by the age of the mice used for investigation. The former study was done on older mice in which mast cells were already saturated with IgE, attributed to increased IgE production in older lyn-/- mice, therefore the effect of IgE could not be observed. However, in the latter investigation, mice of younger age exhibited normal IgE level and responded normaly to exogenous IgE (223-225). In our report we showed that silencing of Lyn kinase did not affect IgE mediated smMLCK protein overexpression, neither positively, nor negatively. This observation, interestingly, presents an

altogether different view that Lyn kinase might not be required for IgE mediated modulation of smMLCK expression. It proposes an additional aspect of involvement of Lyn in IgE signaling pathway indicating that role of Lyn kinase in IgE signaling may be function or cell specific. This is further supported by our other data showing that lyn silencing was unable to abrogate IgE mediated IL-8 production, unlike Syk silencing (data not shown). Moreover, plausible role of other kinases of Src kinase (Lyn kinase) family cannot be denied. Of note, Fyn kinase is known to take over the role of Lyn kinase, downstream to FcεRI in Lyn-/- mice (226). Altogether, our data suggest that IgE mediated smMLCK expression might utilize restricted signaling pathways in HASM cells unlike mast cells or basophils.

In previous studies, asthmatic bronchial rings were found to exhibit augmented contractility (153). Parallel increase in the levels of IgE and smMLCK in asthmatic ASM tissue were associated with this increase in contractile functions. Based on our results, we propose a novel link which suggests that IgE may contribute to hypercontractility by increasing the content of smMLCK protein. Additionally, we also demonstrated that IgE could induce contraction in HASM cells grown on collagen gel matrix. Previously, Margulis *et al*. (227) also quantified the effect of IgE on the contraction of HASM cell seeded collagen gels. Interestingly, they did not observe contraction of HASM cells upon IgE stimulation (227). The conflicting conclusions of Margulis's report and our study can be explained by careful analysis of the contraction data at different time points. They reported the change in collagen gel area after 24 hrs of IgE-anti-IgE treatment whereas contraction shown here is measured from 1 minute to 24 hrs. As shown in figure 16, IgE induces contraction comparable to MCh untill 10 minutes. From 30 minutes to 3 hrs, IgE mediated contraction was at its maximum, after which collagen gels started to relax.

Observation at early time ponts in Margulis's study would shed more light in this direction. Monomeric IgE is a weaker agonist than MCh, as the magnitude of contraction by MCh is greater than that induced by IgE. Prevalent notion suggests that smMLCK is activated by the calcium-calmodulin complex leading to RLC phosphorylation, which induces contraction in HASM cells (186). The phosphorylated form (p-serine 1760) of smMLCK is known as the inactive form. Serine 1760 is located in the calmodulin binding site in the C-terminal domain of smMLCK. Phosphorylation at this site prevents calcium-calmodulin complex from interacting with smMLCK and activating smMLCK (182). Here we were able to show that IgE stimulation induces dephosphorylation of smMLCK and phosphorylation of myosin RLC. It is not impossible that IgE induces contraction by virtue of activation of smMLCK and subsequent phosphorylation of myosin RLC. Recently, it was reported that myosin phosphorylation might also take place independent of smMLCK (228, 229). However, such conclusions were made based on observations from embryonic fibroblasts and bladder smooth muscle cells. Having identified that embryonic fibroblasts do not express smMLCK, it is obvious that such cells contain an alternate mechanism to regulate myosin phosphorylation. Further, whether the contractile apparatus is regulated in a similar fashion in airway smooth muscle cells and bladder smooth muscle cells needs further clarification. Nevertheless, the role of other kinases including PKC and Rho kinase cannot be ignored (230). In the future, it would be interesting to tease out their role downstream of IgE and upstream to myosin in HASM cells. We have further dissected the mechanism and studied the role of Lyn, MAPKs, PI3K substrate Akt and STAT3 in the IgE activated signaling pathway. We found that IgE stimulation could not enhance the phosphorylation level of lyn in HASM cells. This observation further strengthens the model we proposed earlier that Lyn might not be involved in IgE signaling in HASM cells. Further, we

found that IgE stimulation induces the phosphorylation of ERK, P38 and STAT3 but not JNK and Akt. Monomeric IgE regulates the survival of BMMCs through an ERK dependent pathway (231). SPE-7 (IgE) alone is also shown to induce significant phosphorylation of ERK, P38 and JNK (197). However in human basophils, ERK phosphorylation by antigen free myeloma IgE was not detected (232). Furthermore, IgE mediated release of mediators in basophils was reported to occur through a P38 dependent pathway. Here, phosphorylation of neither JNK nor its substrate c-Jun was observed upon stimulation with IgE-anti-IgE (233). Our data in this report show the phosphorylation of ERK and P38 but not JNK upon IgE stimulation supplements the preceeding reports to some extent. Although the precise reason of this discrepancy is not clearly known, differences in the cell types employed in these studies might provide some interesting clues.

Involvement of Akt is clearly shown in pathways regulating cellular processes such as survival, metabolism and cellular growth. It is a downstream effector molecule of PI3K and is known as an index of PI3K activity. IgE-antigen complex is shown to activate Akt, inducing the production of IL-2 and TNF from murine mast cells (234). However, prosurvival effects of IgE alone without antigen is shown to be independent of Akt (235). Also, Feuser *et al.* (236) reported that the IgE-antigen complex alone does not phosphorylate akt by itself. However, priming these cells with IL-4 and SCF before IgE stimulation induced significant Akt phosphorylation. In our study, although we stimulated HASM cells with IgE alone without antigen, we also did not find any change in the phosphorylation status of Akt upon IgE stimulation. Having known that Akt is a downstream effector of PI3K, it was interesting that PI3K is required for the upregulation of smMLCK by IgE but the same IgE does not promote

Akt phosphorylation. We also examined the effect of IgE stimulation on STAT3 phosphorylation. Previously, IgE was also reported to phosphorylate transcriptional factor STAT3 in murine mast cells. In our report we presented a similar observation.

Taken together, our data support a novel model in which IgE augments the expression of smMLCK and also mobilizes the signaling pathway involved in activating the contractile apparatus in HASM cells. However, further studies would validate the role of these IgE activated signaling protein in HASM cells.

5. Future Directions:

In this study we examined the effect of IgE on smMLCK expression. We also determined the signaling proteins which are required by IgE to upregulate the protein expression of smMLCK. Based on our findings we propose that IgE mediated smMLCK upregulation is a mechanism by which IgE contributes to hypertrophy observed in asthmatic condition. According to the current view, accumulation of contractile proteins is indicative of a hypertrophic state of airway smooth muscle cells. Therefore, it would be interesting to determine whether IgE augments the expression of other contractile proteins or its effect is only limited to smMLCK. In our preliminary experiments, we have observed that the content of myosin remains comparable even after treatment with IgE. Also, the effect of IgE on other contractile protein including actin and calponin will be determined. Further, the expression of contractile proteins is known to be regulated by serum response factor (SRF). In future studies, we would like to determine whether IgE mediated regulation of smMLCK protein expression is SRF dependent.

smMLCK is an important regulator of contraction. Conventionally, smMLCK phosphorylates myosin, which interacts with actin and induces contraction of airway smooth muscle cells. Previous studies have shown enhanced contractility of bronchial tissue, which contained increased content of smMLCK. Having identified that myosin phosphorylation can also occur independent of smMLCK, it is not clear whether the increase in contractility was due to an increase in smMLCK content. By examining the effect of IgE on MCh-induced contraction, we will be able to provide precise insight of the relationship between IgE, smMLCK and contraction of HASM cells.

We have also demonstrated that IgE itself can also induce contraction. We identified that IgE stimulation induces the phosphorylation of ERK, P38, STAT3 and myosin, and dephosphorylation of smMLCK. Based on our data we hypothesized that IgE activates MAPK and STAT3 dependent pathways to activate smMLCK (determined by the dephosphorylation of smMLCK), which then phosphorylates myosin regulatory light chain. The results will be further validated by determining the magnitude of IgE induced HASM cells contraction upon inhibition of MAPKs, STAT3, smMLCK and myosin.

IgE plays an important role in the manifestation of inflammation through mast cells, basophils, eosinophils and neutrophils. Its role in other cells, such as airway smooth muscle cells has also been identified. Others and we have previously reported that HASM cells express FceRI and FceRII through which IgE stimulates HASM cells to secrete proinflammatory cytokines. Our study is the first report suggesting the likelihood of a role of IgE in HASM hypertrophy and an aberrant contractile phenotype. Our current data and future experiments will provide a clear view of the multiple functions of IgE on HASM cells. Lately beneficial effects of inhibition of IgE with omalizumab has been shown. Clinically, our data would contribute to our overall understanding of the effect of inhibiting IgE.

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