ABSTRACT

Title of Document: CD23 MEDIATED IGE TRANSCYTOSIS IN AIRWAY INFLAMMATION

Senthilkumar Palaniyandi, Doctor of Philosophy, 2012

Directed By: Xiaoping Zhu, Associate Professor
College of Veterinary Medicine

CD23 (FcεRII), a C-type lectin type II membrane glycoprotein, plays an important role in IgE homeostasis and development of allergic inflammation. I showed that CD23 was constitutively expressed in the established or primary human airway epithelial cells and its expression was significantly up-regulated by IL-4 stimulation. In a transcytosis assay, human IgE or IgE derived immune complex was transported and enhanced by IL-4 stimulation across a polarized Calu-3 monolayer. A CD23 specific antibody or soluble CD23 significantly reduced the transcytosis, suggesting a specific receptor-mediated transport by CD23. Transcytosis of both IgE and the immune complex was further verified in primary human airway epithelial cell monolayers. Furthermore, the transcytosed antigen-IgE complexes were competent in inducing degranulation of the cultured human mast cells. This study implies CD23-mediated IgE transcytosis in human airway epithelial cells may play a critical role in

initiating and contributing to the perpetuation of airway allergic inflammation.

To verify the above results in a mouse model, CD23 expression was detected in epithelial cells lining mouse airway and enhanced by IL-4 exposure as well as in ovalbumin (OVA) sensitized mouse. I showed that CD23 transported IgE and OVA-IgE derived immune complex across airway epithelial cells in wild-type, but not CD23 knockout (KO), mice. The chimeric CD23KO mice repopulated with wild-type myeloid cells, sensitized and challenged with OVA showed significant reduction in siglec-F+ cells, eosinophils, macrophages and IL-4 in bronchoalveolar lavage fluid recovered 24 hours later compared to the wild-type mice.

Our finding of CD23-mediated IgE transport in airway epithelial cells suggest a possibility of CD23 transporting an IgE Fc-fused protein for immunotherapy. CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4) which competitively binds CD80 and CD86 expressed on antigen presenting cells and inhibits CD28 mediated co-stimulation of T cell activation. A CTLA4-Fc (IgE) fusion protein produced in Chinese hamster ovary cells was intranasally administrated into mouse airway for assessing its specific transport by CD23. The effect of this fusion protein on the development of allergic inflammation is being fully investigated in wild-type, CD23-KO, and chimeric mouse model.

CD23 MEDIATED IGE TRANSCYTOSIS IN AIRWAY INFLAMMATION

By

Senthilkumar Palaniyandi

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Advisory Committee:
Dr. Xiaoping Zhu, Chair
Dr. Volker Briken
Dr. David M. Mosser
Dr. Siba K. Samal
Dr. Wenxia Song

Dedication

This dissertation is dedicated to my parents, my sister, my brother, my wife, my son Srikrishna. I couldn’t have done this without your support. Thank you so much for everything you’ve given me. I love you all very much!

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A disintegrin and metalloproteinase 10	ADAM10
Airway hyperresponsiveness	AHR
Antigen presenting cell	APC
Bovine serum albumin	BSA
Bronchoalveolar lavage	BAL
Carbohydrate-recognition domain	CDR
Carbon dioxide	CO_2
Chinese hamster ovary	CHO
Constant epsilon domain	Cε
Constant gamma domain	Cγ
Enzyme-linked immunosorbent assay	ELISA
Fc gamma receptor	FcγR
Fc epsilon receptor	FcεR
Fetal bovine serum	FBS
Fluorescein isothiocyanate	FITC
Fluorescent activated cell sorter	FACS
Horseradish peroxidase	HRP
Immune complex	IC
Immunoglobulin	Ig
Immunoreceptor tyrosine based activation motif	ITAM
Interferon alpha	IFN-α

Interferon gamma	IFN-γ
Interleukin	IL
Kilodaltons	kDa
Knockout	KO
Macrophage inflammatory protein	MIP
Messenger ribonucleic acid	mRNA
Mitogen-activated protein kinase	MAPK
Monoclonal antibody	mAb
Neonatal Fc receptor	FcRn
Nuclear magnetic resonance	NMR
Optimal cutting temperature	OCT
Ovalbumin	OVA
Peripheral blood mononuclear cell	PBMC
Phosphate buffered saline	PBS
Polymeric immunoglobulin receptor	pIgR
Reverse transcription polymerase chain reaction	RT-PCR
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	SDS-PAGE
Soluble CD23	sCD23
Soluble FcεRI	sFcεRI
T helper cell	Th
Transepithelial electrical resistance	TER
Tumor necrosis factor alpha	TNFα
Variable heavy domain	V_H

4-hydroxy-3-nitrophenylacetyl	NP

CHAPTER 1: INTRODUCTION

OVERVIEW

Immunoglobulin E and human diseases

In the developed world, there is an increased incidence of Immunoglobulin E (IgE) mediated diseases like allergy. In February 1968, the name Immunoglobulin E was officially given to the final class of human antibody in the World Health Organization Immunoglobulin Reference Laboratory meeting held in Lucerne, Switzerland (1). Initially, IgE was found to have effector functions against invading parasites and in the controlling of the parasitic diseases. Later on, the beneficial effect of the IgE was overridden by the harmful effects in the body by developing various IgE mediated allergic diseases. The body responds to the exogenous antigen called allergen and develops allergen specific IgE, leading to the exacerbation of the allergic diseases by releasing various allergic mediators. IgE mediated allergic diseases include allergic rhinitis, asthma, food allergy, systemic anaphylaxis, urticaria and atopic dermatitis or eczema. Asthma is a chronic lung disease that inflames and narrows the airways. It affects people of all ages, but it occurs most frequently in childhood. The exact cause of asthma remains elusive. A combination of factors, such as family genetics, respiratory infections, and certain environmental exposures are involved in developing asthma. Ongoing airway inflammation results in abnormal structural changes in the airway due to the epithelial desquamation/denudation and repair processes (2). Food allergy is characterized by the abnormal immune response to the harmless food substance in certain individuals, resulting in production of food allergen specific IgE and characterized by vomiting, diarrhea and mouth itchness. Sometimes patient suffer from breathing

difficulty and hives or eczema. In most cases, avoiding the food allergen may by the best way to prevent the food allergy. However, it is very difficult to find good nutritious food to avoid infant food allergy. The worst case of the allergic diseases can lead to anaphylaxis and if left untreated, they can result in the death due to anaphylactic shock by arresting breathing and blood circulation.

After the first time exposure to an allergen, allergen specific IgE can be developed against that particular allergen. The antigen presenting cells (APCs) can take up and process this allergen and migrate to the draining lymph node where allergens are presented to the naive CD4+ T cells. These T cells will differentiate into helper T (Th2) cells which secrete large quantities of cytokines IL-4 and IL-13. The B cells present in the lymph node also recognize, take up and process the allergens. Under a Th2 environment, B cells can class switch to produce IgE against the allergen. Allergen specific IgE binds to the high affinity receptor for IgE, FcεRI which is typically expressed on the mast cells, eosinophils, and basophils (1). This process is called sensitization. During a subsequent exposure, the same allergen will cross-link IgE/FcεRI resulting in the release of various inflammatory mediators, a phenomenon called degranulation. Degranulation occurs within the minutes of the allergen exposure. Mast cells and basophils release various cytokines, chemokines, histamine, leukotrienes and prostaglandins (3). These mediators subsequesntly bind to their specific receptors expressed on the smooth muscle cells, epithelial cells, and blood vessels, leading to muscle contraction, increased mucus production and vascular permeability. In the latter phase of allergic reaction, the mast cells and basophils synthesize and release various

cytokines and chemokines that recruit eosinophils and Th2 cells (3). The long-lasting effects of these mediators lead to the remodeling of airway that is responsible for various allergic manifestations and symptoms in asthmatic patients.

Discovery of IgE

In 1921, Prausnitz and Kustner originally showed that transfer of serum from an allergic patient to a non-allergic patient resulted in sensitization of the non-allergic patient and they named the molecule responsible for this activity as reagin (4). This test was also known as the Prausnitz-Kustner (PK) test. In 1967, two groups independently identified the protein present in the reaginic preparation. Ishizakas prepared the γE antisera, and with the help of radioimmunodiffusion assay showed γE precipitin bands and purified the protein present in the reagin preparation while, Stanworth et al. discovered a myeloma protein that inhibits the PK reaction (5-8). In 1968, the World Health Organization Immunoglobulin Reference Laboratory meeting named the final and fifth class of human immunoglobulin as IgE.

Structure of IgE

Like other immunoglobulin molecules, IgE consists of two identical heavy and light chains as illustrated in the Figure 1.1. The heavy chain of IgE consists of one variable heavy (V_H) domain and four constant epsilon domains (Cε1–Cε4). The Cε3 and Cε4 are homologous in structure and sequence to the heavy gamma chain domain Cγ2 and Cγ3 in IgG. Interestingly, the Cε2 domain corresponds the hinge region of the heavy gamma chain (9, 10). Remarkably, the crystal structure of the IgE-Fc fragment

including the disulphide-linked Cε2 domain reveals that Cε2 domain is much closer to the Cε3 domain with extensive contact with Cε3 and Cε4 domains, which leads to asymmetrically bent conformation in IgE (11).

RECEPTORS FOR IGE

There are two kinds of membrane receptors for IgE, high affinity receptor for IgE (FcεRI) and low affinity receptor for IgE (FcεRII or CD23). Soluble receptors for IgE includes soluble FcεRI (sFcεRI), soluble CD23 (sCD23) and galectin-3 (1, 12). Only in the mouse, FcγRIV, homologous to the FcγRIIIA in humans is capable of binding IgE with a low affinity; but, hFcγRIIIA does not bind to human IgE. Mouse FcγRIV is expressed in the lung macrophages and can also binds to IgG with intermediate affinity. However; it will be readily displaced by IgE immune complexes in the lung and plays a regulatory role in the lung inflammation (13).

Structure, expression and function of FcεRI.

FcεRI expressed on the mast cells and basophils is responsible for the degranulation and the release of inflammatory mediators responsible for the development of allergy. FcεRI is expressed as a tetrameric complex made of an alpha subunit responsible for binding to IgE, a beta subunit and two gamma subunit linked by a disulphide bond (FcεRI$\alpha\beta\gamma_2$) as shown in the Figure 1.2 (1, 14-18). While the alpha subunit binds to the IgE, FcεRI signals through immunoreceptor tyrosine based activation motif (ITAM) carried in beta and gamma subunits. These two subunits act synergistically to amplify the signaling events. In humans, the FcεRI is also expressed in a trimeric form

($Fc\varepsilon RI\alpha\gamma_2$) that lacks beta subunit in mast cells, basophils and antigen presenting cells, monocytes, smooth muscle cells, eosinophils, neutrophils and platelets. In rodents, the trimeric form of FcεRI ($Fc\varepsilon RI\alpha\gamma_2$) is not detected so far (1, 14-18).

Among all the Fc receptors, FcεRI has the highest affinity for IgE with $K_a \approx 10^{10}$ M^{-1} (1). FcεRI is involved in the development of allergy. Allergen specific IgE binds to the FcεRI expressed on the mast cells and basophils and the cross linking of the IgE-FcεRI complexes by the allergen specific IgE leads to the degranulation, with the release of various inflammatory mediators, like histamine, prostaglandins, serotonin, leukotrienes (3, 19-21) and various cytokines, such as IL-4, IL-3, IL-5, IL-6, IL-13, IFN-γ and granulocyte macrophage colony-stimulating factors (3, 22-24). The release of inflammatory mediators is the hallmark for the development of allergy or immediate type 1 hypersensitivity (1). FcεRI expressed on the APC also takes part in the antigen uptake, processing and presentation to T cells and involved in Th2-dependent allergic inflammation (25- 27).

SOLUBLE RECEPTORS FOR IGE

Soluble FcεRI (sFcεRI)

Soluble form of FcεRI (sFcεRI) is found in the human serum. This 40 kDa molecule of sFcεRI is generated from the alpha chain of FcεRI and it can bind to the human IgE. Since the alpha chain of FcεRI binds to IgE with very high affinity, it is expected that sFcεRI also binds to IgE with a similarily high affinity (28). Recombinant sFcεRI has been shown to prevent anaphylactic shock and allergic disease in a mouse model (29) and passive cutaneous anaphylaxis in rats (30). The possible

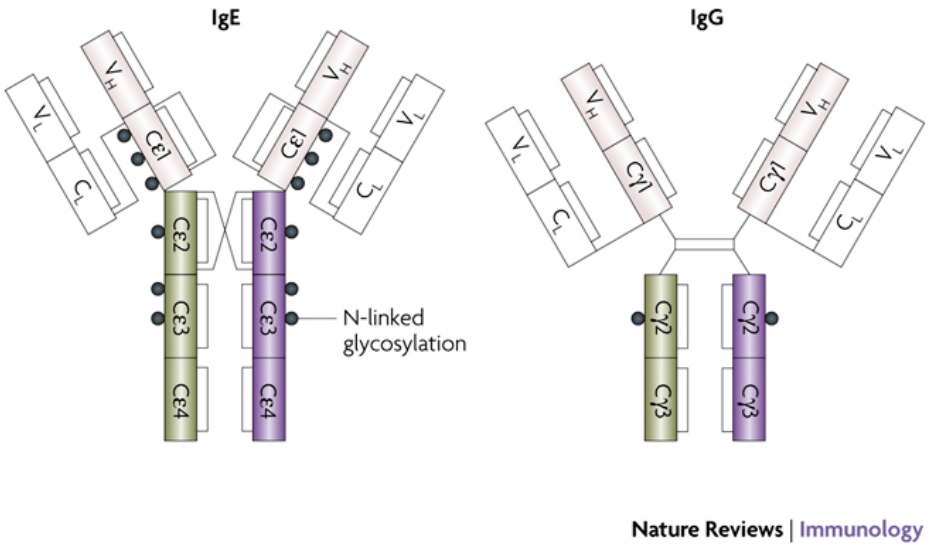


Figure 1.1 Comparison of structure of IgE and IgG. Structural domain organization of human IgE and IgG1 showing intra and inter disulphide linkage bonds and site for N-linked glycosylation. (Taken from reference 1).

mechanism is that sFcεRI can bind to free IgE and IgE immune complexes thereby reducing the availability of these IgE immune complexes for interacting with the FcεRI on the surface of basophils and mast cells (12).

Galectin-3

Galectin-3 previously known as ε–binding protein is a 30 kDa secretory protein and belongs to β-galactose containing oligosaccharide lectin protein (32). It has the ability to bind both IgE and FcεRI and this can activate the FcεRI bound with or without IgE on the mast cells or basophils (33-35). Galaetin-3 consists of a carbohydrate-recognition domain (CDR) with proline and glycine rich repeats at amino terminal through which it can form a pentameric structure similar to IgM as shown in the Figure 1.3 (36). Differentially glycosylated IgE has exhibited distinct binding properties to galectin-3 (37, 38).

Galectin-3 is found on the surface of variety of cells like neutrophils, mast cells, eosinophils, keratinocytes, monocytes, macrophages, T cells and bronchial epithelium of airways (39-46). Murine CD4+ and CD8+ T cells but not the resting T cells express galectin-3 under the influence of IL-2, IL-4, and IL-7 (44). Galectin-3 expressed in T cells contributes to the growth and survival of T cells (45). Galectin-3 is also found to be expressed in the bronchial epithelium of the small airways of patients with chronic obstructive pulmonary disease (COPD) (46). In addition, Galectin-3 plays an important role in the binding to the IgE on the mast cells and activates the mast cells through cross linking of IgE bound to FcεRI or by directly binding to the FcεRI expressed on the mast

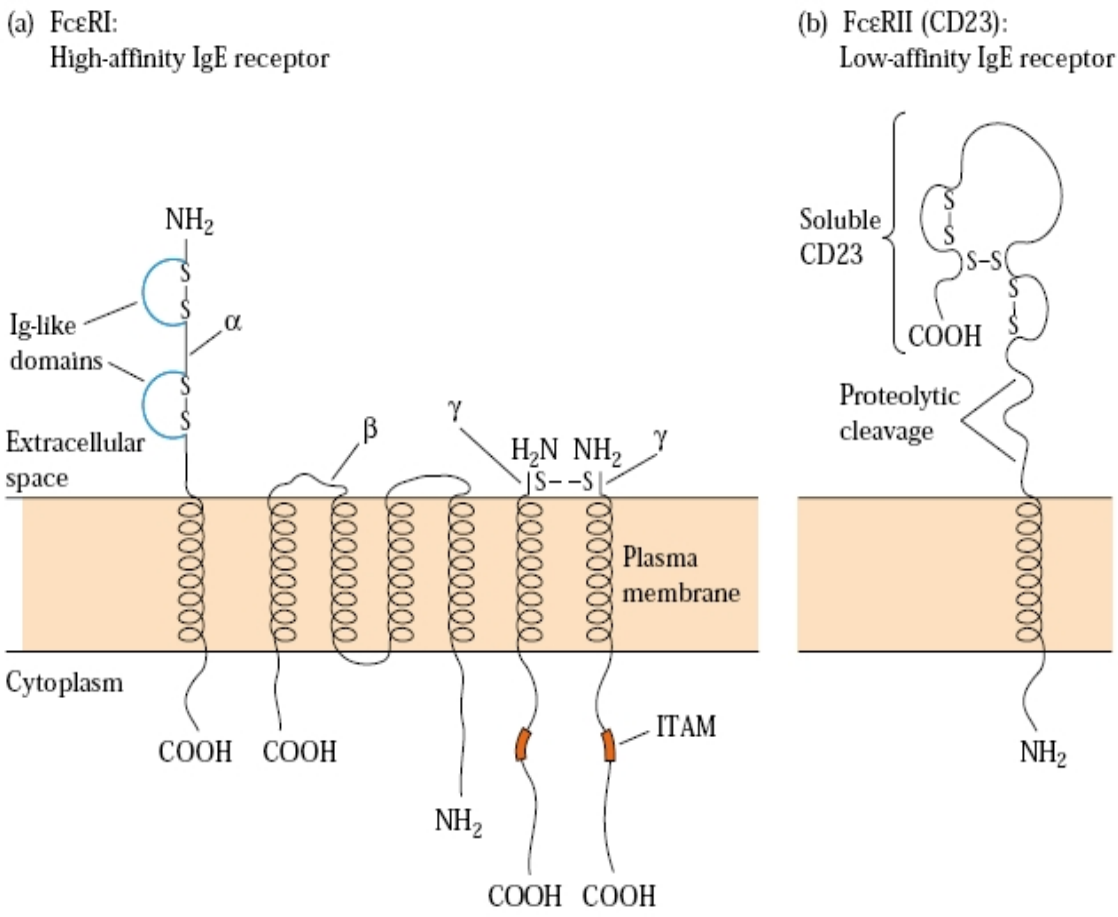


Figure 1.2 Structure of high affinity receptor for IgE, FcεRI and low affinity receptor for IgE, FcεRII or CD23. (Taken from 31).

cells (33). Galectin-3 mediated IgE dependent allergic disease activation and cytotoxicity have also been found in human neutrophils and eosinophils (39, 41).

LOW AFFINITY RECEPTOR FOR IGE, CD23 OR FcεRII

Discovery of CD23

In the year 1975, Lawrence et al. first showed the presence of a receptor for IgE on the human lymphocytes (47). In 1976, IgE myeloma protein was found to bind cultured lymphoblastoid cell lines in a species specific manner (48). IgE bound to the receptor expressed on lymphocytes with a relatively low affinity ($K_a \approx 10^6$ -10^7 M^{-1}) compared to the high affinity receptor FcεRI expressed on the mast cells, hence named as low affinity receptor for IgE or FcεRII. Later two groups independently identified the CD23 molecule. First in 1985, Epstein Barr virus (EBV) activates a cell surface marker in human B cells which is named as BLAST-2 or EBVCS (49). Another group in 1987 showed that EBV infection induced the expression of activation marker CD23 in B cells (50). In 1987, two individual groups with the help of monoclonal antibody (mAb) showed that CD23 was expressed on human lymphocytes and identified that BLAST-2, EBVCS and CD23 are the same (51,52). Cloning of the human CD23 cDNA was later achieved by three different groups. They showed that CD23 is a type II transmembrane protein consisting of 321 amino acids with its N-terminus in the cytoplasm and C-terminus on the cell surface. The amino acid sequence of CD23 shows a marked homology with animal lectin and closely resembling human, rat and chicken asialoglycoprotein receptors (53-55). Unlike other immunoglobulin (Ig) receptors, CD23 is the only Ig receptor that does not belong to the Ig super family. The binding of IgE to

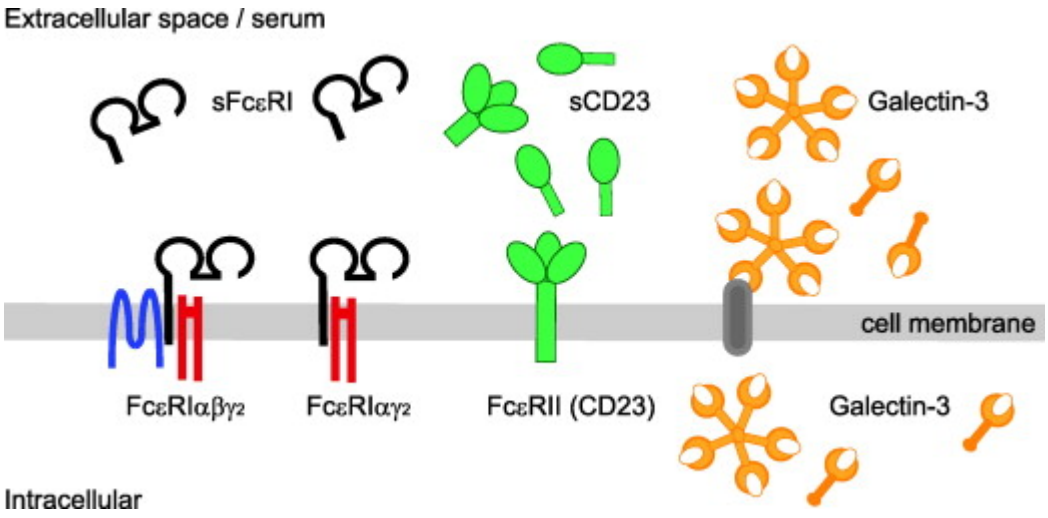


Figure 1.3 Membrane associated receptors for IgE and its soluble forms. FcεRI is expressed as tetrameric FcεRI$\alpha\beta\gamma_2$ or FcεRI$\alpha\gamma_2$ and its soluble form sFcεRI was generated from the alpha chain of FcεRI. Low affinity receptor for IgE, (FcεRI or CD23) expressed as oligomeric or trimeric form on the cell surface and different molecular weight of soluble CD23 was generated by intracellular or extracellular proteolytic cleavage of full length membrane expressed CD23. Galectin-3 is a secretory protein with no trans membrane domain and it can form a pentameric structure (Taken from 12).

CD23 is dependent on calcium, and it recognizes a Cε3 domain of IgE independent of the IgE oligosaccharides (56, 57). Mouse CD23 is also a type II transmembrane protein with 331 amino acids and two N-linked glycosylation sites sharing 57% amino acid sequence identity with human CD23 (58,59).

Structure of CD23

Different from all other immunoglobulin receptors, CD23 belongs to a C-type lectin family consisting of three lectin head domains in the carboxy terminal region followed by a triple α–helical coiled-coil stalk region, transmembrane and short cytoplasmic tail domain as illustrated in the Figure 1.4 (1). The stalk region of the CD23 has a highly similar sequence to tropomyosin that has a α–helical coiled coil structure with heptad repeats (60). Through chemical cross linking studies, it was found that CD23 stalk region containing the α–helical coiled coil stalk region enable the oligomerization of CD23 to form trimer. It was also showed that CD23 can bind IgE with both high and low affinity (61). Development of mAb B3B4 against murine CD23 showed that two lectin head domains of CD23 bind to one IgE cooperatively (62). CD23, the low affinity receptor for IgE, is later considered as a misnomer since CD23 in the monomeric state binds IgE with low affinity ($K_a \approx 10^6$ -10^7 M^{-1}), while the trimeric form of CD23 binds IgE with high affinity ($K_a \approx 10^8$ -10^9 M^{-1}) (1, 63,64). Using the mAb directed against the stalk region of murine CD23, it was demonstrated that CD23 is preassociated as a trimer on the cell surface (65). CD23 trimerization (65,66) brings their lectin domains close to each other leading to cooperative and high affinity binding of single IgE molecule (63,64, 67). One main difference between mouse and human CD23 is that the lectin domain of

human CD23 contains additional C-terminal tail (1). Nuclear magnetic resonance spectroscopy of CD23 revealed the three dimensional structure of the C-type lectin domain. CD23 binds to both the ligands IgE and CD23 simultaneously and does not require calcium for its interaction (64).

Expression and regulation of CD23

Human CD23 is expressed in variety of cells such as B cells, monocytes, eosinophils, platelets (68-71), Langerhans cells and intestinal epithelial cells (72-75). CD23 exists as two isoforms, CD23a and CD23b; they are arised from the alternative RNA splicing with different transcription initiation sites and differing only in the N-terminal cytoplasmic region by 6-7 amino acids (76). In humans, the isoform CD23a is expressed only in the B lymphocytes while CD23b is expressed in variety of cells like monocytes, eosinophils and langerhans cells (77). In mice, CD23 is expressed in B cells, follicular dendritic cells, intestinal epithelial cells (58, 78, 79, 80, 81). Regarding the isoforms, murine CD23a is expressed in the B cells and follicular dendritic cells and CD23b is expressed in follicular dendritic cells and intestinal epithelial cells (77). FcεRII (CD23) is expressed in the B cells based on the differentiation stage, for example, human bone marrow-derived B cells with surface IgM and IgD do not express CD23 whereas circulating B cells with surface IgM and IgD express CD23 (82), and the murine B cells also express CD23 (83). Majority of the CD27+ memory B cells do not express CD23, however a small subset of B cells co- express both CD23 and CD27 (84). Mouse intestinal epithelial cells also express CD23. Mouse CD23 is also expressed as CD23a and CD23b isoforms and additional CD23b isoforms lacking exon 5 or exon 6 is also

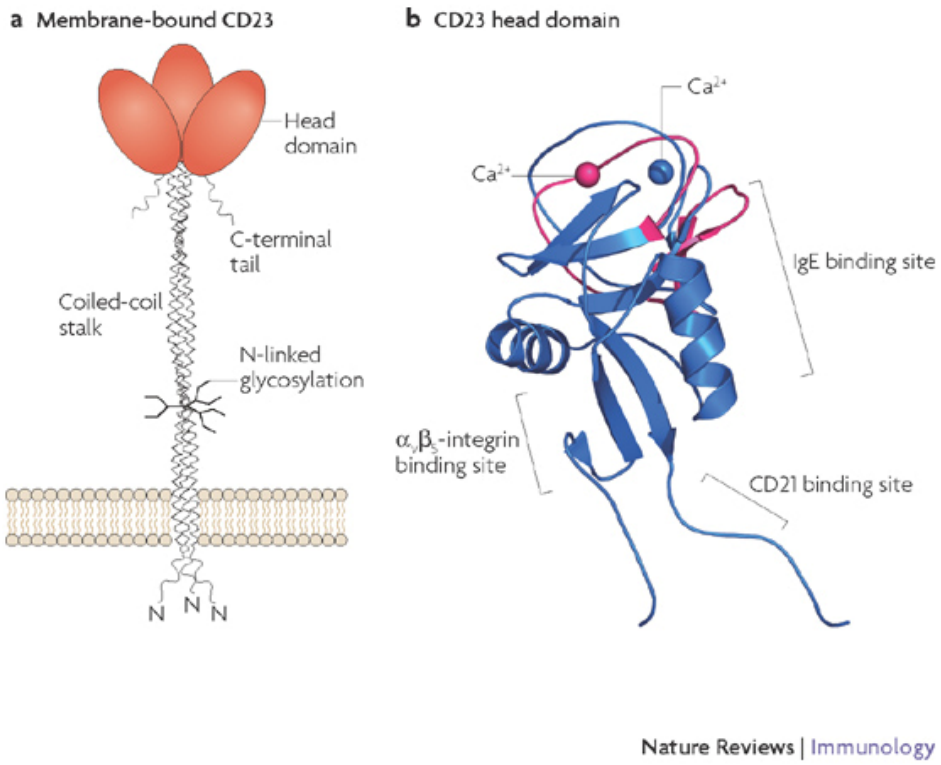


Figure 1.4 Structure of CD23: a. Schematic presentation of membrane bound CD23 showing extracellular trimeric coiled-coil stalk region, head domain, C-terminal tail and site for N-linked glycosylation, transmembrane domain and N terminal region. b. Three dimensional structure of one of the head domain showing the binding sites for IgE, CD21 and $\alpha_v\beta_5$ integrin. (Taken from 1).

documented in the intestinal epithelial cells (78, 79).

CD23 expression is well known to be regulated by cytokines (80, 81, 85-98) and IgE ligands (99, 100). Th2 cytokines like IL-4 and IL-13 increases CD23 expression at both the mRNA and protein levels (94-98). In the human B cells, IL-13 alone can induce the CD23 expression and no synergistic enhancement is observed with IL-4 (98). In normal human peripheral blood T (PBT) cells, IL-7 treatment increased the intracytoplasmic level of the CD23 (88). In human B lymphocytes, TNF-α had an antagonist effect on the IL-4 induced CD23 production and its inhibitory effect is greater than the effect of interferons (IFN) (89). The cytokines IFN-α and IFN-γ have been shown to both enhance and decrease the expression of CD23 (90-94). While IFN-α enhanced the expression of CD23 in U937 cells (92), IFN-α had an opposite effect of suppressing the CD23 expression on lymphocytes in Peyer's patches (93). Likewise, IFN-γ suppressed IL-4 mediated enhancement of CD23 expression in human B cells and monocytes, whereas IFN-γ up-regulated the expression of CD23 in IL-4 treated THP-1 cells and in human tonsillar mononuclear cells (90, 91, 94). Furthermore, IFN-γ treatment alone had either increased the expression of CD23 in the U937 cells or suppressed the expression of CD23 in T lymphocytes (92, 95). It remains unclear how these complicated regulations occur at molecular level. In human monocyte and B cell line, IgE and anti-CD23 mAb increases the surface expression and mRNA of CD23 in a T cell line (99). In rodent B cells, IgE increases CD23 expression on the cell surface by preventing its degradation but does not affect its synthesis (100). In murine follicular dendritic cells, stimulation of CD40 increases mRNA levels of both CD23a and CD23b. Addition of IFN-γ enhances the effect of CD40 stimulation on CD23 expression. Interestingly, IL-4

treatment enhances only CD23a mRNA expression in follicular dendritic cells (81).

Soluble CD23 (sCD23)

Full-length CD23 can be cleaved either extacellularly or intracellularly at different locations of the stalk region, consequently generating various sizes of soluble CD23. The sizes of these sCD23 are 37 and 28 kDa (62,77,101) in humans and 37, 33, 25, and 12 kDa in mouse, respectively (77, 102). Importantly, sCD23 retains the globular lectin head domain thereby retaining its ability to bind IgE. However, sCD23 interacts with IgE with lower affinity ($Ka \approx 10^5$ to 10^6 M^{-1}) compared to the trimeric form of CD23 (102). It was found that CD23 expressed on the surface of RPMI 8866 B cells was cleaved by metalloprotease using various metalloprotease inhibitors (103). Later, a disintegrin and metalloproteinase 10 (ADAM10) was found to be responsible for CD23 cleavage and the releasing of soluble CD23 (104-107). In vivo, ADAM10 is also found responsible for cleaving the CD23 by using selective ADAM inhibitor and ADAM knockout mice (104). Mathews et al. in an elegnant experiment described that CD23 in B cells is also cleaved intracellularly within the endosome by ADAM10. This action occurs in a pH dependent manner and the resultant products are further sorted into the exosomes (107). Further experiments are needed to show the physiological relevance of this cleavage. The house dust mite *Dermatophagoides pteronyssinus* protease der p1 also cleaves the CD23 into a soluble 17 kDa fragment (108-110).

Binding partners for CD23

CD23 has ligands such as IgE, CD21, CD11b/c, CD47-vitronectin, and mannose-

containing proteins (64, 111-116). Recently, through NMR studies, CD23 was found to interact with both IgE and CD21 simultaneously and the binding residues for IgE and CD21 are distinct. More importantly, this kind of interaction has a functional effect on the IgE synthesis and regulation (64). CD21 was found as a ligand for human CD23 by using fluorescent liposomes containing full-length CD23 as a probe and it binds to CD21 on B cells and follicular dendritic cells. This interaction was specifically blocked by either anti-CD23 or anti-CD21 mAb (111). Later the specific site of interaction between CD23 on CD21 is mapped, with, extracytoplasmic short consensus repeats (SCR) 5 to 8 and 1 to 2 on CD21 interacting with human CD23 (112). In addition, CD23 was also found to specifically interact with CD11/b/CD18 and CD11/c/CD18 on human monocytes. This type of interaction was specifically inhibited by mAb directed against CD11/b, CD11/c or CD23. This interaction resulted in significant increase of nitric oxide, hydrogen peroxide and proinflammatory cytokines like IL-lβ, IL-8, and TNF-α (113). Similarly, murine CD23 also specifically interacts with CD11b, the α chain of β_2 integrin adhesion molecule CD11b/CD18 complex expressed on monocytes, which results in up-regulation of IL-6 (114). In human monocytes, sCD23 additionally interacts with vitronectin receptor ($\alpha_v\beta_3$ integrin) and this interaction is further enhanced in the presence of vitronectin receptor associated molecule CD47. The mAbs directed against CD47, β_3 and vitronectin blocked the release of sCD23-mediated proinflammatory cytokines like TNF-α, IL-12, and IFN-γ (115). Human IgE interacts only with human CD23 and does not bind to rodent IgE. The residue Lysine 352 in human IgE is critical for interaction with CD23, its replacement with glycine or glutamate results in a significant reduction in binding to human CD23 (116).

Functions of CD23 and its soluble form, sCD23

CD23 has multiple functions: enhancing IgE-specific antigen processing and presentation in the form of IgE/antigen complexes (117-124), regulating IgE production (63, 64, 125-128), and influencing differentiation, survival and growth of both B- and T-cells and myeloid precursors (51, 130-135). Both human and mouse intestinal epithelial cells expressing CD23 can transport IgE and IgE-antigen immune complexes across the intestinal epithelial barrier (140-147). CD23 expressed on mouse B cells allows antigens more efficiently processed in the form of IgE-immune complexes and presented to T cells when compared to the antigen alone. This enhancement of antigen presentation can be blocked by a mAb against CD23 (117). Human B lymphocytes expressing CD23 also demonstrate an enhanced antigen presentation to the T cells in an IgE dependent manner (118). Furthermore, both *in vitro* and *in vivo* assays demonstrate that covalently coupled antigen-anti-CD23 conjugates targeted to CD23 enhances antigen presentation to T cells, consequently increases the production of antigen specific antibody (119). Similarily, CD23 effectively enhances presentation of the major birch pollen allergen, Bet v 1, to Bet v 1-specific CD4+ T cells and results in increased production of allergen specific IgE antibody. Specific allergen vaccination is successful by controlling CD23 mediated IgE dependent antigen presentation to T cells (120,121).

In CD23 knockout mice, IgE-mediated antigen presentation and enhancement of antibody responses is blocked in vivo. This experiment defintely demonstrated the role of CD23 in IgE-mediated antigen processing and presentation (122). However, enhanced CD23 expression in CD23 transgenic suppresses the IgE immune response and it is

possible that CD23 expressed on the nonlymphoid cells like follicular dendritic cells may contribute to the suppression of IgE responses (123,124).

CD23 has been shown to be involved in IgE regulation and synthesis (63, 64, 125-129), but definite evidence still lacks. Yu et al. shows that thymus dependent antigen immunization produced sustained immune response with increased antigen specific IgE in CD23 knockout mice, suggesting that CD23 may act as negative feedback for IgE regulation (125). On the other hand, CD23 over-expression blocks the IgE production both in vivo and in vitro assays (126). Blocking antibody against CD23 also suppresses the production of IgE in vitro and in vivo (127,128). Hibbert et al. proposed a model for IgE regulation based on NMR studies of interaction between derCD23/IgE and CD21 as shown in the Figure 1.5. Cross-linking of membrane-expressed CD23 and IgE by allergen-IgE leads to the down-regulation of IgE synthesis whereas, cross-linking of membrane expressed IgE and CD21 by trimeric soluble CD23 causes the up-regulation of IgE synthesis (64). In human B cells, soluble CD23 monomers inhibits the IgE synthesis whereas, oligomers of soluble CD23 enhanced the IgE synthesis (63). In murine B cells, CD23 also regulates the serum IgE levels (129).

CD23 is shown to be involved in the differentiation of the B cells and prevents them from apoptosis (51, 130- 133). Swendeman et al. showed the sCD23 acts as an autocrine B cell growth factor for EBV-transformed B cells and normal stimulated B cells (131). Later, it was shown that sCD23 prevents the apoptosis of human pre-B cell line and recombinant 25kDa sCD23 and IL-1α synergistically promoted the survival of

germinal center B cells (132,133) and the maturation and proliferation of early human thymocytes and myeloid precursors (134,135). sCD23 also acts as a proinflammatory cytokine and in combination with IL-2 functions on human peripheral blood mononuclear cells (PBMC) to release IFN-γ, TNF-α, IL-1α, IL-1β and IL-6 (136). Soluble CD23 was also responsible for inducing IL-8, macrophage inflammatory protein (MIP)-1α, and MIP-1β from primary human monocytes (137).

CD23 expression in the intestinal epithelial cells

Ramaswamy et al. first showed the evidence of IL-4 dependent IgE uptake and transport across the rat intestine in a parasitic infection model (138). Two mAb EBVCS1 and EBVCS2 specifically detected CD23 in human intestinal epithelial cells and its expression level was enhanced in enteropathies (139). Yang et al. showed that CD23 expressed in intestinal epithelial cells of allergic rats were responsible for the enhanced transepithelial HRP transport (140). Using IL-4 and CD23 knockout mice, Yu et al. verified that CD23 expressed in the intestinal epithelial cells of the allergic mice is responsible for transport of HRP antigen across the epithelial barrier,which was regulated by IL-4 exposure. CD23b and novel CD23b lacking exon 5 or 6 (CD23bΔ5/6) has been detected in the intestinal epithelial cells of the allergic mice. Interestingly, CD23b isoform is able to transport IgE/allergen complexes whereas CD23bΔ5 only transports free IgE antibody from apical to basolateral direction (141-143). Human intestinal epithelial cells express CD23 and it is capable of transporting human IgE across the intestinal epithelium. In addition, human CD23a and CD23b were also expressed in human intestinal epithelial cells and capable of transporting IgE and IgE-antigen

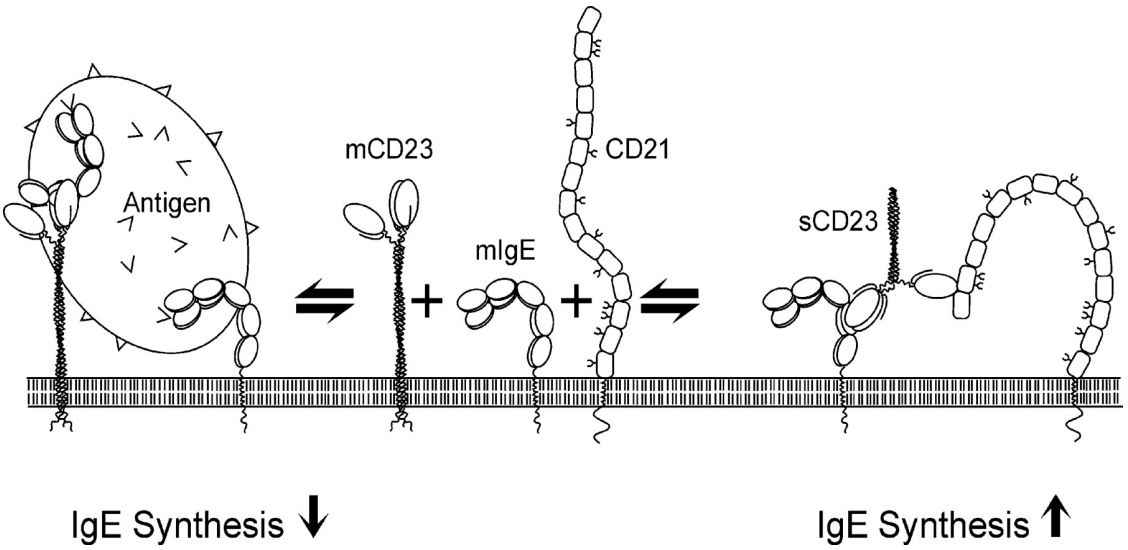


Figure 1.5 Model of IgE regulation by CD23. Cross-linking of membrane expressed CD23 and IgE by allergen-IgE immune complex down-regulate the IgE synthesis and cross-linking of membrane expressed IgE and CD21 by trimeric sCD23 enhance the IgE synthesis (Taken from 64).

complexes as illustrated in the Figure 1.6 (1, 144,145). Interestingly, CD23 expressed by human intestinal epithelial cells can be regulated by p38 MAPK pathway (146). IgE immune complexes engaged to human CD23 in the intestinal epithelial cells causes the release of IL-8 and CCL20 (147).

TRANSCYTOSIS AND RESPIRATORY EPITHELIAL CELL BIOLOGY

Transcytosis

The epithelial monolayer lining the respiratory tract is impervious to macromolecule diffusion in the absence of inflammation due to the presence of intercellular tight junctions at the apical poles. The tight junctions divide polarized epithelial cells into the apical and the basolateral domains (148, 149). These domains further form mucosal barriers that allow for the selective exchange of macromolecules between the lumen and submucosal tissues. Therefore, soluble macromolecules, like immunoglobulins, transported across the mucosal epithelium are mainly mediated by a transcellular transport pathway or transcytosis. Specifically, the transcellular pathway involves the endocytic uptake of macromolecules, generally by receptor-mediatedand/or fluid-phase endocytosis (148, 150), at the apical or basolateral membrane. The molecules are then transported through the cell in endocytic vesicles to the opposite membrane surface, where they are released into the extracellular space.

ALLERGIC DISEASES IN THE RESPIRATORY TRACT:

The bronchial epithelium has multifunctional roles (151,152). For many years, epithelial cells are considered to have the relatively simple roles, being a physical barrier,

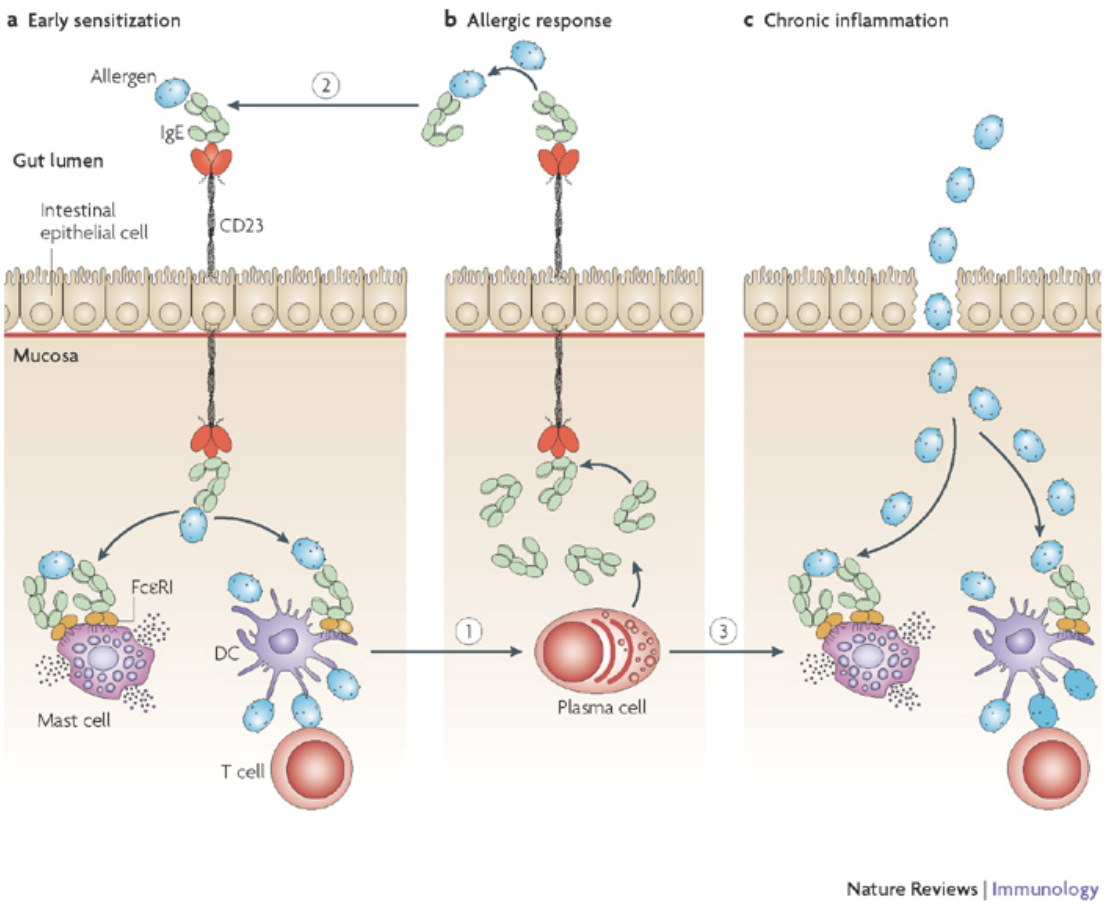


Figure 1.6 Role of CD23 in food allergy. CD23 expressed in the intestinal epithelium binds to IgE in the lamina propria and transport and release into the gut lumen. IgE binds to the allergen and form immune complexes in the luminal side. IgE/allergen immune complexes are transported back by CD23 and release into the lamina propria. FcεRI expressed on mast cells or dendritic cells binds IgE immune complex and cause release of allergic mediators and local IgE synthesis and secretion. In the chronic stage, intestinal luminal barrier is damaged and the allergen can freely move and binds to mast cells and dendritic cells causing severe allergic manifestations (Taken from reference 1).

secreting mucus, and removing noxious agents by cilia between the internal and external milieu of the lungs. More recently, these cells are shown to have a much broad activities in communicating with cells of the immune systems, as shown by their ability to produce inflammatory mediators and variety of immune molecules (153-155). In particular, epithelial cells increase CD23 expression in patients during allergic reactions (156-164). CD23 expression has been reported in human bronchial epithelia cells (165), and however, its function remains to be defined.

Asthma

Asthma is a chronic lung disease that inflames and narrows the airways. The exact cause of asthma is not known. A combination of factors, such as family genetics, respiratory infections, and certain environmental exposures interact to cause asthma to develop, most often in the early life. Ongoing airway inflammation results in abnormal structural changes in the airway during to the epithelial desquamation/denudation and repair processes (166). Bronchial inflammation is characterized by infiltration of mast cells, basophils, eosinophils, monocytes, and Th2 lymphocytes (166-168). Th2 lymphocytes play a central role in the pathogenesis of allergic bronchial asthma, since each of their characteristic cytokines such as IL-4, IL-5, IL-9 and IL-13 contributes to progression of this disease (167,169), including airway eosinophilia, intermittent airway obstruction with increased mucus production, and elevated total serum IgE levels by production of allergen-specific IgE, and development of airway hyper-responsiveness.

CD23 knockout mice have been used to study the development of airway allergy

against chicken ovalbumin (OVA) as an allergen. After sensitization and challenge with OVA, CD23KO mice developed enhanced allergic airway inflammation with increased airway hyperresponsiveness (AHR) when compared to the nonsensitized mice (170, 171). Treatment with anti-CD23 mAb but not the anti-CD23 Fab fragment attenuated the airway inflammation and AHR, and demonstrated that CD23 negatively regulates the airway inflammation. (172). Similarly, after sensitization and challenge with OVA, CD23-KO mice produced increased eosinophils in the bronchoalveolar lavage fluid, enhanced airway inflammation, and bronchial hyperresponsiveness was observed compared to the wild-type mouse control. (173). Sensitization and challenge with OVA in CD23 transgenic mice also developed airway inflammation and hyperresponsiveness and they showed a negative correlation between AHR and eosinophilia with level of CD23 expression in the spleenic T and B cells (171).

Immunoglobulins in mucosal secretions

Immunoglobulins (Igs) have been found in a variety of mucosal secretions and play first line of defense against mucosally-transmitted pathogens**.** The major Ig found in the mucosal surface of the intestine or upper airway is secretory IgA (sIgA). The presence of secretory IgA at the mucosal surfaces is mainly a result of active transport by polymeric IgA receptors (pIgR) through the epithelium (174-176). The IgG is also detected in human and animal mucosal secretions and neonatal Fc receptor for IgG (FcRn) is responsible for IgG transport (177-179).

In addition, IgE is consistently present in nasal washings and bronchoalveolar

lavage (BAL) fluids from human patients with allergic rhinitis and bronchial asthma (180-182) and is detected in nasal washes from allergic patients receiving nasal challenge with specific allergens (180, 182, 183). The functional significance of IgE in airway secretions and mechanism by which IgE is transported to airway secretions are not well understood. IgE synthesis and heavy chain class switching to IgE occur in B cells from the nasal mucosa of allergic individuals (184). Such allergen-specific plasma cells rest in lymphatic organs and can secrete large amounts of IgE antibodies over several years and in the complete absence of allergen (185).

Microbial infection and asthma

Several respiratory viruses and bacteria have been shown to cause exacerbations of asthma (186-191). Virus-induced wheezing in infancy is a risk factor for asthma, and recent studies have highlighted the role of viruses in causing acute illnesses and being a possible contributing factor to chronic asthma. For example, human rhinoviruses (HRVs) is considered as the most common cause of the common cold in infants and children (192- 194). Atypical bacterial infections from *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* have also been linked to chronic asthma and potential asthma exacerbations. The respiratory syncytial virus (RSV) and influenza virus appears to promote specific responses that, on their own, can cause severe pulmonary problems (195-199). However, the pathogenic microbiologic mechanisms involved in this link with asthma have not been well characterized.

Immunotherpy in allergic inflammation

Asthma is a chronic inflammatory disorder of the airways and Th2 inflammation is a key feature of asthma. Immunotherpeutic approaches are proposed and target Th2-type effector cytokines, Th2 cell recruitment and development (1). Alternative strategies use the immunomodulatory potential of tolerance-inducing cytokines such as IL-10 or of cytokines such as IL-12, IL-18 and IFN-γ that are able to induce a counter-balancing Th1 immune response. These are likely to be due to an allergen-blocking effect at the mast cell level and/or at the level of the antigen-presenting cell that prevents IgE-facilitated activation of T cells. In addition, blocking the effects of IgE or CD23 has also been shown to be an effective strategy in the treatment of allergic asthma (1). Therapeutic effect of anti-IgE treatment for allergic disease is based on the ability of anti-IgE antibodies to form immune complexes with free IgE, reducing the degranulation of mast cells and basophils. For example, Omalizumab is an effective humanized anti-IgE mAb showing promising results in controlling allergic inflammation (200). In addition, immunotherapy targeting CD23 molecule with Lumiliximab (IDEC-152) a primatized human anti-CD23 antibody with primate variable region and human constant region is safe and has potential therapeutic effect in the Phase I clinical trial for controlling allergic diseases (201).

SPECIFIC AIMS

Epithelial cells lining the airway, in addition to function as a barrier, are likely to play a more critical role in the initiation of airway allergic inflammation, as shown by increased release of inflammatory mediators and expression of a variety of immune molecules. IgE is present in airway secretions and its level can be enhanced in human

patients with allergic rhinitis and bronchial asthma. For many years, the presence of IgE in airway secretions has been simply considered as IgE transuded passively from the serum. The complete paucity of mechanistic studies IgE transport in airway has seriously hampered the understanding of allergic inflammation in the lung. CD23, an IgE receptor, is shown to regulate IgE synthesis in B lymphocytes and transport IgE across the polarized intestinal epithelial cells during food allergies and infections. Here, we reason that CD23 may also be expressed in the airway epithelial cells regulating IgE transport. Therefore **Specific Aim 1 is to define the role of CD23 expression and CD23-mediated IgE transport in the respiratory tract.**

Similarly, IgE and immune complexes were found to be a potent inflammatory mediator in the murine airway tract during asthma development. Therefore, **Specific Aim 2 is to determine the CD23-mediated IgE/allergy transport and the roles of such transport in asthma development in a murine model.**

CD23 can transport IgE across polarized epithelium. To take advantage of this unique transport pathway, we hypothesized that CD23 may deliver CTLA-4, a negative regulator of T cell immune response, fused to IgE Fc fragment across epithelial barrier to serve as immunotherapeutic purpose for asthmatic allergy. Therefore, the **Specific Aim 3 is to determine the feasibility of CD23 to deliver IgE Fc-fused proteins across airway barrier and further the effect of this fusion protein on the development of allergic inflammation in a mouse model.**

CHAPTER 2: CD23 DEPENDENT TRANSCYTOSIS OF IGE AND IMMUNE COMPLEX ACROSS THE POLARIZED HUMAN RESPIRATORY EPITHELIAL CELLS

(Previously published as: Palaniyandi S, Tomei E, Li Z, Conrad DH, Zhu X. CD23-dependent transcytosis of IgE and immune complex across the polarized human respiratory epithelial cells. J Immunol. 2011,186:3484-96).

ABSTRACT

IgE-mediated allergic inflammation occurs when allergens cross-link IgE's on the surface of immune cells, thereby triggering the release of inflammatory mediators as well as enhancing antigen presentations. IgE is frequently present in airway secretions and its level can be enhanced in human patients with allergic rhinitis and bronchial asthma. However, it remains completely unknown how IgE appears in the airway secretions. In this study, we show that CD23 (FcεRII) is constitutively expressed in established or primary human airway epithelial cells and its expression is significantly up-regulated when airway epithelial cells were subjected to IL-4 stimulation. In a transcytosis assay, human IgE or IgE derived immune complex was transported across a polarized Calu-3 monolayer. Exposure of the Calu-3 monolayer to IL-4 stimulation also enhanced the transcytosis of either human IgE or the immune complex. A CD23 specific antibody or soluble CD23 significantly reduced the efficiency of IgE or immune complex transcytosis, suggesting a specific receptor-mediated transport by CD23. Transcytosis of both IgE and the immune complex was further verified in primary human airway epithelial cell monolayers. Furthermore, the transcytosed antigen-IgE complexes were competent in inducing degranulation of the cultured human mast cells. Since airway epithelial cells are the first cell layer to come into contact with inhaled allergens, our study implies CD23-mediated IgE transcytosis in human airway epithelial cells may play

a critical role in initiating and contributing to the perpetuation of airway allergic inflammation.

INTRODUCTION

Allergic diseases are the most common type of all immunologically mediated disorders affecting 20–30% of the U.S. population and are increasing in incidence in the developed world (202). Airway allergy is one type of allergic disorder that affects both adults and children. Typically, the allergic inflammation in the airway is characterized by a prominent increase in the numbers of immune cells, such as eosinophils, and an imbalance between the Th1 and Th2 cells mediated immune response with a shift towards Th2 cytokine release, such as IL-4, IL-5, and IL-13 and the considerable increase of allergen-specific IgE (1, 180, 203). IL-4 and IL-13 can further promote B cell class switch to increase IgE production (1, 204, 205). IgE is considered a major player in airway allergic inflammation. Clinical studies have found a close association between asthma and serum IgE levels and the amount of allergen specific IgE can be dramatically increased in sensitized or atopy patients (206, 207). Allergen-specific IgE is located in the human airway, such as in nasal mucosal from allergic patients who suffer allergic rhinitis in sinonasal tissue, nasal polyposis, and allergic fungal rhinosinusitis (181, 208-210). This observation is further supported by the fact that B cells from the nasal mucosa of allergic individuals locally synthesize IgE (184, 211). As a result, allergen-specific IgE consistently appears in airway fluids in asthmatic patients obtained with or without bronchial provocation with the allergen. The proposed role of IgE in the initiation of the asthmatic allergy is that the allergy is triggered by antigens cross-linking IgE bound to IgE receptors on the surface of mast cells. This, in turn, triggers degranulation of mast

cells which releases potent inflammatory mediators (1, 202). In the airway mucosa, these inflammatory mediators, including histamine, lipid mediators, proteases, etc, can act on nearby cells, such as epithelial cells and smooth muscle cells and further cause airway obstruction. However, in order for inhaled allergens to gain access to immune effector cells in the lamina propria, they must first cross the respiratory epithelium containing ciliated columnar, mucus-secreting goblet and surfactant secreting Clara cells. These cells can form a highly regulated and impermeable barrier made possible through the formation of tight junctions localized in the apical part of the columnar cells. In general, tight junctions prevent the free uptake and passage of macromolecules such as IgE and allergens. Hence, the exact mechanisms responsible for the cross-talk between allergen/IgE and immune effector cells in the airway remains poorly understood.

Two receptors for IgE, high affinity receptor FcεRI and low affinity receptor FcεRII (CD23), play important roles in the pathogenesis of airway allergic inflammation. FcεRI present in airway mast cells is sensitized by the allergen-specific IgE upon exposure to a specific allergen. FcεRI is activated to signal for the production of potent mediators that are responsible for clinical symptoms in allergic diseases (17). In humans, CD23 has two isoforms-CD23a and CD23b. The amino acid sequences of the CD23a and CD23b proteins differ only in their 6/7 N-terminal residues, a region that corresponds to the cytoplasmic domains (76, 130), suggesting that this region regulates divergent intracellular trafficking and/or signaling pathways. Unlike FcεRI, both CD23 isoforms exhibit a type II membrane glycoprotein structure with a carboxyl terminal C-type lectin head that binds IgE in a calcium-independent manner (18). Although CD23 monomers display lower-affinity binding for IgE, the membrane-bound CD23 can also form trimers,

which enables CD23 to bind IgE with higher-affinity (1, 61, 130). More interestingly, the stalk region of CD23 is susceptible to proteolysis by enzymes, such as the metalloproteinase ADAM10, to release various soluble CD23 fragments (sCD23) (104-106). Soluble CD23 also has IgE binding activity (212) and its level is increased in allergies (157). CD23 is constitutively and inducibly expressed in variety of cell types, including B cells, eosinophils, monocytes and Langerhans cells (76, 77, 82, 164). More interestingly, CD23 has various functions, such as mediating B cell growth, enhancing IgE mediated antigen presentation (122, 213), and regulating IgE homeostasis (63, 64, 125, 127).

The functional significance of IgE found in the mucosal secretions of the human airway remains elusive. Several elegant studies have demonstrated a potential function for CD23 to transport IgE and IgE-derived immune complex across the polarized human intestinal epithelial monolayer (143-145). Furthermore, the facilitated transport of IgE and uptake of antigens by CD23 are essential steps in the initiation of rapid allergic inflammation in the intestine in a murine model (140, 141). All these experiments suggest a role of CD23-mediated IgE transport in food allergy. However, it remains completely unknown if a similar mechanism of transport of IgE or immune complex exists in human airway epithelium. If such a function exists, allergen-specific IgE in the airway may capture airborne allergens and form immune complexes in human airway tract. Subsequently, because of the especially sensitive environment of the airway tract, these immune complexes may function as more potent inducers or triggers of immune responses in the airway and thus, contribute to the perpetuation of airway inflammatory responses. As the first step, we found human CD23 was constitutively expressed in a

variety of epithelial cells derived from human airway. Most importantly, CD23 was capable of transporting human IgE or IgE-derived immune complex across polarized human airway epithelial monolayers. By mimicking the allergic conditions, we also demonstrated that epithelial cell exposure with IL-4 resulted in the up-regulation of CD23 expression accompanied by enhanced transepithelial transport of IgE and, more importantly, of IgE-derived immune complexes. These transported immune complexes were then capable of inducing the degranulation of human mast cells. Our findings may provide important evidences that CD23 in human airway epithelial cell is very likely to have a pivotal role in the initiation and development of airway allergic inflammation.

MATERIALS AND METHODS

Antibodies, cells and reagents.

The human airway epithelial cell lines A549 and Calu-3, intestinal epithelial cell line HT-29, and monocytic cell line U937 were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia). The human nasal epithelial cell Detroit 562 was a gift from Dr. Jing-Ren Zhang (Albany Medical School, NY); human bronchial epithelial cell line 16HBE14o- was from Dr. Dieter Gruenert (University of California, SF). Human B cell line RPMI8866 was from Dr. Lance Bridges (University of Central Arkansas, AK). T84 cells stably expressing human CD23b was a gift from Dr. Cecilia Berin (Mount Sinai School of Medicine, NY). The cells were cultured in DMEM complete medium containing 10% FBS, 1% L-glutamine, non essential amino acids, 100U/ml penicillin and 100 μg/ml streptomycin. U937 and RPMI 8866 cell line were grown using RPMI 1640 complete medium. Human mast cell LAD2 was kindly provided

by Drs. Arnold Kirshenbaum and Dean Metcalfe (National Institutes of Health, Bethesda, MD) and grew in serum free growth medium Stem Pro-34 containing 100 ng/ml of stem cell factor. EpiAirway (AIR-100) tissue model containing normal human tracheal bronchial epithelial cells were obtained from MatTek Corporation (Ashland, MA). The cells were cultured in AIR-100 medium according to the manufacturer's instruction. All cells were grown in a humidified atmosphere of 5% CO_2 at 37ºC.

NP-BSA, 4-hydroxy-3-nitrophenylacetyl (NP) hapten conjugated to bovine serum albumin (BSA), was purchased from Biosearch Technologies (Novato, CA). The anti-human CD23 Ab-secreting murine hybridoma EBVCS1 (67, 214) was a kind gift from Dr. Bill Sugden (University of Wisconsin-Madison, WI). Human IgE was purchased from Scripps (LaJolla, CA), human IgE containing kappa light chains was from Abbiotec (San Diego, CA). Goat anti human IgE and mouse IgG1 were purchased from Sigma Aldrich (St Louis, Mo). Antibody against human IL-4 receptor, chimeric anti-NP IgE, chimeric anti NP-IgG, and mouse anti-human CD23 Ab clone Tu1 were obtained from AbD Serotec Inc (Raleigh, NC). JW8 cells secreting the NP-specific human IgE were obtained from Dr. Hannah Gould (Kings College, London). Mouse anti human IgE, mouse anti-human tryptase specific antibody was from Abcam (Cambridge, MA). Recombinant IL-4 and soluble CD23 were purchased from R & D Systems (Minneapolis, MN). Proteinase inhibitor cocktail was obtained from Calbiochem (San Diego, CA). The CD23bHA plasmid was received from Dr. Zena Werb (Univeristy of California-San Francisco, CA). Antibody IDEC-152 was a gift from Dr. Marilyn Kehry (Biogen-Idec, San Diego, CA).

RT-PCR.

Semi-quantitative RT-PCR was performed as previously described (215). In brief, total RNA was isolated from stimulated human IL-4 (20 ng/ml) and mock-stimulated cells (2×10^6/ml) in TRIzol reagents (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed using a one-step RT-PCR kit (Qiagen). Negative control was set without RNA template. Primers for amplification of CD23a and CD23b (145) and gylceraldehyde-3-phosphate dehydrogenase (GAPDH) (215) have been previously described. For the amplification of CD23 mRNA, 150 ng of total RNA was used. For IL-4 stimulation, 5 ng total RNA from U937 and 40 ng total RNA from A549 or Calu-3 was used for amplification. Thirty-six cycles of PCR amplification were performed in a 20-µl volume. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 20 s. An additional 10 min was applied for the final extension. PCR products were resolved on 1.5 % agarose gels and visualized by ethidium bromide staining. Integrated density values for CD23 band intensities were analyzed by densitometry using Adobe Photoshop and normalized to the GAPDH values to yield a semi-quantitative assessment.

Gel electrophoresis and Western blotting.

Gel electrophoresis and Western blot were performed as previously described (215). Protein concentrations were determined by the Bradford method. The cell lysates were subjected to 12% SDS-PAGE gel electrophoresis under reducing conditions. Proteins were electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked with 5% skim milk powder in PBS. The membranes were probed with anti-CD23 mAb EBVCS1 for 1 hr or overnight followed by incubation with

HRP-conjugated rabbit anti mouse Ab (Pierce). For detecting human IgE, membranes were probed with HRP conjugated goat anti human IgE Ab. For detecting NP antigen in NP-BSA, membranes were first incubated with chimeric anti-NP IgG followed by HRP conjugated rabbit anti human IgG Fc. All blocking, incubation, and washing were performed in PBST solution (PBS and 0.05% Tween 20). Proteins were visualized by an ECL method (Pierce).

Flow cytometry.

Surface and intracellular expression of human CD23 was examined in either fixed or permeabilized A549 or RPMI 8866 cells by flow cytometry. For staining, 1 X 10^6 cells were washed with FACS washing buffer (2% FBS in PBS) and followed by blocking with PBS containing 3% normal goat sera (Rockland, Gilbertsville, PA)/PBS on ice for 20 min. For surface CD23 staining, the cell suspensions were incubated with anti CD23 EBVCS1 or isotype-matched mouse IgG1 (1μg/ml) for 30 min, washed and further incubated with Alexa flour 488 conjugated goat anti mouse (1:500) antibody at 4°C. Surface staining was carried out at 4°C to minimize internalization. For intracellular staining, the cells were first permeabilized and fixed with Cytofix/Cytoperm (BD Pharmingen) on ice for 20 min, then washed with washing/permeabilization buffer and blocked with 3% normal goat serum. The cells were further incubated with EBVCS1 antibody (1μg/ml) diluted in washing/permeabilization buffer, washed and incubated with Alexa fluor 488 conjugated goat anti mouse (1:500) antibody. After washing, cells were analyzed using a FACSAria II and the software FlowJo.

Immunocytochemistry.

Immunocytochemistry was performed as previously described (216). The epithelial cells were grown on coverslips to 70-80 % confluence overnight or in transwell inserts to allow polarization. The cells were rinsed in cold PBS and cold-fixed in 4% paraformaldehyde in PBS for 20 min at 4°C. The cells were washed and quenched with 100 mM glycine for 10 min. After washing, the cells were permeabilized for 30 min with 0.1% Triton X -100 and blocked with 3% normal goat serum. Cells were incubated with affinity purified anti-CD23 EBVCS1 Ab (1μg/ml) in PBST (0.05% Tween 20/PBS) or mouse anti-human IL-4 receptor Ab with 3% normal goat serum in PBS for 1 hr, and further incubated with Alexa flour 488 conjugated goat anti mouse antibody (1:500). The cells grown in the transwell were stained using procedures above with the addition of antibodies on both sides. Finally membrane was cut from the transwell and mounted on the slide and analyzed. Negative control was performed by incubating the isotype-matched normal mouse IgG1 Ab. Cell nuclei were counterstained with DAPI (0.5 g/ml, Molecular Probes) in PBS. After each step, cells were washed with 0.1% Tween-20 in PBS. The ProLongTM antifade kit was used to mount coverslips (Molecular Probes). The images were taken using a Zeiss LSM510 laser scanning confocal microscope (Zeiss Microimaging Inc., NY).

Enzyme-linked immunosorbent assay (ELISA).

The IgE concentration was measured with ELISA. ELISA plates (Nunc) were coated with rabbit anti human IgE Ab (10 μg/ml) overnight at 4ºC. Plates were then washed three times with PBST (0.05% tween- 20) and blocked with 10 % FBS in PBS

for 1 hr at room temperature. The transcytosed samples or IgE standards diluted in 10% FBS in PBS were incubated for 2 hr at room temperature. Mouse anti human IgE (1:10,000, Abcam) and HRP conjugated rabbit anti mouse antibody (1:10,000, Pierce) were used for detection of human IgE. For detection of chimeric IgE, HRP conjugated goat anti-human IgE (1:10,000, Sigma) was used. A colorimetric assay was done with tetra methyl benzidine (TMB) and hydrogen peroxide and a Victor III microplate reader (Perkin Elmer).

Transcytosis assay.

Transcytosis was performed as previously described with modifications (145, 177, 215). Calu-3 cells (1×10^5/ml) were grown onto 0.4 μm-pore size transwell inserts (BD Bioscience) to form a monolayer exhibiting transepithelial electrical resistances (TER, 450-900 Ω/cm^2). TER was measured using a tissue-resistance measurement equipped with planar electrodes (World Precision Instruments, Sarasota, FL). Monolayers were equilibrated in serum free DMEM or stimulated with human IL-4 (20 ng/ml) for 24 hr. The pseudo-stratified normal human tracheal bronchial epithelial tissue (AIR-100) had a TER at least 450 Ω/cm^2 during transcytosis assay. For Western blotting analysis, human IgE or chicken IgY was added either to apical (25μg/250μl) or basolateral (50μg/500μl) side. However, when ELISA is used for detecting transcytosis, the IgE amount loaded was lowered to 5μg/250μl in the apical side or 10μg/500μl in basolateral side, respectively. For IgG transcytosis, human IgG (125μg/250μl) was added to the apical chamber. They were incubated at 37ºC for 2 hr. In addition, protein L beads (Pierce) was used to specifically bind to the transported kappa light chain IgE Ab during

transcytosis. The immune complex or NP-BSA antigen alone (10 μg/ml) was added to apical chamber and incubated at 37ºC for 30, 60, 120 min. The immune complex was formed by incubating chimeric anti NP IgE (20 μg/ml) with NP-BSA (10 μg/ml) at 37ºC for 45 min. For negative control, monolayers were also incubated at 4ºC for 2 hr before transcytosis. The media from the opposite chambers were collected and concentrated using ultracel YM-10 (Millipore, Billerica, MA). Transported proteins were analyzed by reducing SDS-PAGE and Western blot-ECL for detection of NP antigen, IgG, IgE and chicken IgY. IgE was also detected with ELISA. Adobe Photoshop was used to determine relative band intensities of a blot.

Mast cell degranulation assay.

Mast cell degranulation assay was performed as previously described with modification (217). The Calu-3 cells were allowed to get polarized in the transwell insert. Immune complex, antigen alone, and human IgE were respectively added into the apical chamber and transcytosis were performed at 37ºC for 3 hr. Human mast cell line LAD2 was cultured with media in the basolateral chamber and allowed to capture the transported immune complex, antigen or human IgE. In addition, prior to the addition of the transcytosed immune complex, LAD2 cells were also primed with or without 200 ng/ml NP specific IgE or antigen non specific IgE. The degranulation assays were performed in a 96 well plate. Tryptase or β-hexosaminidase was measured in 100 μl supernatants as indicator of mast cell degranulation. As a positive control, immune complexes were directly added to the LAD2 cells for 1 hr. β-hexosaminidase was measured using *p*-nitrophenyl-*N*-acetyl-β-d-glucosaminide (Sigma) dissolved in 0.1

mol/L phosphate buffer (pH 4.5) as substrate for incubation at 37ºC for 1 hr. The reaction was terminated with a 2-M glycine solution (pH 10.7). Tryptase was measured by adding the substrate 1 mM N-Tosylglycyl-L-prolyl-L-lysine 4-nitroanilide dissolved in 50 mM Tris-HCl (pH 7.6), 120mM NaCl and 20 μg/ml heparin solutions and incubated for 1 hr at 37 °C. To quantify the substrate cleavage, the absorbance at 405 nm was measured using a microplate reader. The results were read out at 405 nm. In addition, tryptase in LAD2 mast cells was analyzed by immunofluoresence staining with human tryptase specific antibody in a confocal microscope.

Statistics.

The statistical difference between groups was tested by Student *t* test. A *P* value of less than .05 was considered significant. Data are expressed as mean ± SD.

RESULTS

Human respiratory epithelial cells express CD23.

Epithelial cell lines from human nasal, trachea, and lung were used in studies on human CD23 expression. These cell lines maintain the differentiation characteristics of their tissues of origin with proper polarity. Human CD23 is expressed in two isoforms: CD23a and CD23b (145). Results from RT-PCR amplification showed that all airway epithelial cells expressed the CD23b (Fig. 2.1A), but not the CD23a (Fig. 2.1B), transcript. Mononuclear cell line U937 was used as a positive control. The PCR products were sequenced to verify CD23b isoform specific transcript. To verify CD23 expression at the protein level we blotted the cell lysates from Detroit 562, Calu-3, and A549 cells

with EBVCS1 mAb, a specific IgG1 Ab for human CD23 (67, 214). These airway epithelial cells expressed protein bands identical to 293T and intestinal T84 cells transfected with vectors expressing human CD23b cDNA in a Western blot (Fig. 2.1C, *left panel*). Two bands were identified; however, the top band (50 kDa) was also expressed in cell lysates from Chinese hamster ovary (CHO) cell lysates (Fig. 2.1C, *right panel*), suggesting the lower band (45 kDa) is CD23 specific. In addition, immunofluorescence staining of Calu-3 cells by EBVCS-1 mAb (Fig. 2.1D, *left panel*), but not for isotype-matched IgG1 Ab (Fig. 2.1D, *right panel*), revealed the expression of CD23 protein.

CD23 is known to be expressed intracellularly and/or at the cell surface (218). To show the expression pattern of CD23 in airway epithelial cells, we stained lung epithelial cells A549 with EBVCS1 mAb for cell surface or intracellular expression by flow cytometry (Fig. 2.1E). The specificity and expression pattern of CD23 was, in parallel, performed in a RPMI 8866 human B cell line. In contrast, to approximately 84% human B cell line, only about 36% A549 cells expressed CD23 at cell surface. However, permeabilization of A549 cells revealed substantial increases of CD23 positive cells from approximately 36% to 99%. The distinct cellular distribution of CD23 between airway epithelial and B lymphocytes has not been appreciated in previous study. In addition, activation of human eosinophils leads to increased surface expression of CD23 (218). Our data showed that IL-4 stimulation also increased the surface expression of CD23 in A549 cells (data not shown). Therefore, we conclude that CD23 is mainly expressed intracellularly in lung epithelial cell A549, suggesting CD23 may function as an intracellular IgE receptor in airway epithelial cells.

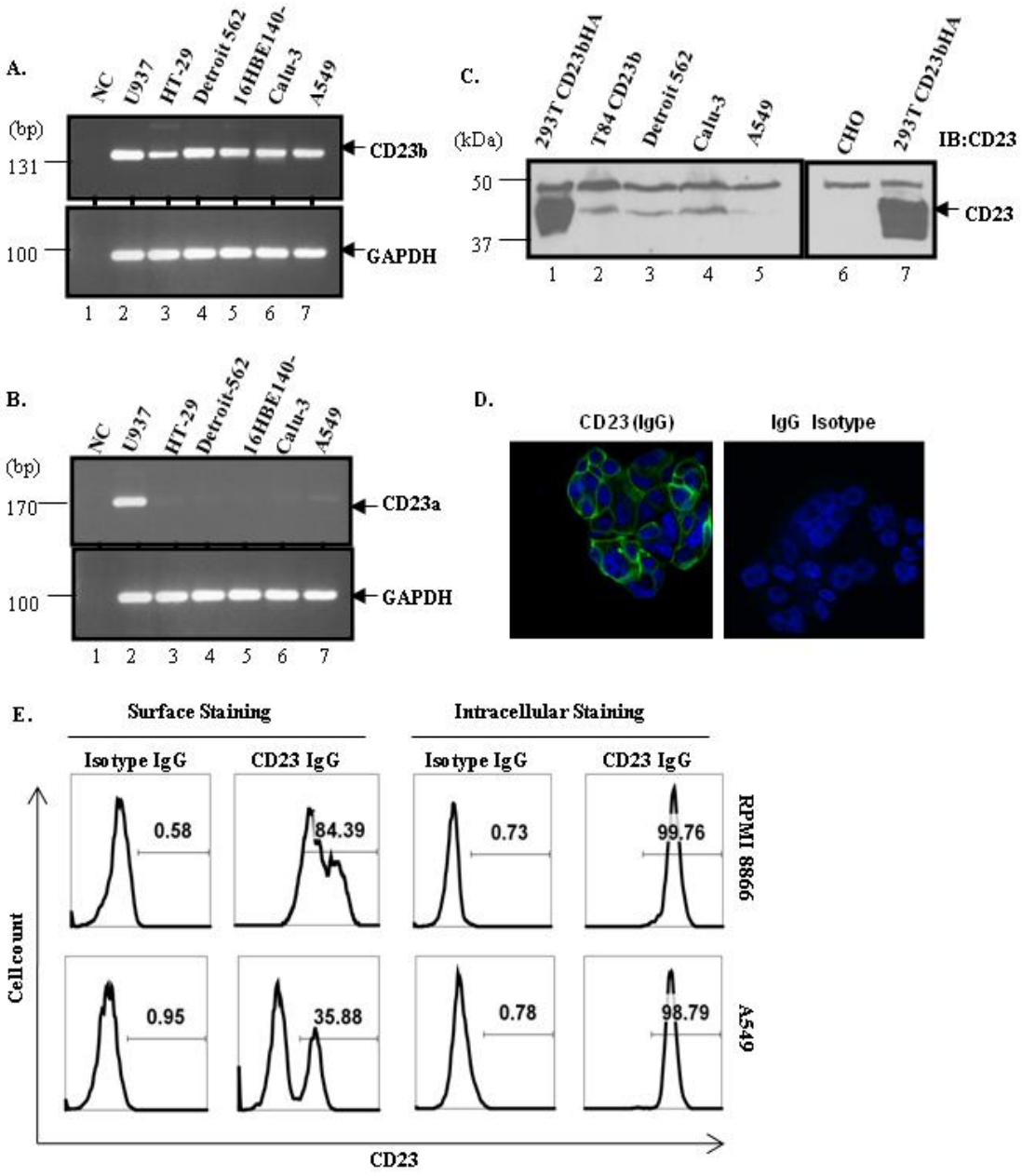

A.
NC
U937
HT-29
Detroit 562
16HBE140-
Calu-3
A549
(bp)
131
100
CD23b
GAPDH
1 2 3 4 5 6 7
B.
NC
U937
HT-29
Detroit-562
16HBE140-
Calu-3
A549
(bp)
170
100
CD23a
GAPDH
1 2 3 4 5 6 7
C.
293T CD23bHA
T84 CD23b
Detroit 562
Calu-3
A549
CHO
293T CD23bHA
(kDa)
50
37
IB:CD23
CD23
1 2 3 4 5 6 7
D.
CD23 (IgG)
IgG Isotype
E.
Surface Staining
Intracellular Staining
Isotype IgG
CD23 IgG
Isotype IgG
CD23 IgG
0.58
84.39
0.73
99.76
RPMI 8866
0.95
35.88
0.78
98.79
A549
Cell count
CD23

Figure 2.1 Human airway epithelial cell lines express CD23.
(**A** + **B**) CD23b, but not CD23a, isoform was expressed in human airway epithelial cells. Total RNA was extracted by TRIzol reagent from airway epithelial cells Detroit 562 (lane 4), 16HBE14o- (lane 5), Calu-3 (lane 6), A549 (lane 7). Monocyte cell line U937 (lane 2) and intestinal epithelial cell HT-29 (lane 3) were used as a positive control. RT-PCR was performed by using CD23a or CD23b isoform specific primers as described in *Materials and Methods*. Amplified PCR products, 170 bp for CD23a and 131 bp for CD23b, were electrophoresed in 1.5% agarose gel and stained with ethidium bromide. NC represents a negative control without template in RT-PCR reaction. Similar PCR products amplified with a GAPDH-specific primer pair was used as internal controls. The arrow indicates the location of the amplification products for CD23 and GAPDH. The molecular weight in base pairs (bp) is indicated on the left.
(**C**) CD23 protein was expressed by human airway epithelial cells. The cell lysates (60 μg) from 293T-CD23bHA (lanes 1 & 7) and T84-CD23b (lane 2), Detroit 562 (lane 3), Calu-3 (lane 4), A549 (lane 5), and CHO (lane 6) were separated by electrophoresis on 12% SDS-PAGE gel under reducing conditions. The proteins were transferred onto nitrocellulose membrane and blotted with mouse anti-human CD23 EBVCS1 mAb. The blots were incubated with HRP-conjugated rabbit anti-mouse IgG and the protein bands were visualized by ECL method. The arrow indicates the location of the CD23.
(**D**). Immunofluorecence staining of Calu-3 cell line. The Calu-3 cells were grown on glass coverslips, fixed with 4% *para*-formaldehyde and permeabilized in 0.1% Triton X-100. Subsequently, the cells were incubated with goat serum for blocking and affinity-purified mouse anti-CD23 EBVCS1 mAb, followed by staining with an alexa flour 488-conjugated goat-anti mouse antibody. The Calu-3 cell was stained with an isotype matched mouse IgG1 Ab as negative control (*right panel*). The nucleus was stained with DAPI and photographed through a fluorescence microscope. Samples were viewed using the same contrast and brightness settings.
(E). Cellular distribution of human CD23 expression patterns in lung epithelial cell and B lymphocytes. Cell surface and intracellular expression patterns of CD23 in either fixed or permeabilized lung epithelial cell A549 and B lymphocyte RPMI 8866 were measured by flow cytometry analysis. Cells were stained as described in the Materials and Methods. Results are expressed as histograms of fluorescence intensity (log scale). The histograms represent staining of cells with anti-CD23 EBVCS1 mAb or isotype-matched mouse IgG1 and then with Alexa fluor 488 conjugated goat anti mouse IgG Ab. Values in each rectangle correspond to the proportion of cells stained with the anti-CD23 Ab relative to the control Ab. The staining for A549 and RPMI 8866 was conducted three times with similar results.

IL-4 stimulation up-regulates the expression of CD23 in human airway epithelial cells.

The Th2 type T cells mediate airway allergic inflammation by producing IL-4 and IL-13. IL-4 and IL-13 can enhance the expression of CD23 in both B cells, monocytes, and intestinal epithelial cells (219, 220). The results showed that when airway epithelial cells A549 and Calu-3 cells were subjected to IL-4 (20 ng/ml) stimulation for 24 hr, CD23b mRNA expression, which was measured by semi-quantitative RT-PCR, was significantly enhanced (Fig 2.2A, lanes 5 & 7). In contrast, CD23a mRNA expression was not induced by 24 hr IL-4 stimulation in A549 and Calu-3 cells (Fig. 2.2B, lanes 5 & 7). As expected, mRNA expression of both CD23a and CD23b isoforms in U937 were significantly enhanced after IL-4 stimulation. Furthermore, as shown in Fig. 2.2C, expression of IL-4 receptor was shown by immunofluorescence staining with IL-4 receptor mAb, but not with isotype matched IgG antibody when Calu-3 cells were grown in transwell inserts and polarized.

CD23 transports human IgE bidirectionally across the polarized Calu-3 epithelial cells.

The human airway epithelial cell, Calu-3, was polarized and used to transcytose IgG bidirectionally when grown on both sides of filter supports (215). Hence, we used Calu-3 as a model cell line to examine transcytosis of human IgE. First, CD23 expression was verified by immunofluorescence staining with EBVCS1 mAb when Calu-3 cells were grown on 0.4 μ pore size inserts (Fig. 2.3A). CD23 staining was mainly on the brush border apical membrane with some staining also evident on the basolateral membrane. To show interaction of CD23 and IgE within the Calu-3 cells, IgE (0.5 μmol/L) was added into the apical chamber of the transwell. As shown in Fig. 2.3B,

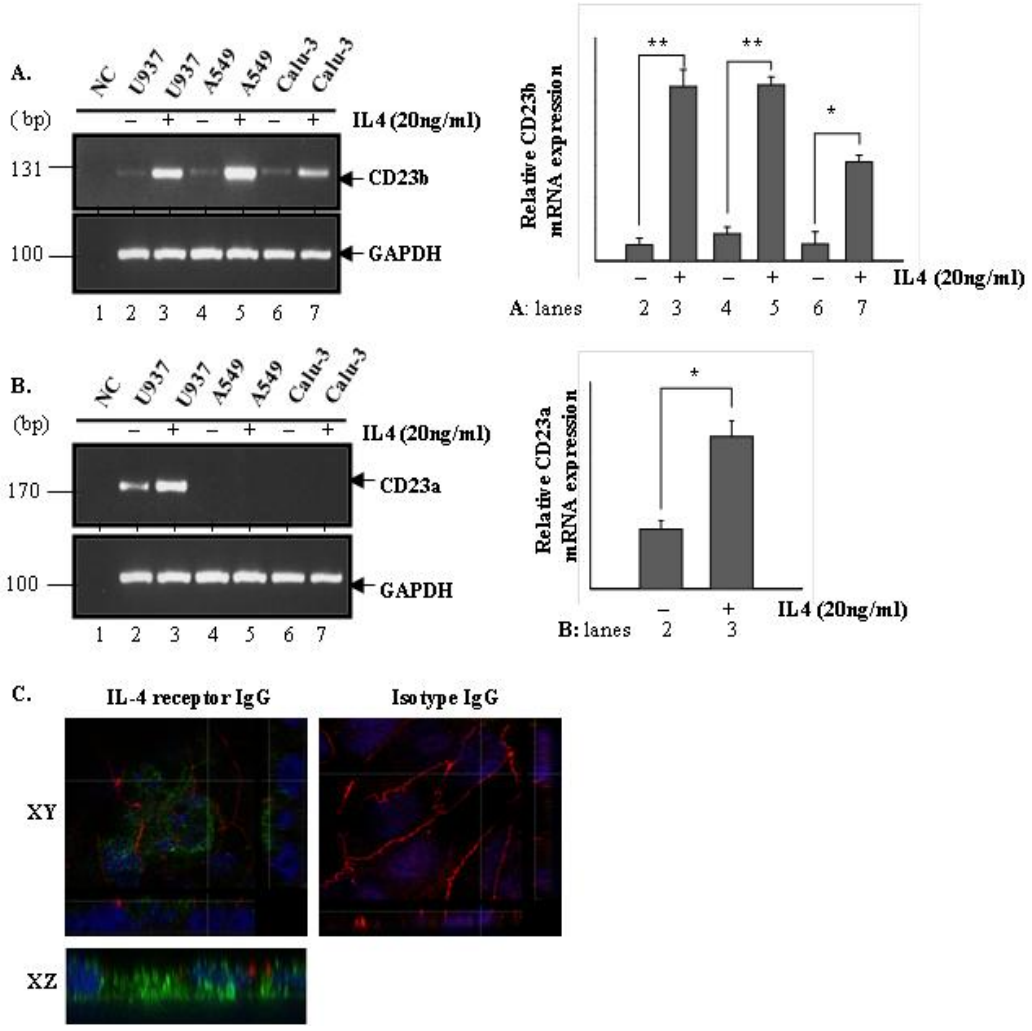


Figure 2.2 CD23 expression was up-regulated in human airway epithelial cells following exposure to IL-4 treatment.

(**A** + **B**). Human airway epithelial cells Calu-3 and A549 were incubated with or without IL-4 (20 ng/ml) for 24 hrs. Monocytic cell line U937 was used as a positive control. At the end of incubation period, the cells were washed and total RNA was extracted by TRIzol reagent. Total RNA was subjected to semi-quantitative one-step RT-PCR with CD23b (A) or CD23a (B) specific primers. GAPDH was used as internal control. PCR products were run in 1.5% agarose gels and stained with ethidium bromide. Densitometry analysis of CD23b (A, *right panel*) or CD23a (B, *right panel*) band intensities which were normalized to GAPDH was presented. NC: a negative control without template RNA in RT-PCR reaction. *P<0.05, **P<0.01.

(**C**). Confocal microscopy detection of IL-4 receptor. Calu-3 cells (1×10^5/ml) were grown on 0.4 μm pore size inserts. Calu-3 cells were polarized, fixed and permeabilized in 0.1% Triton X-100. IL-4 receptor was detected by immunostaining with Alexa flour 488 (green). Cells were stained with ZO-1 (red) to show the level of the tight junction and DAPI to stain nuclei (blue). The XY sections are taken at the level of the ZO-1 staining, and the XZ sections are shown at the bottom. The red staining indicates the tight junctions at the apical pole of the Calu-3 cells.

CD23 (green) and IgE (red) were co-localized (merged yellow). To show whether Clau-3 cell transcytoses IgE, human IgE was added either to apical or basolateral side and further incubated at 37ºC for 2 hr. As a negative control, the Calu-3 cells were also incubated at 4ºC for 2 hr. The results showed that intact IgE applied to either apical (Fig. 2.3C, lane 3) or basolateral (Fig. 2.3C, lane 4) chamber was transported in the opposite directions across the Calu-3 monolayer as assessed by blotting of IgE heavy chain. Transport of IgE was not detected in monolayers incubated at 4°C (Fig. 2.3C, lane 2), ruling out the leaking possibility of IgE Ab across Calu-3 monolayers. Since CD23 was able to transport IgE bidirectionally across the polarized epithelial cells, we also employed protein L beads that can specifically bind to kappa light chain IgE Ab in order to prevent the transcytosed IgE Ab from re-entering the transcytosis. This trapping method increased sensitivity and allowed ELISA to detect 1-1.5 ng IgE Ab that was transcytosed bidirectionally when either 75 ng or 150 ng of IgE was applied in apical or basolateral chamber respectively (Fig. 2.3D). It should be noted that this IgE Ab concentration represented a physiological level of IgE Ab in human sera although the amount of allergen specific IgE can be considerably augmented in sensitized or atopy patients. It remains to be known exactly how CD23 transports IgE Ab across the airway epithelial cell monolayers.

It is possible that expression of FcεRI in airway epithelial cells may result in IgE transcytosis. To exclude this possibility we performed RT-PCR analysis for the expression of the alpha chain of FcεRI. Our results showed that airway epithelial cells failed to express FcεRI (data not shown) at 35 cycles of PCR amplifying condition. To show whether IgE transcytosis is dependent on the expression of CD23 in Calu-3 cells,

two experiments were performed. First, IgE incubation with soluble CD23 (sCD23) significantly inhibited the amount of IgE transcytosed (Fig. 2.3E). Second, either apically or basolaterally-applied CD23 specific Ab IDEC152 (1 μg/ml) significantly inhibited the transcytosis of IgE from the apical to basolateral (Fig. 2.3F) or vice versa (data not shown). IDEC-152 mAb is anti-human CD23 mAb (IgG1) consisting of primate (cynomolgus macaque) variable regions and human constant regions, for this reason, we did not include an isotype-matched IgG1 mAb as a control. IDEC-152 has been shown to block synthesis of IgE from human B cells in vitro (201). The protein concentrations for block were previously determined in a serial dilution. Both experiments demonstrated the specificity of CD23-mediated IgE transport in Calu-3 cells. We further defined the role of endosomal trafficking in CD23-mediated IgE transcytosis. Calu-3 cell inserts were incubated with or without bafilomycin A1 (0.1 μmol), which interferes with the intracellular trafficking by inhibiting endosome acidification as shown in transcytosis of IgE or IgG in intestinal epithelial cells (145, 177). As shown in Fig. 2.3G, bafilomycin significantly inhibited apical to basolateral IgE transcytosis although it was unable to completely block CD23-mediated IgE transcytosis. As an internal control, the bafilomycin completely blocked IgG transcytosis across Calu-3 monolayer, which is in agreement with the previous finding (Fig. 2.3H) (177). Hence, we conclude that IgE entering both apically- and basolaterally-directed transcytotic pathways in lung epithelial cells is dependent on CD23 expression.

CD23 transcytoses IgE-derived immune complexes across polarized Calu-3 cells.

Previous studies have shown that IgE is frequently present in the mucosal secretions of airway tract and its level can be enhanced in human patients with asthma (207, 209). Transferring of the intact allergen molecule across the airway epithelial barrier may be an important event in priming a host for an allergen and subsequent exposure of same allergen leads to the development of allergic inflammation. CD23 mediated IgE transport across the polarized Calu-3 epithelial cells suggests allergen-specific IgE may also capture airborne allergen and form immune complexes in the lumen of airway tract. Therefore, we further examined whether CD23 could facilitate transepithelial transcytosis of IgE-derived immune complex across Calu-3 monolayers. We used chimeric IgE that consists of mouse Fab directed against 4-hydroxy-3-nitrophenylacetyl hapten (NP) molecules and human Fc fragment. Chimeric IgE can well bind to human CD23 as previously demonstrated (145). Immune complex was formed by incubating the chimeric IgE with NP-BSA at 37ºC. Transcytosis of immune complexes across polarized Calu-3 epithelial cells was examined from the apical (lumenal) to basolateral direction to mimic the in vivo shown in Fig. 2.4A, the apically applied IgE-NP-BSA complex was transported after 2 hr in the basolateral direction across Calu-3 monolayer at 37ºC incubation (lane 4), as assessed by blotting of NP antigen. Immune complex incubated at 4ºC (Fig. 2.4A, lane 2) condition where the airway epithelial cells are usually exposed to the inhaled antigens. As or NP-BSA antigen alone (Fig. 2.4A, lane 3) was not transcytosed across polarized Calu-3 cells. The chimeric IgE in the immune complex was also detected at 37ºC, but not at 4ºC, by ELISA (Fig. 2.4B). Furthermore,

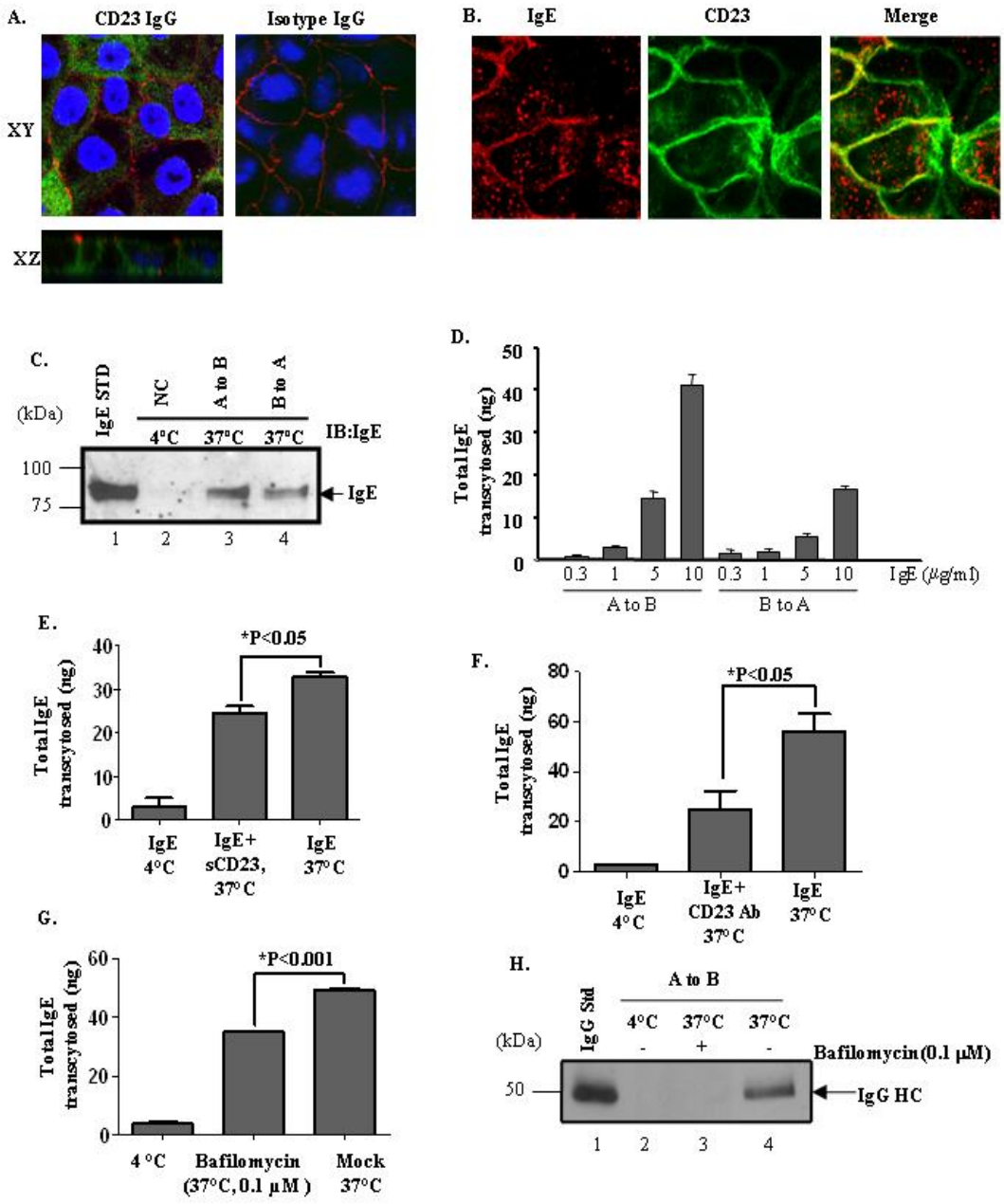

A.
CD23 IgG
Isotype IgG
XY
XZ
B.
IgE
CD23
Merge
C.
IgE STD
NC
A to B
B to A
(kDa)
4°C
37°C
37°C
IB:IgE
100
75
IgE
1
2
3
4
D.
Total IgE transcytosed (ng)
50
40
30
20
10
0
0.3
1
5
10
0.3
1
5
10
IgE (μg/ml)
A to B
B to A
E.
*P<0.05
Total IgE transcytosed (ng)
40
30
20
10
0
IgE 4°C
IgE+ sCD23, 37°C
IgE 37°C
F.
*P<0.05
Total IgE transcytosed (ng)
80
60
40
20
0
IgE 4°C
IgE+ CD23 Ab 37°C
IgE 37°C
G.
*P<0.001
Total IgE transcytosed (ng)
60
40
20
0
4 °C
Bafilomycin (37°C, 0.1 μM)
Mock 37°C
H.
A to B
IgG Std
4°C
37°C
37°C
(kDa)
-
+
-
Bafilomycin (0.1 μM)
50
IgG HC
1
2
3
4

Figure 2.3 CD23 transcytoses human IgE in Calu-3 cells.
(**A**). Immunofluorecence staining of CD23 expression. Cells were grown on transwell insert, fixed and permeabilized in 0.1% Triton X-100. Subsequently, the cells were incubated with mouse anti-CD23 EBVCS1 mAb (green) and ZO-1 (red), followed by staining with Alexa flour 488 or 555 conjugated secondary Ab. The nucleus was stained with DAPI. The XY (top) or XZ (bottom) sections are taken.
(**B**). Colocalization of CD23 and IgE in Calu-3 cell lines. Calu-3 cells were grown on transwell inserts. The cells were allowed to get polarized and the human IgE (0.5 μmol/L) was added to apical chamber and incubated at 37ºC for 30 min. Cells were stained by anti-CD23 EBVCS1 mAb and anti-human IgE Ab, followed by Alexa flour 488 or 555 conjugated IgG. CD23 (green) and IgE (red) were visualized by confocal microscope and yellow indicates the colocalization.
(**C**). Calu-3 cells (1×10^5/ml) were grown on 0.4 μm pore size transwell inserts. The cells were allowed to get polarized and the transcytosis assay was performed when the TER value reached at least 450-900 Ω/cm^2. The human IgE (0.5 μmol/L) was added either to apical or basolateral chamber and incubated at 37ºC for 2 hr. For blocking IgE transcytosis, the Calu-3 cells in individual inserts were incubated at 4ºC for 2 hrs. The IgE was subsequently added and transcytosis assay was performed for additional 2 hrs at 4ºC. The medium from opposite chamber was collected, concentrated and blotted to detect IgE heavy chain. A: apical; B: basolateral.
(D) Effect of IgE concentration on the transcytosis. Human IgE at indicated concentrations were applied to the apical (75-2500 ng/250 μl) or basolateral (150-5000 ng/500 μl) chamber and incubated at 37ºC for 2 hr. Protein L beads were used to specifically bind to the transported IgE Ab during transcytosis. The supernatants from the opposite chamber were collected at the end of 2 hr. The protein L beads were pull down, IgE was eluted and total IgE was measured by ELISA.
(E + F). Effect of soluble CD23 (sCD23) or IDEC152 mAb on IgE transcytosis in Calu-3 cells. Human IgE was preincubated with sCD23 (E) and IDEC152 mAb was added to the transwell at 4ºC for 1 hr (F) before addition of IgE to the transwell at 37ºC. The protein concentrations of either sCD23 or mAb were optimized in a pilot experiment. Transcytosis of human IgE at 4ºC (lane 1) and 37ºC (lane 3) without sCD23 or IDEC152 mAb was used as control. Supernatant from the opposite transwell was sampled 2 hr later. Total IgE was measured by ELISA. Mean ± SD of three experiments is shown.
(G + H). Calu-3 cells were incubated in the presence or absence of bafilomycin A1 (0.1 μM), and basolaterally directed transport of IgE and IgG was measured after 2 hr incubation. Transcytosed total IgE was measured by ELISA; total IgG was measured in a Western blot. Results were representative of three individual experiments. A: apical; B: basolateral.

the immune complexes transcytosed across Calu-3 monolayer were detectable within 30 min after transcytosis initiation (Fig. 2.4C, lane 3). The transcytosis of immune complexes was also decreased when the apically applied immune complexes were serially diluted (Fig. 2.4D, lane 3-4); however, non-linearity was detected between the applied immune complex and the transcytosis efficiency in the assay.

To further show whether this transport is dependent on CD23, two experiments were performed. First, we examined the influence of sCD23 on transcytosis of NP-BSA antigens in the presence of IgE. The incubation of soluble sCD23 with the immune complex before adding it to the Calu-3 monolayer significantly reduced the transcytosis of NP-BSA antigen in a sCD23 protein concentration (10-20 μg/ml) dependent manner (Fig. 2.4E, lane 4 & 5) in comparison with untreated immune complex at 37ºC (Fig. 2.4E, lane 3). This suggests that sCD23 renders the immune complex unavailable for membrane CD23 on the Calu-3 monolayer. Second, we determined that the transcytosis of the immune complex was significantly reduced by CD23-specific IgG Ab IDEC 152 (Fig. 2.4F, lanes 3 & 4). In a parallel experiment, IgE in immune complex was also significantly reduced (Fig. 2.4G).

IL-4 enhances the transcytosis of IgE or immune complex in polarized Calu-3 cells.

IL-4 treatment enhanced CD23b expression in the airway epithelial cells (Fig. 2.2). We further examined whether IL-4 treatment stimulates the transcytosis of either IgE or immune complexes across the Calu-3 epithelial cells. To show this effect, the polarized Calu-3 monolayers were treated with or without IL-4 (20 ng/ml) exposure before either IgE or immune complex was added. As shown in Figure 2.5A and 2.5B, the

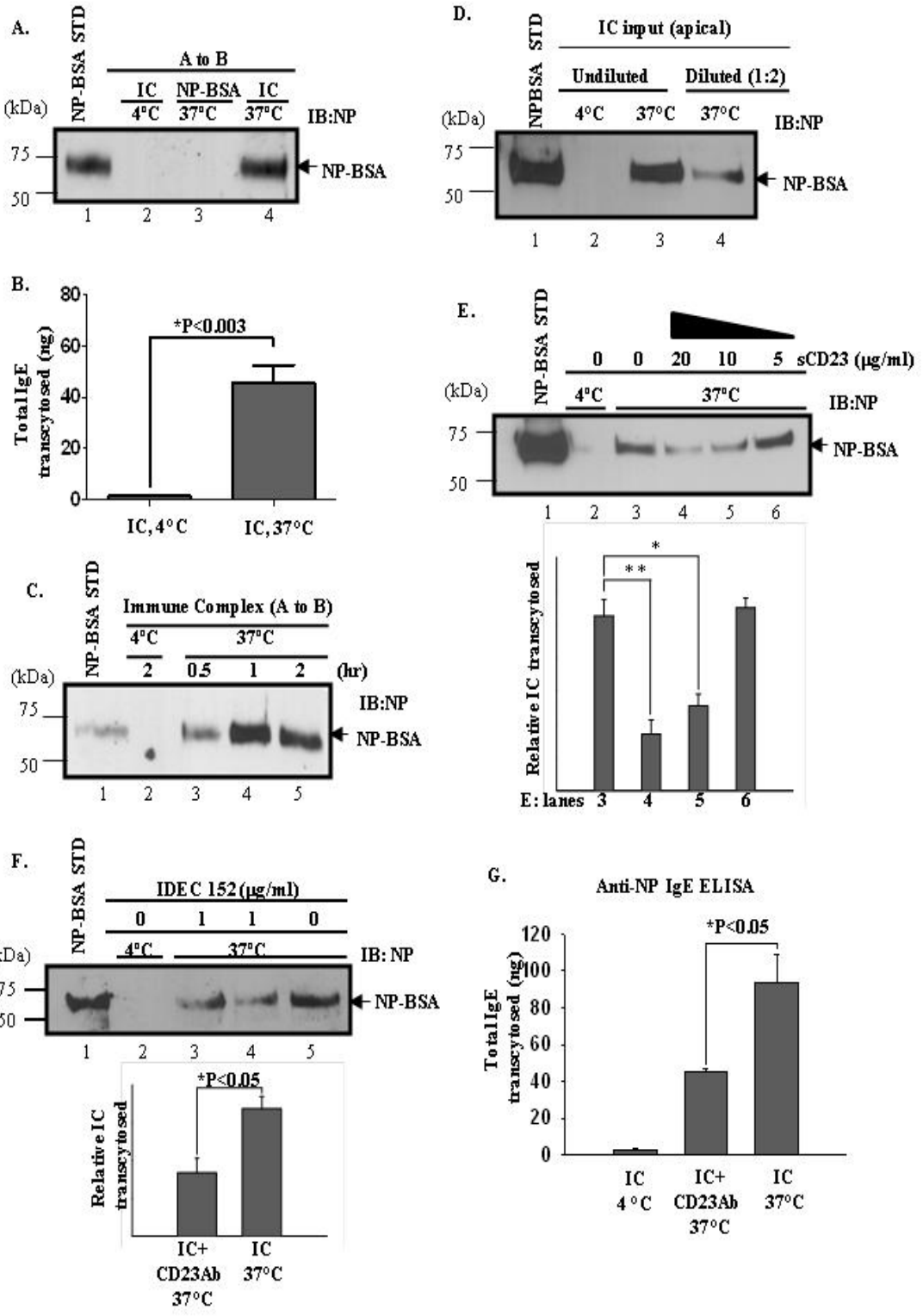

A.
NP-BSA STD
A to B
IC 4°C
NP-BSA 37°C
IC 37°C
(kDa)
IB:NP
75
50
NP-BSA
1 2 3 4
B.
Total IgE transcytosed (ng)
*P<0.003
IC, 4°C
IC, 37°C
C.
NP-BSA STD
Immune Complex (A to B)
4°C
37°C
2
0.5
1
2
(hr)
(kDa)
IB:NP
75
50
NP-BSA
1 2 3 4 5
D.
NPBSA STD
IC input (apical)
Undiluted
Diluted (1:2)
4°C
37°C
37°C
(kDa)
IB:NP
75
50
NP-BSA
1 2 3 4
E.
NP-BSA STD
0 0 20 10 5 sCD23 (μg/ml)
4°C
37°C
(kDa)
IB:NP
75
50
NP-BSA
1 2 3 4 5 6
Relative IC transcytosed
*
**
E: lanes 3 4 5 6
F.
NP-BSA STD
IDEC 152 (μg/ml)
0 1 1 0
4°C
37°C
(kDa)
IB: NP
75
50
NP-BSA
1 2 3 4 5
Relative IC transcytosed
*P<0.05
IC+ CD23Ab 37°C
IC 37°C
G.
Anti-NP IgE ELISA
Total IgE transcytosed (ng)
*P<0.05
IC 4°C
IC+ CD23Ab 37°C
IC 37°C

Figure 2.4 Transepithelial transport of immune complex in Calu-3 cells. Calu-3 cells (1×10^5/ml) were grown on 0.4 μm pore size transwell filters. The cells were allowed to get polarized and the transcytosis was performed when the TER value reached at least 450-900 Ω/cm^2. The immune complex was formed by incubating chimeric anti-NP IgE (20 μg/ml) with NP-BSA (10 μg/ml) at 37ºC for 45 min. A: apical; B: basolateral; STD: standard; IC: immune complex.
(**A+B**). Transcytosis of immune complex (IC) in the Calu-3 cell line. The NP-BSA Ag alone (lane 3) or IgE-NP-BSA immune complex (lanes 2 & 4) was added to the apical chamber for incubation at 4ºC (lane 2) or 37ºC (lanes 3 & 4) for 2 hr. The media from the basolateral chamber were collected and concentrated for detecting NP-BSA Ag in a Western blot (A) or total IgE in an ELISA (B).
(**C+D**). Effect of incubation time or concentration of the immune complex on the transcytosis. Immune complex was added to the apical chamber at different time (C) or serially diluted with normal medium (D), and then incubated at 4ºC (lane 2) or 37ºC (C, lanes 3-5) or 37ºC (D, lanes 3-4) to allow transcytosis as indicated. The medium from the basolateral chamber was collected, concentrated, and blotted for detection of NP-BSA antigen.
(**E**). Effect of soluble CD23 (sCD23) on the transcytosis of immune complex. The immune complex was preincubated with sCD23 at indicated concentration before addition to the apical chamber. Then, the immune complexes were incubated at 4ºC (lane 2) or 37ºC (lanes 3-6) for 2 hr to allow transcytosis. Supernatant from the basolateral chamber was sampled and blotted for NP antigen (E). The graph (E, *bottom panel*) represents the densitometry analysis of NP-BSA bands obtained from the transcytosed immune complex treated with (lanes 4-6) or without (lane 3) soluble CD23 proteins. Results were representative of three individual experiments. *P<0.05, **P<0.01.
(**F+G**). Effect of CD23 specific Ab on the transcytosis of immune complex. CD23-specific IDEC152 mAb at indicated concentration was incubated with Calu-3 cells at 4ºC for 1 hr before the addition of immune complex to the apical chamber. Then, the immune complexes were incubated at 4ºC (lane 2) or 37ºC (lanes 3-5) for 2 hr to allow the IC transcytosis. Supernatant from the basolateral chamber was sampled and measured for NP antigen in a Western blot (F) or total IgE in an ELISA (G). The graph (F, *bottom panel*) represents the densitometry analysis of NP-BSA bands obtained from the transcytosed immune complex with samples treated with (F, lanes 3-4) or without (F, lane 5) IDEC152 Ab. Results were representative of three individual experiments. *P<0.05.

transcytosis of human IgE was significantly enhanced in both apical to basolateral (Fig. 2.5A) and basolateral to apical (Fig. 2.5B) directions with IL-4 exposure at 37ºC in comparison with that of mock-treated cells. Kinetics of IgE transcytosis from the apical to basolateral direction in Calu-3 monolayer were shown in Fig. 2.5C. In a similar way, the transcytosis of IgE immune complex was also significantly enhanced in the presence IL-4 stimulation at 37ºC in comparison with that of mock-treated cells (Fig. 2.5D). The amount of IgE immune complex transported also exhibited a time course dependent manner (Fig. 2.5E). In addition, the immune complex was barely detectable when Calu-3 monolayers were incubated at 4ºC (Fig. 2.5D, lane 2) suggesting Calu-3 cells under 24 hr exposure of IL-4 stimulation did not significantly alter the transepithelial resistance. Taken together, IL4 treatment increased the transcytosis of both IgE and immune complex across Calu-3 cells.

Primary human tracheal and bronchial epithelial cells can transcytose both IgE and immune complex.

To further verify the transcytosis results of either IgE or immune complexes in human Calu-3 cell lines, we used commercially available primary human tracheal-bronchial epithelial tissues which were originally obtained from a human donor. Human tracheal/bronchial cell cultures (EpiAirway^{TM} tissues, AIR-100-SNAP) closely resemble in-vivo conditions of human tracheal/bronchial cells. As the first step, CD23b, but not CD23a, expressions were verified by RT-PCR amplification with CD23 specific primers (Fig. 2.6A), Western blot (Fig. 2.6B) and immunoflouresence staining (Fig. 2.6C, *left*

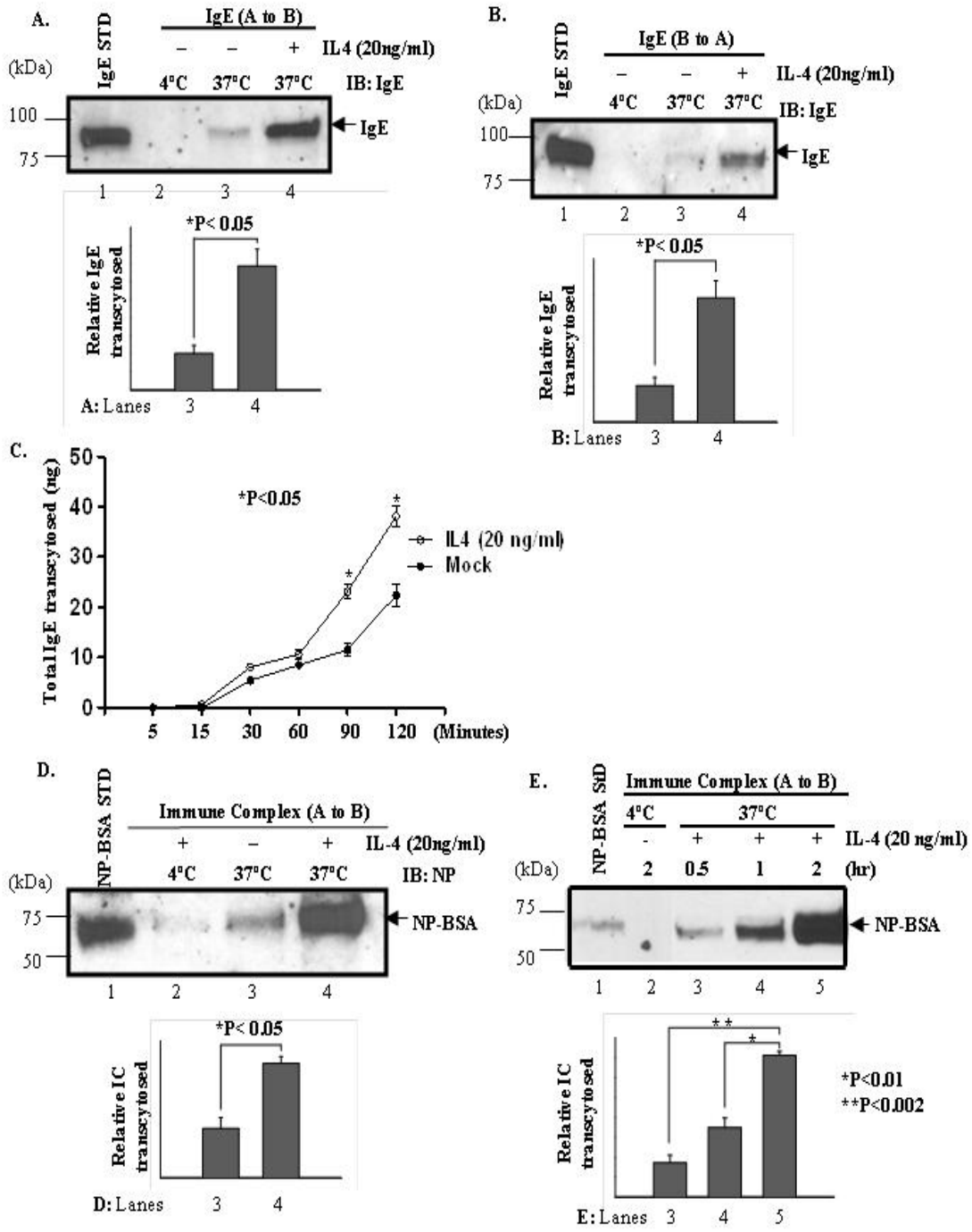
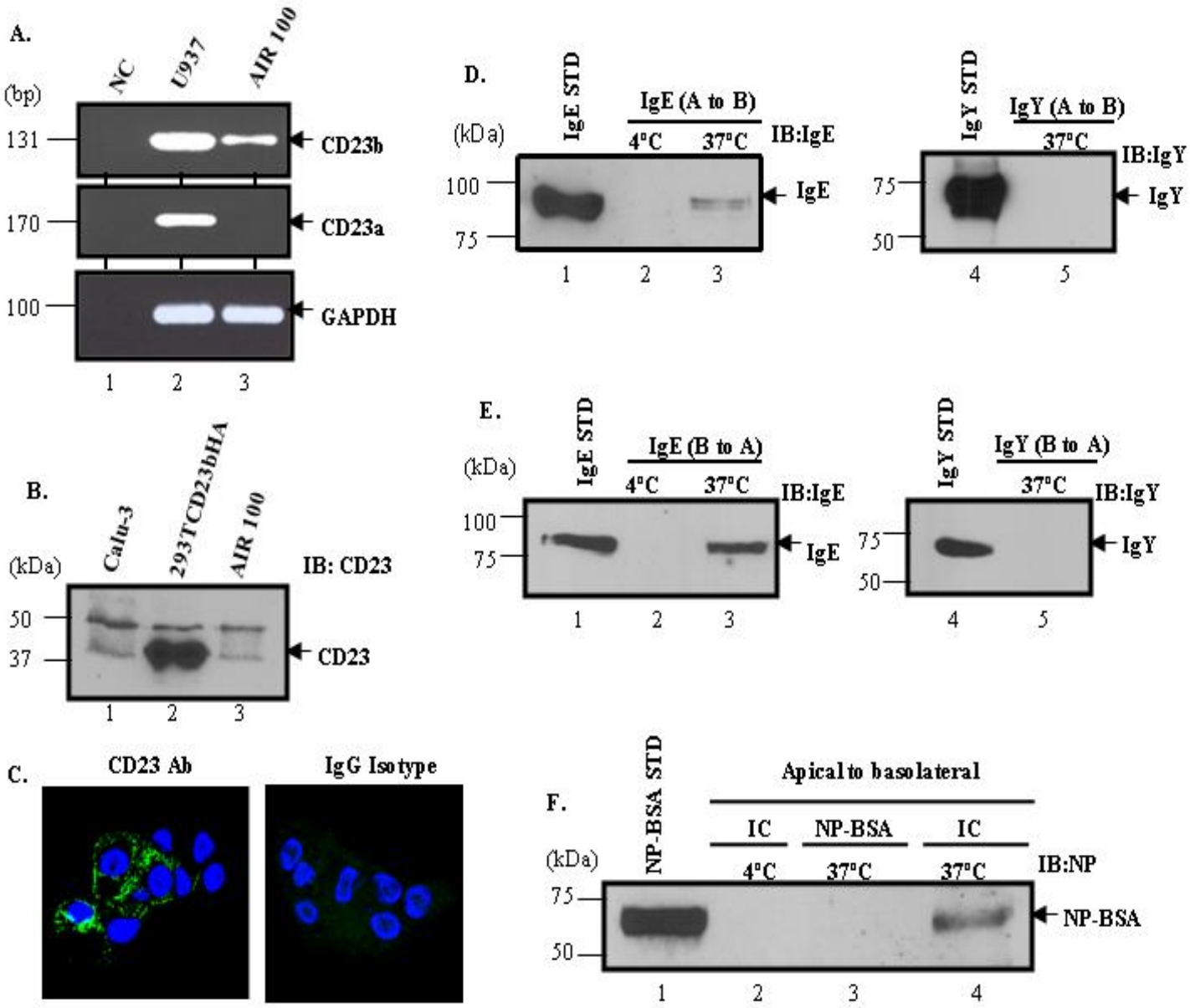

A.
IgE STD
IgE (A to B)
– – + IL4 (20ng/ml)
(kDa)
4°C 37°C 37°C IB: IgE
100
75
IgE
1 2 3 4
*P< 0.05
Relative IgE transcytosed
A: Lanes 3 4
B.
IgE STD
IgE (B to A)
– – + IL-4 (20ng/ml)
(kDa)
4°C 37°C 37°C IB: IgE
100
75
IgE
1 2 3 4
*P< 0.05
Relative IgE transcytosed
B: Lanes 3 4
C.
Total IgE transcytosed (ng)
50
40
30
20
10
0
*P<0.05
IL4 (20 ng/ml)
Mock
5 15 30 60 90 120 (Minutes)
D.
NP-BSA STD
Immune Complex (A to B)
+ – + IL-4 (20ng/ml)
(kDa)
4°C 37°C 37°C IB: NP
75
50
NP-BSA
1 2 3 4
*P< 0.05
Relative IC transcytosed
D: Lanes 3 4
E.
NP-BSA StD
Immune Complex (A to B)
4°C 37°C
- + + + IL-4 (20 ng/ml)
(kDa) 2 0.5 1 2 (hr)
75
50
NP-BSA
1 2 3 4 5
**
*
*P<0.01
**P<0.002
Relative IC transcytosed
E: Lanes 3 4 5

Figure 2.5 Effect of IL-4 treatment on transcytosis of IgE and immune complex in the polarized Calu-3 cells. Calu-3 cells (1×10^5/ml) were grown on 0.4 μm pore size transwell filters. The cells were allowed to get polarized and the transcytosis was performed when the TER value reached at least 450-900 Ω/cm^2. Calu-3 cells were treated with or without IL4 (20 ng/ml) for 24 hr. A: Apical; B: Basolateral.
(**A+B**). Human IgE (0.5 μmol/L) was added into the apical (A) or basolateral (B) chamber to allow transcytosis for 2 hr at 4°C (lane 2) and 37°C with or without IL-4 treatment (lanes 3 & 4). Supernatant from the opposite chamber was sampled, concentrated and blotted for IgE. The relative integrated band intensities of the IgE transcytosed across the Calu-3 monolayer from the top panel were calculated by densitometry analysis (*bottom panels*).
C. Kinetics of IgE transcytosis in lung Calu-3 monolayer. Human IgE was added to the apical chamber of polarized Calu-3 epithelial cells that were pretreated with (open circle) or without IL-4 (solid circle) for 24 hr and incubated at 37ºC for additional 2 hr at indicated time points. The medium from the basolateral chamber was collected and concentrated for measuring total IgE by ELISA.
(**D+E**). IgE derived immune complex was added into the apical chamber to allow transcytosis for 2 hr at 4°C (lane 2) and 37°C in the presence or absence of IL-4 treatment (D, lanes 3 & 4). In panel E, Calu-3 monolayers were treated with IL-4 in indicated time (E, lanes 3-5). Supernatant from the basolateral chamber was sampled and blotted for NP-BSA antigen. The relative integrated band intensities of the immune complex transcytosed across the Calu-3 monolayer from the top panel were analyzed by densitometry (*bottom panels*). The image in panel E was processed by Photoshop

panel) with EBVCS1 mAb in these primary human epithelial cells. The specificity of the staining was confirmed with isotype matched IgG control (Fig. 2.6C, *right panel*). CD23 expression was up-regulated in the primary human tracheal bronchial epithelial cells in response to IL4 treatment (data not shown). When human IgE was loaded onto either apical or basolateral chambers, these human primary tracheal-bronchial epithelial tissues were capable of transcytosing IgE from either the apical to basolateral or the basolateral to apical directions (Figs. 2.6D+2.6E). IgE Ab failed to be detected in both directions when tissues were incubated at 4ºC. In addition, chicken IgY closely resembles human IgE in structure (221) was not transcytosed in both directions (Figs. 2.6D+2.6E) demonstrating that the human IgE detected in transcytosis assay was the specific transport, but not passively diffused through paracellular pathway or due to leakiness in model tissue. To further show whether the primary human tracheal and bronchial epithelial cells can also transcytose immune complex, we incubated these cells with IgE derived immune complex in the apical chamber for 2 hr. As shown in Fig. 2.6F, antigen-IgE complexes were transcytosed from the apical-to-basal at 37°C, but not at 4°C, as detected by Western blotting of NP-BSA antigens. Antigen NP-BSA alone was undetectable in transcytosis by primary human tracheal and bronchial epithelial cells further demonstrating the specificity of IgE-CD23 mediated transcytosis. Overall, all these evidences strongly indicate that IgE or immune complex can enter the transcytotic pathways in primary human airway epithelial cells.


A.
NC
U937
AIR 100
(bp)
131
170
100
CD23b
CD23a
GAPDH
1
2
3
B.
Calu-3
293TCD23bHA
AIR 100
(kDa)
IB: CD23
50
37
CD23
1
2
3
C.
CD23 Ab
IgG Isotype
D.
IgE STD
IgE (A to B)
4°C
37°C
(kDa)
IB:IgE
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75
IgE
1
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IgY STD
IgY (A to B)
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IgE STD
IgE (B to A)
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(kDa)
IB:IgE
100
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IgE
1
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IgY STD
IgY (B to A)
37°C
IB:IgY
75
50
IgY
4
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F.
NP-BSA STD
Apical to basolateral
IC
NP-BSA
IC
4°C
37°C
37°C
(kDa)
IB:NP
75
50
NP-BSA
1
2
3
4

Figure 2.6 The primary human tracheal bronchial epithelial tissue transcytoses IgE and immune complex. All protein samples were separated on 12% SDS-PAGE gel under reducing condition, proteins transferred to nitrocellulose membrane, blotted with protein specific antibody, and followed by HRP conjugated secondary antibody. The blot was visualized by ECL method.

(**A**) The primary normal human tracheal bronchial epithelial cells (TBEC, AIR-100) expressed CD23b but not CD23a mRNA. Total RNA was extracted by TRIzol reagent from primary epithelial cells that were grown in transwell inserts consists of TBEC forming highly differentiated and pseudo-stratified polarized epithelial tissue. The purified RNA was subjected to one step RT-PCR with CD23 specific primers. The PCR products were electrophoresed on 1.5% agaorse gel and DNA stained and identified with ethidium bromide. U937 cells (lane 2) were used as a positive control. NC, a negative control without RNA template in RT-PCR reaction. GAPDH was used as internal control.

(**B**) CD23 protein was expressed in AIR-100 cells. The cell lysates (60 μg) from Calu-3 (lane 1), 293TCD23bHA (lane 2) and human tracheal bronchial epithelial cells (TBEC) (lane 3)
were subjected in a Western blot by blotting with mouse anti-CD23 EBVCS1 mAb.

(**C**) Immunofluorecence staining of TBEC cells. The TBEC cells were removed from cultivated inserts by EDTA and allowed to grow on glass coverslips overnight, fixed with 4% *para*-formaldehyde and permeabilized in 0.1% Triton X-100. Subsequently, the cells were incubated with goat serum for blocking anti-human CD23 EBVCS1 mAb, followed by staining with an Alexa flour 488-conjugated goat-anti mouse Ab. The cells were also stained with an isotype-matched mouse IgG1 as negative control (*right panel*). The nucleus was stained with DAPI and photographed through a fluorescence microscope.

(**D**+**E**). IgE transcytosis in primary TBEC. Polarized AIR-100 tissue from human donors grown on cell culture inserts was used. The cells were allowed to get polarized. The human IgE (*left panel*) or chicken IgY (*right panel*) (0.5 μmol/L) was added to either the apical (D) or basolateral (E) chamber and incubated at 37ºC (lanes 3 & 5) or 4ºC (lane 2) for 2 hr. The medium from opposite chambers was collected, concentrated and blotted to detect IgE or IgY heavy chain in a Western blot. A: Apical; B: Basolateral. The image in Figure 6D was processed by the Photoshop.

(**F**) Transcytosis of immune complex in primary TBEC. TBEC cells were grown on transwell filters. The cells were allowed to get polarized. The NP-BSA antigen alone (lane 3) or immune complex (lanes 2&4) was added to the apical chambers and incubated at 37ºC or 4ºC for 2 hr. The medium from the basolateral chamber was collected, concentrated, and blotted for NP to detect NP-BSA Ag in a Western blot. STD: standard; IC: immune complex.

CD23-mediated transcytosis of immune complex induces human mast cell degranulation.

IgE mediated hypersensitivity reactions are mediated by antigen cross-linking of IgE antibodies bound to its high affinity receptor (FcεRI) on immune cells, such as mast cells (1, 206). Because mast cells reside in close proximity to airway epithelial cells in the respiratory tract, we tested the ability of transcytosed antigen–IgE complexes to trigger the degranulation in human mast cells. To mimic the natural condition we cultured LAD2 cells (1 X 10^5/ml) in basolateral chambers when Calu-3 cells were grown in the transwell inserts. The LAD2, is an human mast cell analogue that can be stimulated to degranulate in an IgE-dependent manner (222). The supernatant from the basolateral chamber was obtained 3 hr later after apical addition of the immune complex, IgE, and antigen alone. Although mast cell degranulation occurs rapidly, the supernatant sampled 3 hr after addition of the immune complex ensured enough antigens to be transported in the experiment. The degranulation of LAD2 cells were assessed by both tryptase and β-hexosaminidase assays. As shown in Fig. 2.7A, as a positive control, direct incubation of LAD2 mast cells with immune complex induced high levels of mast cell degranulation. Also, LAD2 cells exhibited significant degranulation which was triggered by the transcytosed immune complex in comparison with that of IgE or antigen alone in either tryptase or β-hexosaminidase analyses. When LAD2 cells were primed with NP specific IgE prior to addition of the transcytosed immune complex, the level of degranulation was significantly enhanced in comparison with that of LAD2 cells primed with antigen non-specific IgE Ab (Fig. 2.7A). The degranulation was also demonstrated by the immunofluorescence staining of tryptase in LAD2 cells; the intensity of tryptase staining

was greatly reduced in the presence of the transcytosed immune complex in comparison with IgE or antigen alone (Fig. 2.7B). However, it would be informative to use an antigen without multiple identical epitopes in degranulation assay. Therefore, IgE released from the airway epithelial cells together with the antigen in an immune complex form is capable of activating effector mast cells.

DISCUSSION

The early inflammatory response during allergic rhinitis and asthma is strongly associated with inhaled allergens cross-linking specific IgE on the surface of mast cells, leading to their activation and release of primary inflammatory mediators (202, 223). The late phase reaction is characterized by recruitment, activation and tissue infiltration of leukocyte populations, including lymphocytes, eosinophiles, basophiles, and neutrophiles (1, 202). Antibody-antigen interaction in the airway drives early granulocyte recruitment (224). Recently basophiles were also found to function as antigen-presenting cells for an allergen-induced Th 2 response (225). Therefore, one of the major roles of the airway epithelium is to act as a barrier to restrict the antigens and other potentially noxious materials from penetrating into the mucosa laminar propria of the airway. It has been known that some allergens with protease character penetrate mucosa or dendritic cells directly sample the inhaled antigens from the respiratory lumen (226). Here we found that CD23 is capable of transport of IgE and the immune complex across polarized human airway epithelial monolayer.

CD23 expression has been shown in many immune cells. In this study, we have systemically demonstrated that the constitutive expression of human CD23 in epithelial

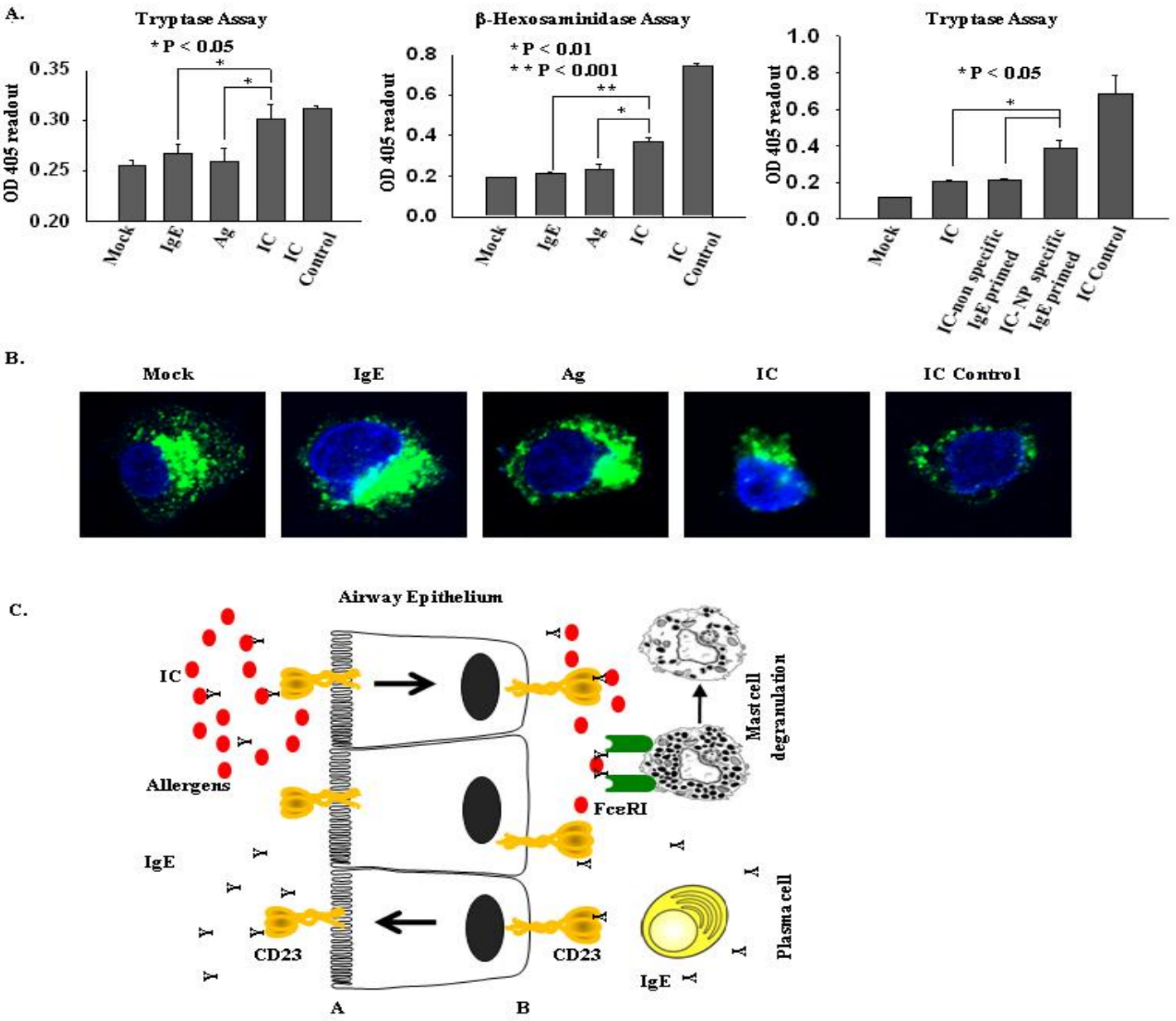

A.
Tryptase Assay
* P < 0.05
OD 405 readout
0.35
0.30
0.25
0.20
Mock
IgE
Ag
IC
IC Control
β-Hexosaminidase Assay
* P < 0.01
* * P < 0.001
OD 405 readout
0.8
0.6
0.4
0.2
0.0
Mock
IgE
Ag
IC
IC Control
Tryptase Assay
* P < 0.05
OD 405 readout
1.0
0.8
0.6
0.4
0.2
0.0
Mock
IC
IC-non specific IgE primed
IC-NP specific IgE primed
IC Control
B.
Mock
IgE
Ag
IC
IC Control
C.
Airway Epithelium
IC
Allergens
IgE
CD23
CD23
IgE
FcεRI
Mast cell degranulation
Plasma cell
A
B

Figure 2.7 Immune complex transported by CD23 in polarized Calu-3 cells facilitated mast cell degranulation.
A. Effect of transcytosis of the immune complex on degranulation of mast cells. Calu-3 cells (1×10^5/ml) were grown on 0.4 μm pore size inserts. The cells were allowed to get polarized. The immune complexes were made by incubating chimeric anti-NP IgE with NP-BSA. Supernatants from the basolateral chamber containing mast cell LAD2 ($1 \text{ X } 10^5$/ml) were sampled 3 hr later after addition of IgE, NP-BSA (Ag), immune complex (IC) to the apical chamber. In a 96 well plate, the transcytosed immune complexes were also incubated with LAD2 cells that were primed with or without NP specific IgE or non specific IgE (*right panel*). The tryptase (*left panel*) or β-hexosaminidase (*middle panel*) activity in the supernatant was measured as described in Materials and Methods. Medium was used as a negative control (mock). LAD2 mast cells ($1 \text{ X } 10^5$/ml) that were directly incubated with the immune complex were used as a positive control (IC control).
B. Representative immunofluorecence staining of LAD2 mast cell for tryptase expression during degranulation. LAD2 cells from the above experiments (A) were fixed and permeabilized in 0.1% Triton X-100. Subsequently, the cells were incubated with affinity-purified mouse anti-tryptase mAb (green), followed by staining with an Alexa flour 488-conjugated rabbit anti-mouse Ab. The nucleus was stained with DAPI (blue). Experimental conditions are displayed in the top.
C. Schematic description of mast cell degranulation by transcytosed immune complex in a transcytosis model. IgE from mucosal plasma cells or from the blood are transported by CD23 into the airway lumen. Upon meeting with airborne allergens, the immune complexes (IC) are formed. CD23 acts as a bidirectional IgE transporter and facilitates apical (luminal)-to-basal transport of IgE-allergen complexes. At the subepithelial side, airborne allergens or the immune complexes engage with FcεRI on the surface of mast cell, to trigger release of mast cell mediators.

cell lines derived from the nose, trachea, and lung (Fig. 2.1). We were not able to obtain respiratory biopsy samples and therefore examined a model tissue as a measure of airway CD23 expression. The association between CD23 and IgE was also demonstrated in a CD23 dependent IgE uptake assay in the human airway Calu-3 cells proving that CD23 is structurally intact in this cell type. Therefore, our results support and extend the previous finding that CD23 is constitutively expressed in human intestinal epithelial cells (140, 145). We further found that human airway epithelial cells expressed CD23b, but not CD23a, mRNA (Fig. 2.1). The absence of CD23a protein expression by airway epithelial cells may indicate lack of constitutive expression, but it may also be due to the absence of a required growth factor in cell culture media. Our study does not rule out expression of CD23a in intact tissues in vivo. Clearly, in situ hybridization studies are necessary for conclusive evidence regarding CD23 isoforms expressed by specific cell types in vivo. The expression of isoform specific CD23 mRNA in human intestinal epithelial cells remains controversial (144, 145). It is possible that cells grown in non-polarized or polarized conditions might affect the expression of CD23 isoform in polarized epithelial cells (143). Interestingly, intracellular expression of CD23 in airway epithelial cells suggests CD23 functions as an intracellular receptor for IgE.

CD23 transports human IgE in polarized airway epithelial monolayer. The function of CD23 has been most extensively studied on B cells, where it is involved in IgE-mediated antigen capture and facilitated presentation to T cells or regulation of IgE homeostasis (77, 130). There is a paucity of information on the link between engagement of CD23 on the airway epithelial cells and the functional outcome. In airway epithelial cells, CD23 protein was visualized on both the apical and basolateral membrane

suggesting that IgE may be transported in both directions across the epithelium (Fig. 2.3A). In fact, we demonstrated that human lung epithelial cell Calu-3 cell line transcytosed the exogenously applied IgE which occurred in both apical-to-basal and basal-to-apical directions (Fig. 2.3C). Most importantly, human IgE was transported bidirectionally at concentrations representing at both physiological and allergy conditions (Fig. 2.3D). In our studies, IgE was clearly released after transcytosis although we did not determine the relative quantity of free IgE against that which remains associated with its receptor. To confirm the specific transport, we conducted additional experiments using either soluble CD23 or CD23 specific Ab. We showed that either of them was able to significantly decrease IgE transport across the Calu-3 cell monolayer (Fig. 2.3). These results, taken together with the temperature sensitivity of IgE transport, provide evidence that the transepithelial transport of IgE is mediated by the CD23 expressed in the airway epithelial cells. The IgE transport in airway epithelial cells was partially sensitive to bafilomycin, suggesting the transcytosis occurs also through endosome. Both IgA and IgG transcytosis by the polymeric immunoglobulin receptor (pIgR) and FcRn have also been shown to be sensitive to agents that interfere with pH gradients (150, 177). In contrast to FcRn-mediated IgG and pIgR-mediated IgA transcytosis, it remains unclear how CD23 releases IgE at cell surface after transcytosis. Although the intracellular trafficking mechanism of CD23 has just begun to be explored, the similar ability of CD23 and FcRn to mediate bidirectional transcytosis suggests possible similarities in their transport mechanism.

CD23 transports IgE-derived immune complex across the airway epithelial barrier. The evidence of the airway epithelial cells transporting the IgE suggests that

when a host inhales allergen, it may combine with the allergen specific IgE present on the lumen or cell surface to form immune complexes and it can then be transcytosed across the airway epithelial barrier. To mimic the natural condition, we performed the transcytosis of immune complex from the apical to basolateral direction and found that Calu-3 monolayer efficiently transcytosed the immune complex, but not antigen alone (Figs. 2.4A & 2.4B). In confirmation of the specificity of CD23 in transepithelial antigen transport, sCD23 or CD23 specific Ab blocked antigen uptake and transport of antigen in a concentration-dependent manner (Fig. 2.4). Furthermore, primary human tissues could also transcytose IgE or immune complex (Fig. 2.6). This is, however, not surprising given the fact that CD23 has been described previously as mediating endocytosis of immune complex in B cells (119, 213, 227). However, several issues should be noticed. First, in our experiment, an intact antigen was detected in the transport of the immune complex. This is consistent with the previous finding that CD23 mediated transepithelial transcytosis of the immune complex diverts antigen from lysosomal degradation in intestinal epithelial cells (145, 228). It is possible that by protecting antigen from degradation, CD23-mediated transcytosis of the immune complex may increase quantity of antigen delivered across the airway epithelium into the body. Second, the transport of the immune complex in the apical to basolateral direction was much more efficient than that of IgE alone since we were able to easily detect the immune complex in our assay. The rationale for this discrepancy may be due to the sensitivity of detection methods for NP antigens. Alternatively, immune complex bound to CD23 airway epithelial cells may cross-link CD23 molecule, initiate signaling cascade, and subsequently result in enhanced transcytosis of the immune complex. Studies in human intestinal epithelial cells have

already found that the immune complexes on the intestinal epithelial monolayers preferentially activate MAPK signaling pathway and subsequent release of chemokines IL-8 and CCL20 (146, 147). Although the exact mechanisms of this antigen transport system need to be further investigated, our results provide convincing evidence that human airway cell lines and primary airway tissue can transcytose IgE-derived immune complex, which is mediated by IgE and CD23. Therefore, CD23 can function as an antigen-sampling mechanism by transporting intact allergen-IgE complexes across human airway epithelia.

What might be the clinical implication and significance of CD23 mediated IgE and immune complex transport in human airway epithelial cells? Expression of CD23 and IgE level is enhanced in asthma patients (156, 165). CD23 is involved in regulating IgE synthesis in B cells (125), developing extrinsic allergic alveolitis in alveolar macrophages (164), and reacting to stimulations in airway smooth muscle cells (161). Our study enlightens the role of CD23 in transepithelial transport of IgE and immune complex which may have significant role in the development of airway allergic inflammation. We speculate that this may be an important step in the amplification of adaptive immune responses to allergen ingestion or may be a critical step in the initiation of a local late-phase airway inflammatory response. Since IL-4 is elevated in all allergic conditions, our results have clearly demonstrated that airway epithelial cell CD23b mRNA appeared to be IL-4 dependently up-regulated. The increased expression of induced CD23 is most likely due to activation of the IL-4 enhancer element on the CD23 gene (219). More importantly, exposure of IL-4 up-regulated CD23 expression and enhanced the transcytosis of either IgE or the immune complexes across the polarized

Calu-3 monolayer (Fig. 2.5). It should be noted IL-4 treatment did not lead to the transport of immune complex at 4 ºC, suggesting that IL-4 influences IgE transport by stimulating expression of CD23 rather than by inducing nonspecific increases in permeability of the epithelial monolayer. Although we did not obtain respiratory biopsy samples from patients with IgE-mediated asthmatic allergies, IgE presence in human airway secretions of individuals with asthma has been documented. It is very likely that CD23 might enhance transport function on airway epithelial cells in asthmatics. Our discovery was in agreement with findings from IL-4 enhanced IgE and immune complex transcytosis in the intestinal epithelial cells (138, 144, 145). Transport of the immune complex may lead to several immunological consequences in shaping the airway allergy. First, binding to IgE/CD23 protects antigen from degradation during transepithelial transport resulting in large quantities of allergens gaining access to the lamina propria in a short period. This allergen or immune complexes is available to activate mast cells, basophiles, through cross linking FcεRI and initiate the hypersensitivity reaction. We have shown that IgE-Ag complexes that were transcytosed across human airway epithelial cells triggered the degranulation of mast cells (Fig. 2.7A+2.7B). Therefore, it is conceivable that the degranulation subsequently leads to the release of various inflammatory and lipid mediators contributing to airway perturbation and hypersensitivity reactions. Second, the transport of the immune complexes may be accompanied by cytokine release from immune cells in the mucosa, which may enhance the expression of CD23 on the airway epithelial cell layer. Therefore, each exposure to immune complex could potentially enhance CD23 expression on the epithelial cell layer by activating mucosal immune cells. Third, the transported immune complex may also be

delivered to antigen-presenting cells such as dendritic cells or basophiles for presentation to T cells. These cells can use FcεRI receptor for capturing IgE complexed antigen. More interestingly, the sensitization can increase uptake of specific antigen via an endosomal transcellular pathway across tracheal and jejunal epithelium (229, 230). Further, in vitro-formed IgE immune complexes, when administered through the airways, are more potent than antigen alone in inducing airway inflammatory responses in mice previously sensitized with the antigen (231). However, these studies did not reveal the CD23 dependent transport of the immune complex on airway epithelial cells. Therefore, our study may reveal a novel antigen transport or capture mechanism leading to an allergic response to inhaled allergens in an atopy individual.

Although CD23 expression has been noted previously in intestinal epithelial cells, to our knowledge, this is the first study to identify that CD23 expressed in human airway epithelial cells is a receptor involved in the binding and bidirectional transport of IgE across polarized airway epithelial monolayers. Taken together, the results obtained in this study demonstrate that CD23, expressed on human airway epithelial cells, acts as a bi-directional IgE transporter, which is regulated by IL-4. Both directions fit well with the model that IgE produced within the body is transported to the apical membrane of airway epithelial cells and released into the lumen, resulting in binding inhaled allergens. It is possible that free allergen can attach to IgE-CD23 complex too. CD23 facilitates apical-to basal transcytosis of functional IgE/antigen complexes, which are capable of triggering underlying effector cells, such as mast cells for degranulation (Fig. 2.7C). Such activation may provide feedback to the airway epithelium to signal cytokine or chemokine release to support the influx of a new wave of inflammatory and adaptive immune cells into the

airway mucosa. We, therefore, speculate that CD23 is a critical receptor in the initiation of allergic responses in human airway allergic inflammation. Besides unraveling these basic mechanisms of CD23-mediated IgE and immune complex transport in humans, this study also suggests that blocking CD23-mediated IgE transport is a potentially important target to interfere with allergen-induced respiratory symptoms.

CHAPTER 3: CD23 MEDIATED TRANSCYTOSIS OF IGE AND IMMUNE COMPLEX ACROSS THE MOUSE RESPIRATORY TRACT AND ITS ROLE IN ALLERGY DEVELOPMENT

ABSTRACT

IgE is one of the major contributing factors for the development of allergic diseases and its exact role in airway mucosal secretions remains elusive. The presence of IgE in mast cells and basophils cross linked by the allergen and trigger the release of variety of inflammatory mediators associated with allergic inflammation. We have previously showed that human CD23 is capable of transporting IgE and immune complex across the polarized respiratory epithelial cells, implying CD23 may play a critical role in initiating and contributing to the airway allergic inflammation. To verify this in a mouse model, we at first showed that mouse CD23 was expressed in airway epithelial cells. Sensitization of Balb/c mice with allergen ovalbumin (OVA) augmented CD23 expression in airway epithelial cells. Transcytosis of OVA-specific IgE across mouse airway was demonstrated bidirectionally from the apical to basolateral or the basolateral to apical and exhibited in a time- and dose-dependent manner. The OVA was also detected by transcytosis across airway barrier when mice were intranassally inoculated with OVA immune complex. The CD23 mediated specific transport of either IgE or immune complexes was further verified in a CD23 knockout mice. A further experiment for showing the roles of epithelial CD23 in regulating the airway allergic inflammation by transporting allergens in an immune complex form is being investgated in an OVA-based allergy model in mice

INTRODUCTION

Allergic diseases are one of the most common immunological disorders which include allergic asthma, allergic rhinitis, food allergy, atopic dermatitis, and anaphylaxis affecting approximately 25% people in the developed world (202). The airway allergic inflammation is commonly characterized by presence of eosinophils and CD4+ T cells (232, 233). Allergen inhalations into patients with atopic asthma activate the Th2 type CD4+ T cells and enhance cytokine expression of IL-4, IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF) and eosinophil accumulation (233). These Th2 cells play an important role in the pathogenesis of allergic asthma (202). Airway inflammation is predominantly associated with IgE and characterized by the presence of allergen specific IgE in the airway tract (1, 234), although IgE independent allergy has also been documented (235). Local production of allergen specific IgE by class switch in B cells leads to IgE appearance in the airway secretion (236, 237). Allergen specific IgE binds high affinity receptor for IgE (FcεRI) on the mast cells, eosinophils and basophils and plays a very important role in the pathogenesis of airway allergic inflammation. Allergen cross-linking of surface IgE leads to cell degranulation and the release of various allergic mediators (1, 202, 237). These include proteases, biogenic amines, prostaglandins, leukotrienes, etc. and acts on the local tissue like epithelial and smooth muscle cells (202). In order for inhaled allergens to interact with immune cells, allergens have to access and activate the immune cells in the lamina propria of the airway tract. However, the epithelial cells lining the airway form a mucosal barrier by forming tight junctions between the adjacent cells. Hence macromolecules, like inhaled allergens, have to first cross the mucosal barrier by a process called transcytosis to trigger the allergy.

CD23, a receptor for IgE is the only Fc receptor that belongs to a C-type lectin family consisting of three lectin head domain in the C-terminal that can bind to IgE followed by a triple α–helical coiled-coil stalk region, transmembrane and short cytoplasmic tail domain (1). CD23 binds IgE with a dual affinity, a low affinity ($K_a \approx 10^6$ -10^7 M^{-1}) in the monomeric state and high affinity to IgE ($K_a \approx 10^8$ -10^9 M^{-1}) in a trimeric form (1, 63, 64). CD23 is susceptible to proteolytic cleavage by enzymes like ADAM10 and house dust mite protease der p1 at the stalk region, which is responsible for releasing various soluble CD23 (104, 105, 109). Soluble CD23 has multiple functions involved in IgE homeostasis and various cytokine like activities (12, 63, 64). CD23 exists as two isoforms, CD23a and CD23b. They have different transcription initiation sites and differ only in the N-terminal cytoplasmic region by 6-7 amino acids (76, 77). In humans, the isoform CD23a is expressed only in the B lymphocytes while CD23b is expressed in variety of cells like monocytes, eosinophils, langerhans, respiratory and intestinal epithelial cells (77, 144, 145, 238). In mice, CD23 is expressed in B cells, follicular dendritic cells, and intestinal epithelial cells (58, 80, 81, 140-142). CD23 functions in enhancing IgE-specific antigen processing and presentation in the form of IgE/antigen complexes (117-120), regulation of IgE production (63, 64, 125, 126, 128), and influencing cell differentiation, survival and growth of B- and T-cells and myeloid precursors (131, 133-135). Several studies showed that human and murine intestinal epithelial cells express CD23 receptors and are capable of transporting either IgE or IgE-allergen immune complex across the polarized epithelial cells (140-142, 144, 145).

Recently, we showed that human respiratory epithelial cells express CD23 and IL-4 enhanced the expression of CD23 and transport of IgE and IgE derived immune

complexes (238). Furthermore, CD23 enhanced intestinal transepithelial antigen transport in a sensitized allergic rat and mouse model (140-142). All of these experiments strongly showed a role for CD23 in aiding allergy by enhanced IgE or antigen transport across the polarized epithelial cells lining the mucosal surfaces. However, the expression of CD23 and its function in murine respiratory tract remain elusive. Here we showed that CD23 was expressed in mouse respiratory tract and was capable of transporting mouse IgE or IgE-derived immune complexes.

MATERIALS AND METHODS

Antibodies, mice, cells and reagents. The murine lung epithelial cell line LA4 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in Ham's F12K complete medium containing 15 % FBS, 1% L-glutamine, non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were grown in a humidified atmosphere at 37ºC containing 5% CO_2. Chicken ovalbumin (OVA, Grade V), was purchased from Sigma Aldrich (St. Louis, MO). The anti-murine CD23 antibody-secreting hybridoma B3B4, rabbit anti-murine CD23 antibody, CD23 knockout (KO) mouse on a *Balb/c* background were kind gifts from Dr. Daniel Conrad (Virginia Commonwealth University School of Medicine, VA). Six- to eight-week-old inbred female *Balb/c* mice were purchased from the National Cancer Institute (Frederick, MD).

Mouse anti-OVA IgE was purchased from Bio X-cell (West Lebanon, NH). Rabbit anti-mouse IgE-HRP, normal rabbit IgG, mouse anti-chicken OVA mAb and FITC-conjugated pan anti-cytokeratin mAb were purchased from Sigma Aldrich (St

Louis, Mo). Mouse anti-β-tubulin antibody was purchased from Developmental Studies Hybridoma Bank (The University of Iowa, Iowa City, IA). Rabbit anti-mouse IgG HRP conjugated antibody was obtained from Pierce (Rockford, IL). FITC- conjugated polyclonal goat anti-mouse IgE and normal goat anti-mouse IgE antibody were obtained from Novus Biologicals (Littleton, CO). Alexa fluor 555 conjugated Goat anti-rabbit or anti-mouse IgG and Alexa fluor 633-conjugated goat anti-rabbit IgG were purchased from Invitrogen. HRP conjugated bovine anti-goat IgG antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FITC conjugated rat anti-mouse B3B4 mAb and rat anti-mouse CD16/CD32 antibody were obtained from BD Pharmingen (San Diego, CA). APC conjugated rat anti-mouse CD45R (B220) and FITC conjugated isotype rat IgG2a antibody were obtained from Caltag laboratories. Biotin-labelled mouse anti-OVA IgE antibody was generated by EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) following manufacturer's instruction. Recombinant murine IL-4 was from R&D Systems (Minneapolis, MN). Proteinase inhibitor cocktail was purchased from Calbiochem (San Diego, CA).

RT-PCR. Semi-quantitative RT-PCR was performed on total RNA extracted from lung epithelial cell line LA4 as previously described (238). Total RNA was isolated from 2 x 10^6/ml LA4 cells either mock stimulated or stimulated with murine IL-4 (20 ng/ml) using TRIzol reagents (Invitrogen) according to the manufacturer's instructions. One-step RT-PCR kit (Qiagen) was used to perform semi-quantitative RT-PCR. Negative control was performed without addition of RNA template. Primers used for amplification of murine CD23a, CD23b (142) and murine CD23bΔ5 lacking exon 5 (143) were described

previously. Primers used for the amplification of gylceraldehyde-3-phosphate dehydrogenase (GAPDH) were GAPDH-F 5'-ACC CAG AAG ACT GTG GAT GG -3' and GAPDH-R 5'-CAC ATT GGG GGT AGG AAC AC-3'. Amplification of CD23 mRNA and GAPDH were performed with 450 ng of total RNA in 20-µl volume. One step RT-PCR amplification cycle consisted of reverse transcription at 50°C for 30 min, initial PCR activation at 95°C for 15 min, followed by PCR cycle of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min for 40 cycles and final extension at 72°C for 10 min. Annealing temperature for GAPDH was 55°C for 1 min and the rest of the amplification conditions are same. RT-PCR products were analyzed by resolving on 1.5 % agarose gel and DNA visualized by ethidium bromide staining. Semi-quantitative analysis of CD23 band intensities of RT-PCR products normalized to GAPDH values were analyzed by densitometry using Adobe Photoshop.

Genotyping of CD23 knockout (KO) mice. Genotyping of the CD23KO mice on *Balb/c* background was performed with genomic DNA isolated from mouse tail using Puregene DNA isolation kit (Qiagen) following manufacturer's protocol. Primers used for genotyping were previously described (239). PCR amplification cycle consisted of initial PCR activation at 94°C for 3 min, followed by PCR cycle of denaturation at 94°C for 30 sec, annealing at 63°C for 1 min, extension at 72°C for 2 min for 35 cycles and final extension at 72°C for 10 min. PCR products were analyzed by resolving on 1.5 % agarose gel and DNA bands were visualized by ethidium bromide staining.

SDS-PAGE and Western blotting. SDS-PAGE and Western blot were performed as described previously (238). Protein concentrations of cell lysates were determined by Bradford method. The cell lysates were resolved on 12% SDS-PAGE gel electrophoresis under reducing conditions. Proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell) and the membrane was blocked with 5% skim milk in PBS. The membranes were probed separately with rat anti-murine CD23 mAb B3B4 or murine anti-β-tubulin antibody for 1 hr at room temperature or overnight at 4°C and then incubated with HRP-conjugated rabbit anti mouse antibody. All blocking, incubation, and washing procedures were performed in PBST solution (PBS and 0.05% Tween 20). Proteins were visualized using ECL method (Pierce).

Preparation of primary bronchoalveolar and tracheal epithelial cells. The bronchoalveolar epithelial cells were isolated as previously described (240). The tracheal epithelial cells were isolated by following the procedures as described previously (241). Briefly, the bronchoalveolar and tracheal epithelial cells were isolated by enzymatic digestion of lung with dispase (2.4 units/ml in DMEM) and trachea with pronase (1.5 mg/ml in Ham's F-12 pen-strep) and the remaining procedures were performed as described previously (240, 241).

Immunohistochemistry. Immunohistochemistry was performed as previously described (238). Mouse lung and trachea were inflated and embedded with OCT medium and cryosectioned at 5 μM thickness. Serial cryosections of the frozen tissue were fixed and permeabilized with ice-cold acetone for 20 min and blocked with 10% normal goat serum

for 1 hr at room temperature. Tissue sections were incubated with affinity purified rabbit anti-murine CD23 antibody, FITC-conjugated pan-cytokeratin antibody, FITC-conjugated goat-anti mouse IgE antibody or mouse anti-chicken OVA antibody in a humidified chamber overnight at 4°C followed by alexa-fluor 555-conjugated goat anti mouse antibody or alexa fluor 555- or 633-conjugated goat anti-rabbit antibody. All incubations were performed with 3% normal goat serum and finally nuclei were counterstained with DAPI (0.5 g/ml, Molecular Probes) in PBS. Negative controls were performed by incubating the isotype-matched mouse or normal rabbit IgG as primary antibody. Cover slips were mounted on the tissue sections with Prolong antifade reagent (Molecular probes, Invitrogen) and visualized and images were taken using Zeiss LSM510 laser scanning confocal microscope (Zeiss Microimaging Inc., NY). Images were processed by Zen 2007 software and Adobe Photoshop.

In-vivo transcytosis and enzyme-linked immunosorbent assay (ELISA). The Ova-specific IgE and chicken OVA were measured with ELISA. ELISA plates (Nunc) were coated with rabbit anti chicken OVA antibody (10 μg/ml) for detecting OVA or coated with chicken OVA (10 μg/ml) for detecting OVA-specific IgE overnight at 4ºC. Plates were then washed three times with PBST (0.05% tween- 20 in PBS) and blocked with 10 % FBS in PBS for 1 hr at room temperature. In-vivo transcytosis was performed with a set of WT and CD23KO mice on *Balb/c* background. Mouse anti-OVA IgE (50 μg/40μl) in PBS was given either intranasally or intraperitoneally and serum samples or bronchoalveolar lavage (BAL) fluid were collected 4, 8 or 24 hr later, respectively. Immune complex formed with mouse anti-OVA IgE (20 μg) and chicken OVA (10 μg/40

μl) in PBS for 30 min at room temperature or chicken OVA antigen alone (10 μg/40 μl) in PBS was given intranasally and 8 hr later serum samples were collected. The transcytosed serum or BAL samples, IgE or OVA standards diluted in 10% FBS in PBS were incubated for 2 hr at room temperature. Goat anti-mouse IgE (1:1000, Novus Biologicals) and HRP-conjugated bovine anti goat antibody (1:10,000, Santa cruz) were used for detection of mouse IgE. For detection of chicken ovalbumin, biotinylated mouse anti-OVA IgE (1:10,000) and HRP-conjugated Streptavidin (1:250, BD) were used. Total IgE was calculated by using OptEIA mouse IgE ELISA kit following manufacturer's protocol (BD). A colorimetric assay was done with tetra methyl benzidine (TMB) and hydrogen peroxide and a Victor III microplate reader (Perkin Elmer).

Bone-marrow chimera model development. CD23KO mice on *Balb/c* background were irradiated with 800 Rads and repopulated with 5 x 10^6 WT bone-marrow derived cells given intravenously via tail vein. Repopulation with WT cells in the reconstituted mice was estimated by analyzing CD23 expression in the B cells, total T cells in spleen or B cells in bone marrow by flow cytometry as above described.

Flow cytometry. Surface expression of CD23 on the fixed splenic B cells isolated from either *Balb/c* WT or CD23KO mice were analyzed by flow cytometry. Presence of CD11c and Siglec-F +/- cells in the brochoalveolar lavage obtained from OVA sensitized and challenged *Balb/c* WT or chimeric CD23KO mice were also analyzed by flow cytometry. For staining, 1 X 10^6 spleen B cells or 1 X 10^5 BAL cells (pooled from 5 mice) were washed with FACS washing buffer (2% FBS in PBS) and followed by

blocking mouse Fc binding with mAb to CD16–CD32 on ice for 30 min. The cell suspensions were respectively incubated with murine FITC conjugated anti-CD23 B3B4, FITC-conjugated rat IgG2a, APC-Cy7-conjugated anti-B220 antibody, and APC-Cy7-conjugated rat IgG2a antibody, FITC hamster anti mouse CD11c, PE rat anti mouse siglec-F, FITC hamster IgG1, PE rat IgG2a for 30 min at 4°C, then washed and analyzed using a FACSAria II and the software FlowJo.

OVA sensitization and challenge: The mice were sensitized with chicken ovalbumin by mixing with alum and given intraperitoneally. The sensitization consists of 100 μg OVA mixed with 4 mg Alum given on day 0 and 100 μg ova diluted in PBS on day 7 and 14. In some experiments, the mice were sensitized for single time with 100 μg OVA mixed with 4 mg Alum given on day 0. On day 21, the mice were challenged with 1% aerosol OVA and 24 hr later, the mice were sacrificed to collect BAL fluid, serum, and lung tissue for histopathology. BAL fluid was subjected to cytospin and stained with modified wright-giemsa stain (Sigma) for counting differential cells.

Statistics. The statistical difference between groups was tested by Student *t* test. A *P* value of less than .05 was considered significant. Data are expressed as mean ± SD.

RESULTS

Mouse respiratory epithelial cells express CD23. Epithelial cells isolated from mouse lung, trachea, and lung epithelial cell line LA4 were used in the analysis of mouse CD23 expression. CD23 protein was detected by blotting with CD23-specific B3B4 antibody

(242). The lung and tracheal airway epithelial cells isolated from naive mice expressed a protein band (49 kDa) identical to that of spleen positive control (Figure 3.1A). CHO cell lysate was included as a negative control (Figure 3.1A). Similar to the human CD23, mouse CD23 also exists in two isoforms, CD23*a* and CD23*b* (76, 142). To verify which isoform is expressed, the analysis of mouse CD23 mRNA expression by RT-PCR in mouse lung epithelial cell line LA4 was performed. Results showed that CD23b, but not CD23a mRNA, was detected (Figure 3.1B). The PCR products were further sequenced to confirm the specificity of CD23b mRNA by sequence analysis. The expression of CD23b mRNA in mouse lung epithelial cells is in agreenment with CD23 expression pattern in human airway epithelial cells (238). Since airway allergic inflammation is mediated by Th2 cytokine IL-4 and IL-13, for example, IL-4 has been shown to enhance the expression of CD23 in B cells, respiratory and intestinal epithelial cells (141, 219, 238). Mouse lung epithelial cells LA4 treated with IL-4 resulted in the enhanced expression of CD23b specific mRNA (Figure 3.1B, lane 3, top panel). Mouse intestinal epithelial cells expressed CD23b mRNA lacking exon 5 (CD23bΔ5) and binds to the IgE with high affinity (142, 143). However, we failed to detect the expression of CD23bΔ5 mRNA in mouse lung epithelial cells LA4 (Figure 3.1B, middle panel). Immunohistochemistry analysis of positive control, spleen, revealed the expression of CD23, but not for the normal rabbit IgG (Figure 3.1C) confirming the specificity of CD23 antibody used in this experiment. Further, CD23 was expressed in the naive lung and trachea; however, CD23 staining was negative when normal rabbit IgG was used (Figure 3.1D & 3.1E). Cytokeratin staining was used as a specific marker for lung and tracheal epithelial cells. As shown in the Figure 3.1D & 3.1E, cytokeratin (green) and mouse CD23 (red) were

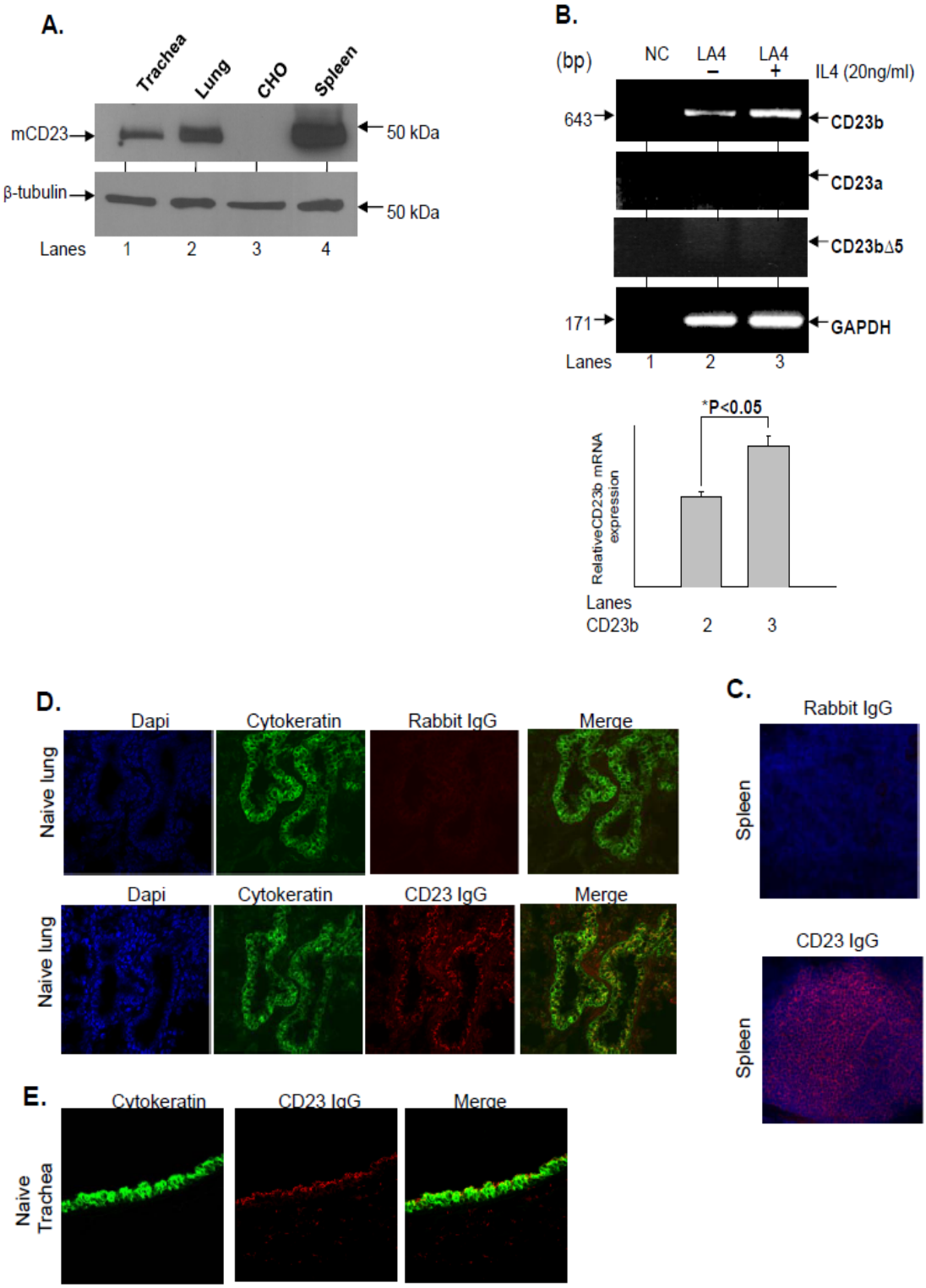

A.
Trachea
Lung
CHO
Spleen
mCD23
50 kDa
β-tubulin
50 kDa
Lanes 1 2 3 4
B.
(bp)
NC
LA4 –
LA4 +
IL4 (20ng/ml)
643
CD23b
CD23a
CD23bΔ5
171
GAPDH
Lanes 1 2 3
*P<0.05
RelativeCD23b mRNA expression
Lanes
CD23b 2 3
D.
Dapi
Cytokeratin
Rabbit IgG
Merge
Naive lung
Dapi
Cytokeratin
CD23 IgG
Merge
Naive lung
C.
Rabbit IgG
Spleen
CD23 IgG
Spleen
E.
Cytokeratin
CD23 IgG
Merge
Naive Trachea

Figure 3.1 Mouse respiratory epithelial cells express CD23.
(**A**) CD23 protein was expressed by mouse respiratory tracheal and lung epithelial cells. The cell
lysates (50 μg) from naïve tracheal epithelial cells (lane 1), naïve lung epithelial cells (lane 2), CHO cells (lane 3) and spleen (lane 4) were gel electrophoresed and separated on 12 % SDS-PAGE gel under reducing condition. The separated proteins were transferred on nitrocellulose membrane, blocked and blotted with rat anti mouse CD23 antibody B3B4. The blots were washed and further incubated with HRP conjugated rabbit anti rat IgG antibody and the protein bands were visualized by ECL method. The arrow indicates the location of mouse CD23 and β-tubulin.
(**B**) CD23b specific mRNA but not CD23a, isoform was expressed in mouse lung epithelial cells LA4. TRIzol reagent was used to extract total RNA from untreated lung epithelial cells LA4 (lane 2), IL4 (20 ng/ml) treated LA4 cells (lane 3), negative control without the addition of mRNA template (lane 1). RT-PCR was performed to amplify CD23a, CD23b, CD23bΔ5 and GAPDH using gene specific primers as described in *Materials and Methods*. Amplified PCR products were electrophoresed in 1.5% agarose gels and stained with ethidium bromide. The arrow indicates amplification products for mouse CD23 and GAPDH. Densitometry analysis of CD23b band intensities normalized to GAPDH was presented in the bottom panel. *P<0.05.
(**C-E**) Immunohistochemistry staining of mouse spleen, lung and trachea. The naive mouse spleen, lung and tracheal tissue were mounted in OCT medium and cryosectioned at 5 μM thickness. The frozen tissue sections were fixed and permeabilized with ice-cold acetone and blocked with 10% normal goat serum. For the positive control, spleen sections were stained. The sections were incubated with rabbit anti CD23 antibody or normal rabbit IgG, followed by staining with alexa flour 555-conjugated goat anti rabbit antibody and FITC conjugated anti cytokeratin antibody. The nucleus was stained with DAPI and imaged using LSM510 confocal microscope. Samples were visualized under same contrast and brightness setting.

colocolized and merged (yellow), suggesting CD23 specific expression in epithelial cells.

Characterization of OVA-sensitized mice. Wild-type mice were sensitized with OVA or left untreated, followed by challenge with 1 % aerosol OVA in PBS and characterized for the development of allergy. Results from PAS (Periodic Acid Schiff) staining showed the presence of PAS-positive mucin staining in epithelial cells only from the sensitized mice in comparison with the epithelial cells from naive animals (Figure 3.2A). Total IgE detected in the serum was significantly higher in the sensitized mice compared with the naive mice; in addition, ova specific IgE was detected in the serum of sensitized mice but not in that of the naive mice (Figure 3.2B & 3.2C). Bronchoalveolar lavage (BAL) fluids were collected either 24 or 72 hr later from OVA sensitized or naive mice which were aerosolly challenged with 1% OVA. At 24 hr time point, significant amount of total IgE, OVA specific IgE and cytokine IL-4 were detected only in the OVA sensitized and challenged mice, when compared to the non-sensitized and OVA challenged mice (Figure 3.2D, 3.2E & 3.2F). 72 hr later after challenge, BAL fluid contains significantly higher amount of total leukocytes, eosinophils, total and OVA specific IgE in the OVA sensitized and challenged mice, when compared to the non-sensitized and OVA challenged mice (Figure 3.2G, 3.2H, 3.2I & 3.2J). OVA specific IgE was completely absent in the non-sensitized and OVA challenged mice. Taken together, sensitization of mice with OVA leads to the development of allergic inflammation in a mouse model.

CD23 expression is enhanced in the sensitized mice. *Balb/c* mice were left untreated or sensitized with OVA. In general, sensitization activates the Th2 type immune response

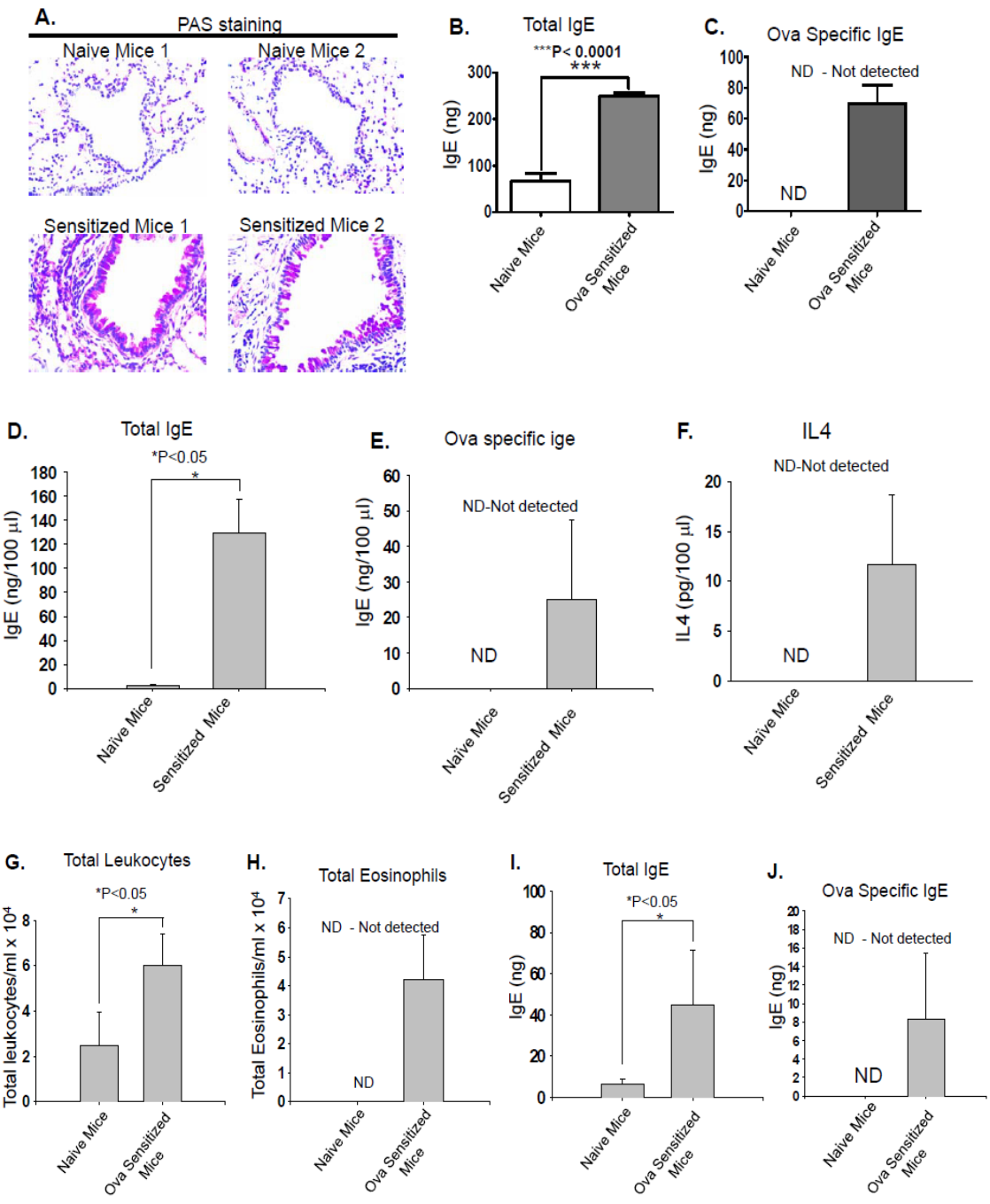

A.
PAS staining
Naive Mice 1
Naive Mice 2
Sensitized Mice 1
Sensitized Mice 2
B.
Total IgE
***P< 0.0001
***
IgE (ng)
Naive Mice
Ova Sensitized Mice
C.
Ova Specific IgE
ND - Not detected
IgE (ng)
ND
Naive Mice
Ova Sensitized Mice
D.
Total IgE
*P<0.05
*
IgE (ng/100 µl)
Naive Mice
Sensitized Mice
E.
Ova specific ige
ND-Not detected
IgE (ng/100 µl)
ND
Naïve Mice
Sensitized Mice
F.
IL4
ND-Not detected
IL4 (pg/100 µl)
ND
Naive Mice
Sensitized Mice
G.
Total Leukocytes
*P<0.05
*
Total leukocytes/ml x 10⁴
Naive Mice
Ova Sensitized Mice
H.
Total Eosinophils
ND - Not detected
Total Eosinophils/ml x 10⁴
ND
Naive Mice
Ova Sensitized Mice
I.
Total IgE
*P<0.05
*
IgE (ng)
Naive Mice
Ova Sensitized Mice
J.
Ova Specific IgE
ND - Not detected
IgE (ng)
ND
Naive Mice
Ova Sensitized Mice

Figure 3.2 Characterization of OVA-sensitized mice.
(**A**). PAS staining of sensitized and naive lung sections. Balb/C mice were sensitized with ova or left untreated and challenged with 1% aerosol ova. Lung tissue was fixed with 10 % neutral buffered formalin and paraffin embedded sections at 5 μM thickness, followed by deparaffinization and hydration. The sections were oxidized with periodic acid solution and stained with Schiff reagent. Samples were visualized and imaged with 40x oil immersion microscope.
(**B**)-(**C**). Detection of total and ova-specific IgE. Serum was collected from the sensitized and naïve mice
21 days after the first immunization with ova and alum. Serum were tested and measured for total and ova-specific IgE by ELISA.
(**D**)-(**F**). Detection of total IgE , ova-specific IgE and IL4 in BAL. Mice were sensitized with ova or left untreated. On day 21, the mice were challenged with 1% aerosol ova and 24 hours later, mice were sacrificed with avertin and BAL fluid were collected. Total IgE, Ova-specific IgE and IL4 were measured in the BAL fluid by ELISA.
(**G**)-(**J**). Detection of total leukocytes, eosinophils, total IgE, ova-specific IgE and IL4 in BAL. Mice were sensitized with ova or left untreated. On day 21, the mice were challenged with 1% aerosol ova and 72 hours later, mice were sacrificed with avertin and BAL fluid were collected. BAL fluids were cytospin centrifuged followed by staining with modified wright-giemsa stain and total leukocytes and eosinophils were counted. Total IgE, Ova-specific IgE and IL4 were measured in the BAL fluid by ELISA.

with the elevated level of IL-4, IL-13, and allergen specific IgE. The IL-4 enhances the expression of CD23 in B cells, monocytes, intestinal and respiratory epithelial cells (105, 141, 219, 238). To verify this enhancement in mouse airway, the lung and tracheal airway epithelial cells freshly isolated from the sensitized and naive mice were blotted for the CD23. Results from the western blot showed that CD23 protein was significantly up-regulated in the lung and tracheal epithelial cells from the OVA sensitized, but not the naive mice (Figure 3.3A & 3.3B). Immunohistochemistry analysis of the lung and trachea in the sensitized mice also revealed that expression of the CD23 protein (red) co-localized with epithelial cell marker cytokeratin (green), which showed by a yellow color (Figure 3.3C).

Genotyping and characterization of CD23 KO mice. CD23KO mice on *Balb/c* background were received from Dr. Daniel Conrad (Virginia Commonwealth University, VA). Genotyping of the CD23KO mice was performed by PCR amplifications. In previous study, two specific primer pairs were designed to differentiate the wild-type and CD23 KO mouse (239). The genomic DNA isolated from mouse tail was used as a template. PCR reaction amplified a 1033 bp DNA fragment indicative of wild-type CD23 allele, whereas 1104-bp DNA band represented the genotype from CD23 KO mice (Figure 3.4A). Surface expression of the CD23 in mouse spleen B cells was further analyzed by flow cytometry. CD23-positive B cells are defined as $B220^{+}CD23^{+}$. They were expressed only in the wild-type splenocytes and but not in B cells from CD23KO spleen, confirming that CD23KO mice does not express CD23 (Figure 3.4B).

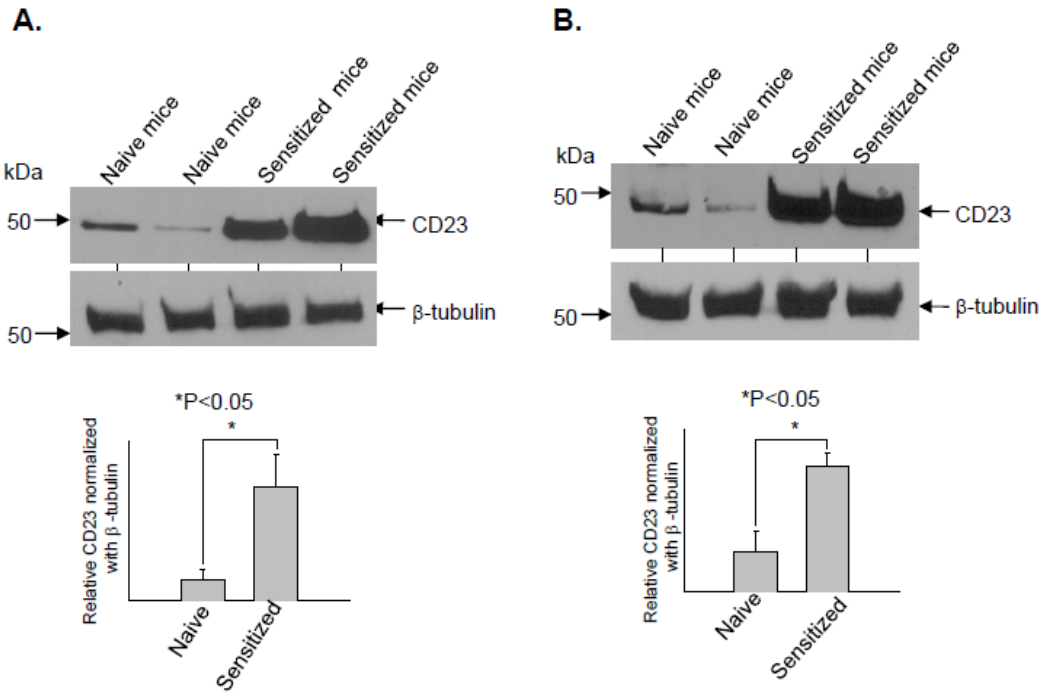

A.
Naive mice
Naive mice
Sensitized mice
Sensitized mice
kDa
50
CD23
50
β-tubulin
*P<0.05
*
Relative CD23 normalized with β -tubulin
Naive
Sensitized
B.
Naive mice
Naive mice
Sensitized mice
Sensitized mice
kDa
50
CD23
50
β-tubulin
*P<0.05
*
Relative CD23 normalized with β -tubulin
Naive
Sensitized
C.
CD23
Cytokeratin /CD23
Sensitized Lung
CD23
Cytokeratin/CD23
Sensitized Trachea

Figure 3.3 Sensitization augments CD23 expression.
(**A**)-(**B**). Mice were sensitized with ova or left untreated. Epithelial cells of lung and trachea from the naive and sensitized mice were isolated as described in Materials and methods. The cell lysate (50 μg) from naïve trachea or lung (lanes 1 &2) and sensitized trachea or lung (lanes 3 & 4) were gel electrophoresed and separated on 12 % SDS-PAGE gel under reducing condition. The separated proteins were transferred on nitrocellulose membrane, blocked and blotted with rat anti mouse CD23 antibody B3B4. The blots were washed and further incubated with HRP conjugated rabbit anti rat IgG antibody and the protein bands were visualized by ECL method. The arrow indicates the location of mouse CD23 and β-tubulin. Densitometry analysis of CD23 band intensities normalized to the β-tubulin band intensities were presented in the bottom panel. *$P<0.05$.
(**C**). Immunohistochemistry staining of CD23 expression. The sensitized mouse lung and tracheal tissue were mounted in OCT medium and cryosectioned at 5 μM thickness. The frozen tissue sections were fixed and permeabilized with ice-cold acetone and blocked with 10% normal goat serum. The sections were incubated with rabbit anti CD23 antibody followed by staining with alexa flour 555-conjugated goat anti rabbit antibody and FITC conjugated anti cytokeratin antibody. The nucleus was stained with DAPI and imaged using LSM510 confocal microscope. Samples were visualized under same contrast and brightness setting.

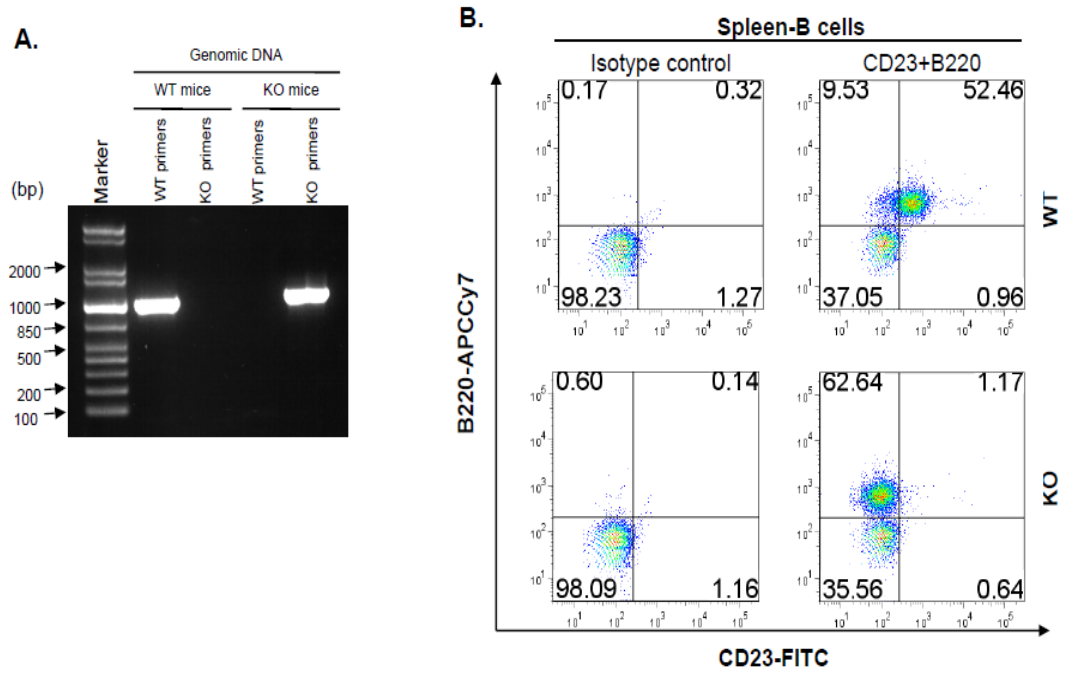
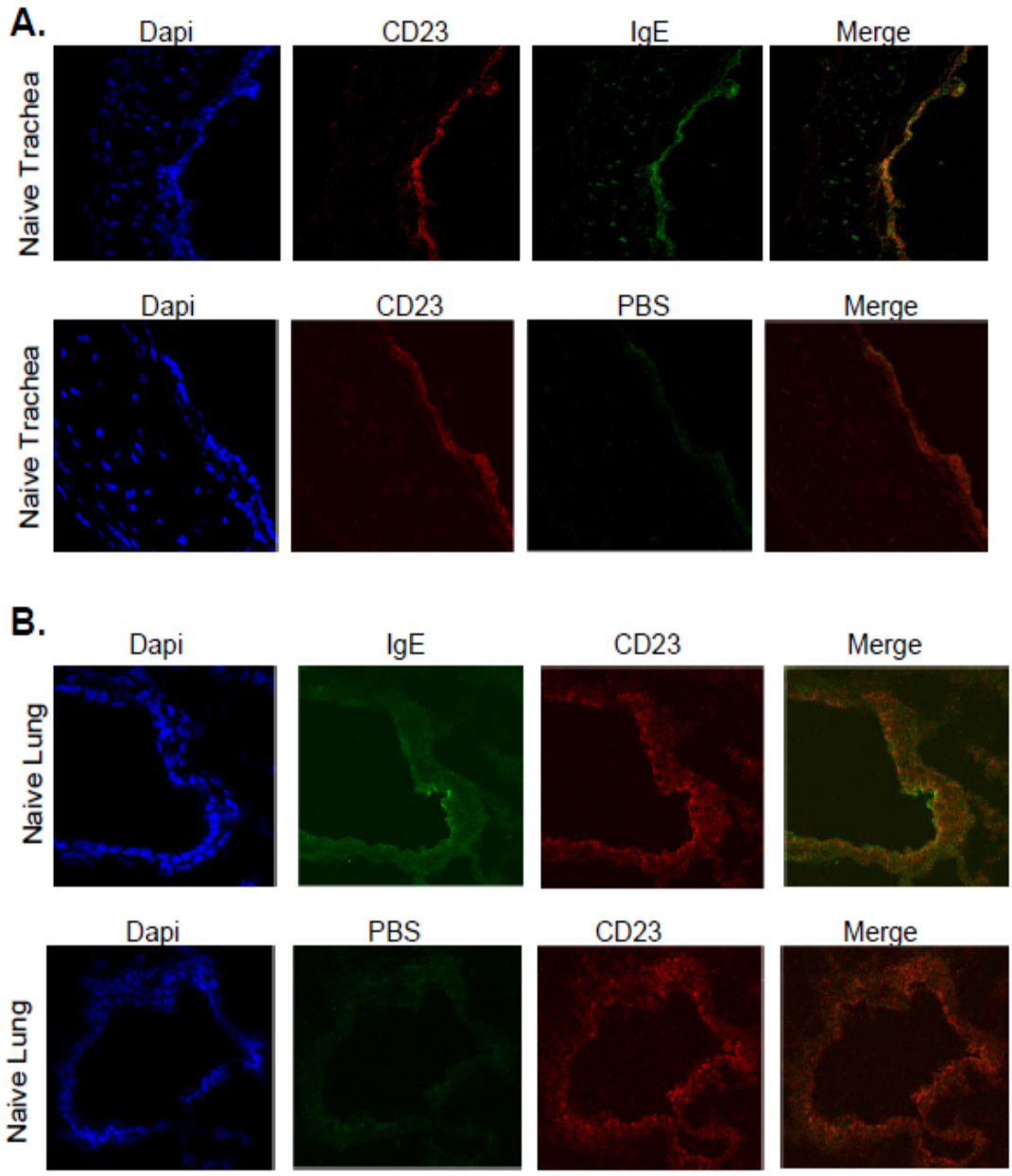


Figure 3.4 Genotyping and characterization of CD23 KO mice.
(**A**). Genotyping of CD23KO mice. Genomic DNA was isolated from the tail of wildtype and CD23KO mice. PCR was performed with Wild type and CD23KO mice genomic DNA using either wild type or Knockout primers as described in materials and methods. Amplified PCR products were electrophoresed in 1.5% agarose gels and stained with ethidium bromide.
(**B**). Flow cytometry analysis of CD23 expression. Cell surface expression of mouse CD23 in fixed spleen B cells of Wild type or CD23KO mice was measured by flow cytometry analysis. CD23 positive B cells, defined as $B220^{+}CD23^{+}$ cells were analyzed by FACS. Spleenocytes (1×10^{6}) were stained with FITC-conjugated B3B4 and APC-Cy7 conjugated B220 antibody or its respective isotype control antibodies. The values in the quadrant represent the percentage of $B220^{+}CD23^{+}$ spleen B cells.

Mouse CD23 transcytoses IgE in vivo. Human CD23 has been shown to bidirectionally transport IgE in the polarized airway epithelial cells (238). To further show IgE transcytosis in mouse airway, mouse was inoculated intra-nasally with IgE or PBS (20 μg/ml). 20 min later, the lung and trachea tissue were sampled for cryosections. The interaction between CD23 and IgE was shown by immunohistochemistry staining. As shown in Figure 3.5A, CD23 (red) and mouse IgE (green) were detected and colocalized (yellow) in a merged picture. In a PBS control group, we failed to detect such co-localization. The green color represented mouse IgE was also detected in the lamina propria (Figure 3.5A), suggesting an IgE antibody transferring the airway barrier. Similar to the tracheal staining CD23 (red) and mouse IgE (green) in the lung tissue were also colocalized (yellow) and such colocalization signal was absent in PBS control group (Figure 3.5B). To further show mouse CD23 transcytoses IgE, different amounts of mouse IgE were intra-nasally administrated and serum collected at specific time points. To mimic the apical to basolateral transcytosis, 20 or 50 μg/ml OVA-specific IgE or PBS was applied intra-nasally to wild type and CD23 knockout mice, 4, 8, or 24 hr later, serum was collected and tested for appearance of OVA specific IgE by ELISA. When 20 μg/ml level of IgE was applied intra-nasally, approximately 22 ng/ml and 18 ng/ml amount of mouse IgE were detected 4 hr and 8 hr later respectively only in wild type mice but not in the CD23 knockout mice. 24 hr later, no IgE was detected in both wild-type and CD23 knockout mice (Figure 3.5C, top panel). IgE transcytosis in vivo exhibited in a dose dependent manner. When 50 μg/ml mouse IgE was applied intranasally, there was significantly increased amount of mouse IgE detected in sera of the wild type mice compared to that of CD23 knockout mice. At 4 and 8 hr time point,

significantly higher amount of 90-100 ng/ml mouse IgE was detected in the wild type mice compared to 20 ng/ml of mouse IgE in the CD23 knockout mice. However, at 24 hr time point, 2 out of 5 mice sera were positive and 3 ng/ml of IgE was detected in wild type mice but not in CD23 knockout mice (Figure 3.5C, lower panel). To show whether mouse IgE is transcytosed from basolateral to apical direction, 20 or 40 μg/ml mouse anti-OVA IgE was injected intra-peritoneally and 8 hr later bronchoalveolar lavage fluids were collected for measuring mouse IgE by ELISA. When 20 and 40 μg/ml amount of mouse IgE were applied intra-peritoneally, significantly increased amount of mouse IgE was detected in the BAL in the wild type mice compared to that of CD23 knockout mice (Figure 3.5D). PBS was used as a background control in both wild type and CD23 knockout mice (Figure 3.5C & 3.5D).

CD23 transcytoses IgE derived immune complex across the epithelial barrier. CD23 and IgE are involved in the enhanced transepithelial antigen transport in intestinal epithelial cells of allergic rat and mice (140-142). In previous study, IgE immune complex is found in the BAL fluid of OVA sensitized and challenged mice and they are potent inducers of airway inflammation in comparison with antigen alone (231). However, this study did not show whether CD23 molecule is involved. To prove this, mice were intrnasally adminstrated with IgE/OVA immune complex. PBS was used as a negative control. To show the co localization of CD23 with IgE and Ova, an immunohistochemical staining was performed in lung tissue. The results showed that pair-wise co-localization of CD23 (red) with either IgE (green) or ova (green) produces the yellow in a merged picture (Figure 3.6A). Likewise, mouse IgE (green) co-localized


A.
Dapi
CD23
IgE
Merge
Naive Trachea
Dapi
CD23
PBS
Merge
Naive Trachea
B.
Dapi
IgE
CD23
Merge
Naive Lung
Dapi
PBS
CD23
Merge
Naive Lung

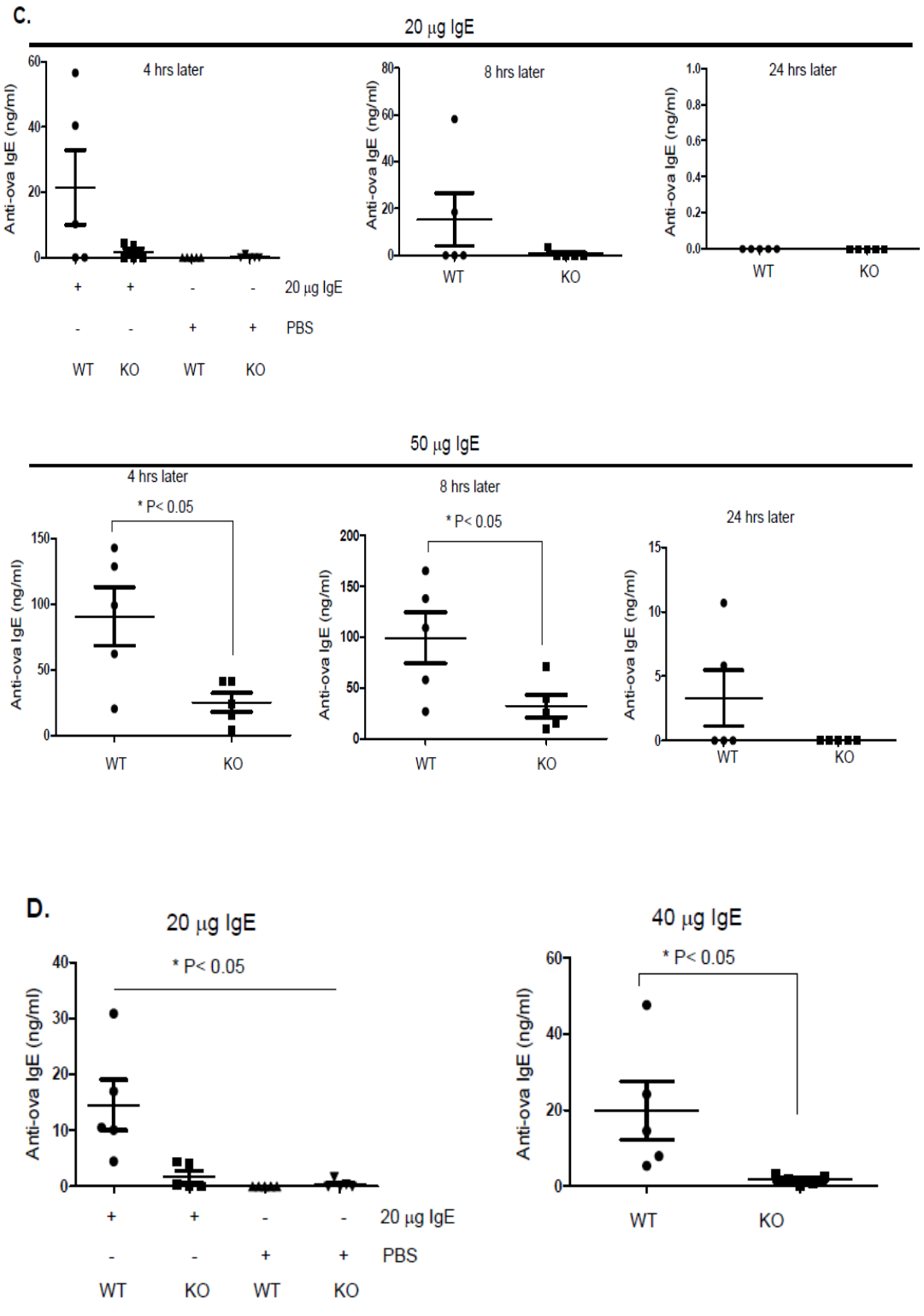
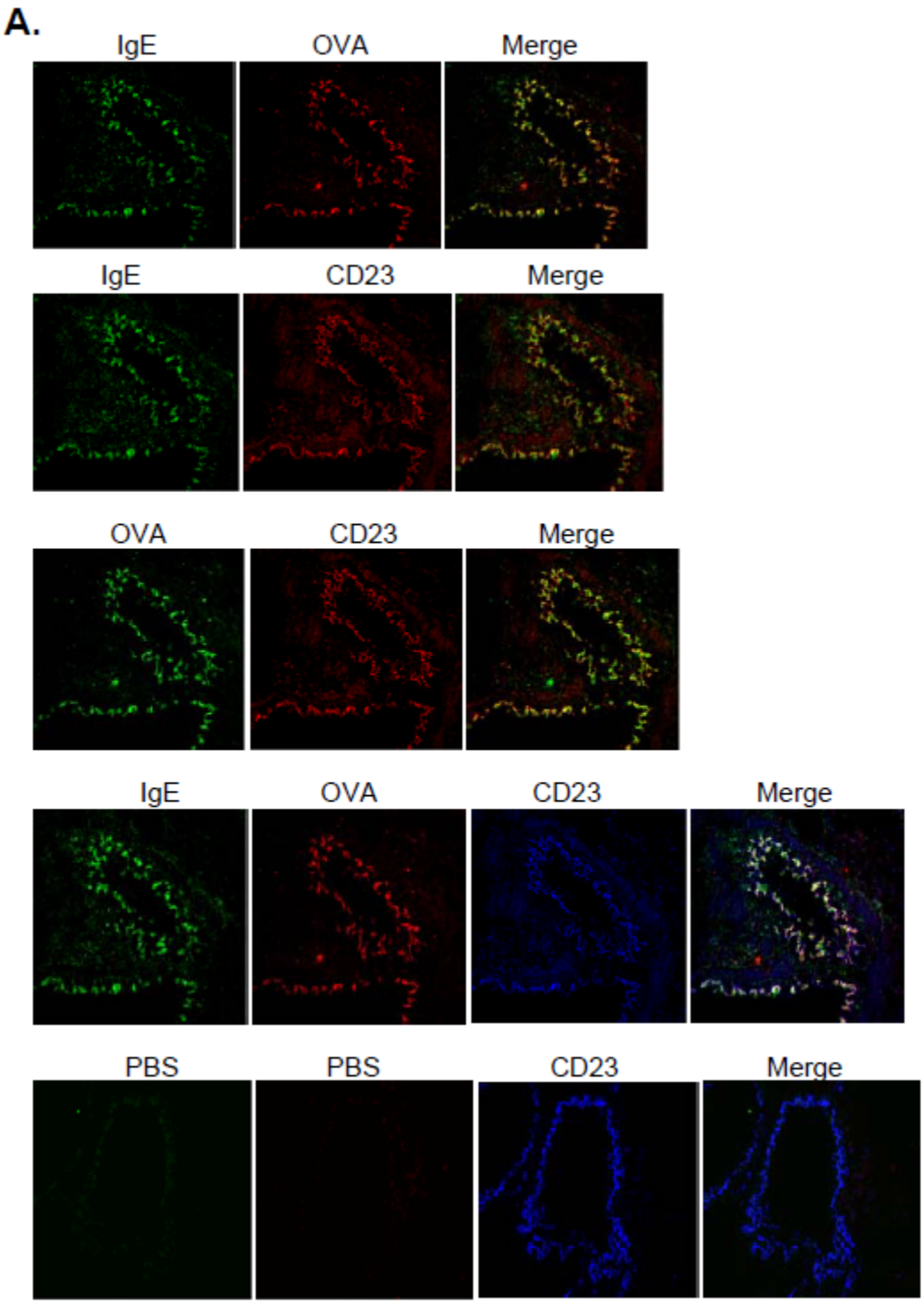

C.
20 μg IgE
4 hrs later
8 hrs later
24 hrs later
Anti-ova IgE (ng/ml)
20 μg IgE
PBS
WT
KO
50 μg IgE
* P< 0.05
D.
40 μg IgE

Figure 3.5 Mouse CD23 transcytoses IgE in vivo.
(**A**) & (**B**). Colocalization of CD23 and IgE in naive lung and trachea. Naïve mice were anaesthetized with avertin and 20 μg of mouse IgE or PBS was inoculated intranasally. Twenty minutes later, the mice were sacrificed and trachea and lung tissue were mounted in OCT medium and cryosectioned at 5 μM thickness. The frozen tissue sections were fixed and permeabilized with ice-cold acetone and blocked with 10% normal goat serum. The sections were incubated with rabbit anti CD23 antibody followed by staining with alexa flour 555-conjugated goat anti rabbit antibody and FITC conjugated goat anti mouse IgE antibody. The nucleus was stained with DAPI and imaged using LSM510 confocal microscope. Samples were visualized under same contrast and brightness setting.
(**C**). Apical to basolateral transcytosis of IgE in naive mice. Wildtype and CD23KO mice were inoculated with 50 μg of mouse anti-ova IgE or PBS intranasally. At specified time points like 4 hrs, 8 hrs and 24 hrs later, serum was collected from these mice and measured for the amount of anti ova IgE transcytosed by ELISA.
(**D**). Basolateral to apical transcytosis of IgE in naive mice. Wildtype and CD23KO mice were inoculated with 20 μg, 40 μg of mouse anti-ova IgE or PBS intraperitoneally. BAL fluid was collected 8 hours later from these mice and measured for the amount of anti ova IgE transcytosed by ELISA.


A.
IgE
OVA
Merge
IgE
CD23
Merge
OVA
CD23
Merge
IgE
OVA
CD23
Merge
PBS
PBS
CD23
Merge

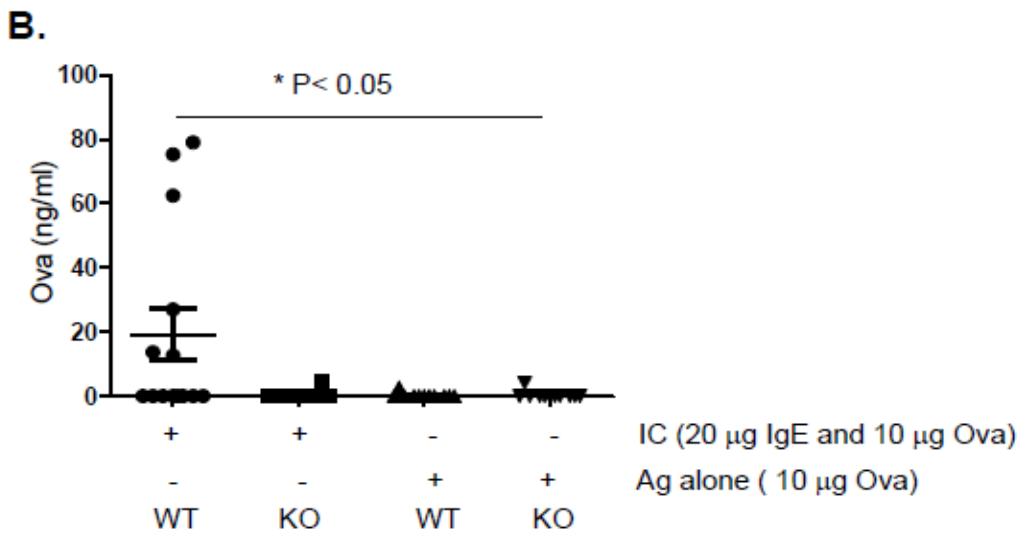


Figure 3.6 CD23 transcytoses IgE derived immune complex across the epithelial barrier.

(A). Colocalization of CD23 and immune complex in naive lung. Immune complex was formed with 20 μg anti Ova IgE and 10 μg of ovalbumin at room temperature for 30 minutes. Naive mice were anaesthetized with avertin and immune complex or PBS was inoculated intranasally. Twenty minutes later, the mice were sacrificed and lung tissue was mounted in OCT medium and cryosectioned at 5 μM thickness. The frozen tissue sections were fixed and permeabilized with ice-cold acetone and blocked with 10% normal goat serum. The sections were incubated with rabbit anti CD23 antibody and mouse anti chicken ovalbumin antibody followed by staining with alexa flour 633-conjugated goat anti rabbit antibody, alexa flour 555-conjugated goat anti mouse antibody and FITC conjugated goat anti mouse IgE antibody. The nucleus was stained with DAPI and imaged using LSM510 confocal microscope. Samples were visualized under same contrast and brightness setting.

(B). Apical to basolateral transcytosis of immune complex in naive lung. Immune complex was formed with 20 μg anti Ova IgE and 10 μg of ovalbumin at room temperature for 30 minutes. Naive mice were anaesthetized with avertin and immune complex or 10 μg of antigen ovalbumin or PBS was inoculated intranasally. Eight hours later serum was collected and quantified for the presence of antigen ovalbumin by ELISA.

with Ova (red) produces the yellow (Figure 3.6A). Furthermore, co-localizations of mouse CD23 (blue), IgE (green), and OVA (red) were shown in white color (Figure 3.6A) by analyzing with three color confocal microscopy. As expected, PBS did not give any background staining (Figure 3.6A). Immune complex or antigen alone was applied intra-nasally, 8 hr later serum was tested for antigen OVA by ELISA. As results shown in Figure 3.6B, significant amount of OVA/IgE immune complex was transported in wild type mice compared to that of CD23 knockout mice (Figure 3.6B). However, OVA alone were not detected in both wild-type and CD23 knockout mice (Figure 3.6B). Taken together, these experiments showed that CD23 is required for specific transport of IgE complexed antigen.

Role of CD23 in allergy development. CD23 was shown to be upregualted in allergic patients (156, 161). Sensitization of mice also augmented the CD23 expression (Figure 3.3A & 3.3B). To investigate the role of CD23 in allergy development, wild-type and CD23 knockout mice were sensitized and challenged with OVA and BAL fluids were collected and analyzed. The level of IL-4 detected in wild type mice was significantly higher than that of CD23 KO mice (Figure 3.7A). However, there was no significant difference in the level of OVA specific IgE and the number of total leukocytes and eosinophils between wild type and CD23 knockout mice (Figure 3.7B & 3.7C). Because CD23 is widely expressed in a variety of cells, especially in myeloid cells, the expression pattern of CD23 in vivo may influence the experimental results.

To further show functional difference of CD23 in airway epithelial cells, between wild type and CD23 knockout mice in allergy development, we generated bone marrow

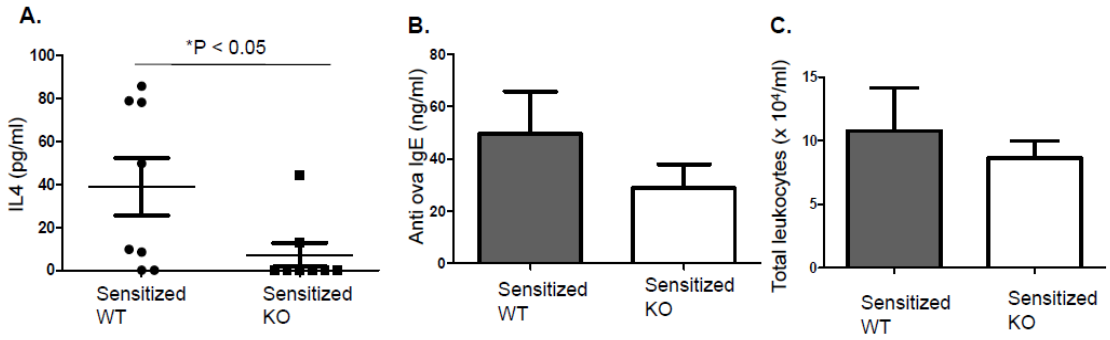


Figure 3.7 Role of CD23 in allergy development.
(A)- (C). Detection of IL4, anti-ova IgE, total leukocytes in BAL fluid. Wild type and CD23KO mice were sensitized with ova as described in materials and methods. On day 21, the mice were challenged with 1 % aerosol ova for 30 minutes and 24 hours later BAL fluid were collected and measured for IL4 and anti-ova IgE by ELISA. Total leukocytes present in the BAL fluid were counted. Cytospin centrifuge the leukocytes, stained with modified wright-giemsa stain and eosinophils were counted.

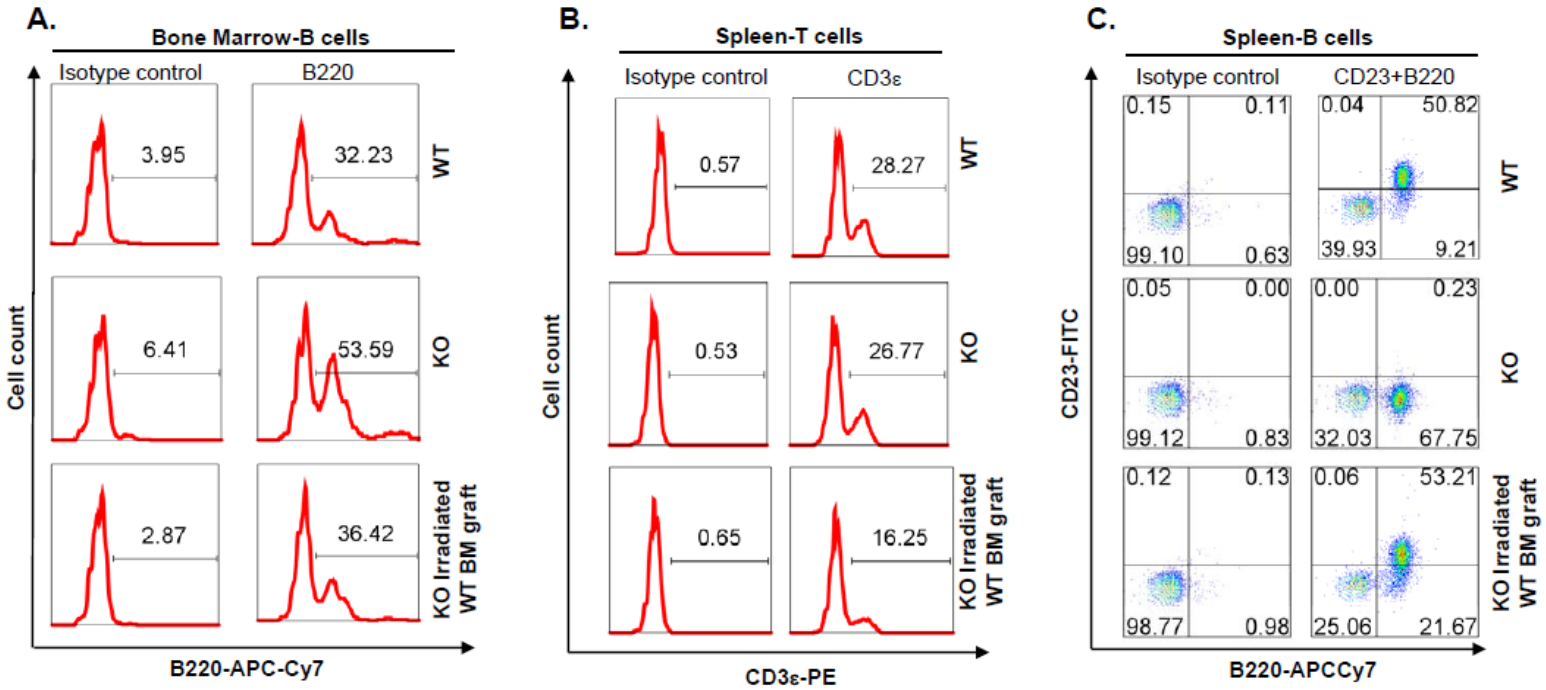

A.
Bone Marrow-B cells
Isotype control
B220
3.95
32.23
6.41
53.59
2.87
36.42
WT
KO
KO Irradiated WT BM graft
Cell count
B220-APC-Cy7
B.
Spleen-T cells
Isotype control
CD3ε
0.57
28.27
0.53
26.77
0.65
16.25
WT
KO
KO Irradiated WT BM graft
Cell count
CD3ε-PE
C.
Spleen-B cells
Isotype control
CD23+B220
0.15
0.11
99.10
0.63
0.04
50.82
39.93
9.21
0.05
0.00
99.12
0.83
0.00
0.23
32.03
67.75
0.12
0.13
98.77
0.98
0.06
53.21
25.06
21.67
WT
KO
KO Irradiated WT BM graft
CD23-FITC
B220-APCCy7

Figure 3.8 Characterization of irradiated CD23 KO mice repopulated with wild-type bone marrow cells.

(A)-(B). Flow cytometry analysis of B cells in bone marrow, T cells in spleen. Amount of B cells repopulated in bone marrow and T cells in the spleen of irradiated CD23KO mice grafted with 5 x 10^6 wild type bone marrow cells was measured by flow cytometry. Normal wild type and CD23KO mice were included as controls. Bone marrow cells or spleenocytes (1 x 10^6) were stained with APC-Cy7 conjugated B220 or PE conjugated CD3ε or its isotype controls, AP-Cy7 rat IgG2a or PE hamster IgG1. Results are expressed as histograms of fluorescence intensity (log scale). Values in the top right of each rectangle correspond to the proportion of cells stained positive with anti-B220 or anti CD3ε antibody relative to the control antibody.

(C). Flow cytometry analysis of CD23 surface expression in spleen B cells. Cell surface expression of mouse CD23 in fixed spleen B cells of irradiated CD23KO mice grafted with 5 x 10^6 wild type bone marrow cells were measured by flow cytometry. Normal wild type and CD23KO mice were included as controls. CD23 positive B cells, defined as $B220^+CD23^+$ cells were analyzed by FACS. Spleenocytes (1 x 10^6) were stained with FITC-conjugated B3B4 and APC-Cy7 conjugated B220 antibody or its respective isotype control antibodies. The values in the quadrant represent the percentage of $B220^+CD23^+$ spleen B cells.

chimera wherein, CD23 knockout mice were lethally irradiated and repopulated with wild-type bone marrow-derived myeloid cells, so that the chimera are expected to be CD23 negative in epithelium but their hematopoietic cells are positive in CD23 expression. Eight weeks after the irradiated CD23KO mice were repopulated with wild-type bone marrow cells, B cells present in spleen were stained positive for CD23 and its expression level was similar to that of wild type mice (Figure 3.8C). T cells in spleen and B cells in bone marrow (Figure 3.8A & 3.8B) were also detected.

To demonstrate the role of CD23 expressed exclusively in airway epithelial cells, the chimeric mice (WT→CD23KO) along with wild-type mice were sensitized and challenged. To maintain the intactness of the airway epithelial barrier, sensitization and challenge were performed in a mild condition only for single time. Although both groups of mice had equal number of total leukocytes present in the BAL by counting, flow cytometry analysis revealed significantly higher amount of approximately 36 % siglec-F+, 33 % CD11c+ Siglec-F+ macrophages and 4 % CD11c low/- SiglecF+ eosinophils in BAL obtained from wild-type mice when compared to the chimeric mice (WT→ CD23KO). More importantly, Th2 cytokine IL-4 was also significantly higher in wild-type mice BAL compared to the chimeric mice (WT→CD23KO). However, there was no significant difference between total and OVA specific IgE detected in the BAL fluids from both groups of mice. This may be due to the mild sensitization and challenge protocol employed to maintain the epithelial barrier. Take together, these experimental data suggests that CD23 expressed in the airway epithelium may be involved in the development of allergy and contributes to the early stages of allergy development. However, further experiments are needed to verify this conclusion.

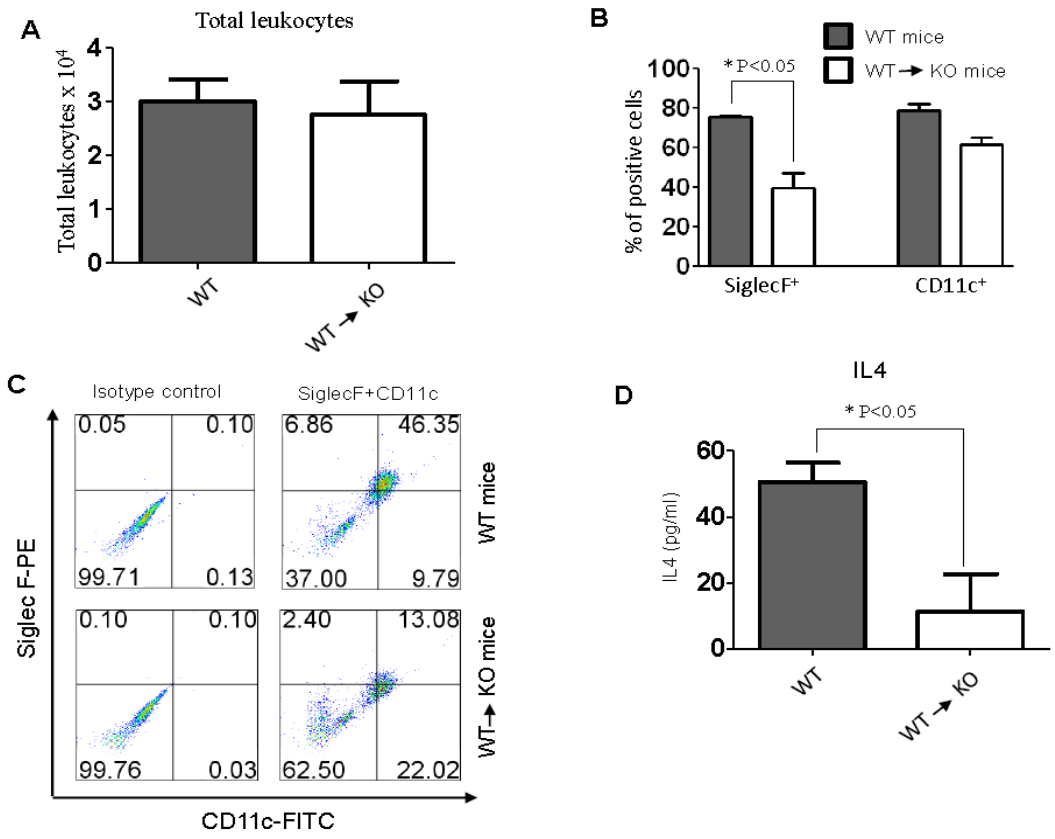


Figure 3.9 CD23 expressed in the respiratory epithelial cells enhances allergy. (A)- (D). Wild type and chimeric WT→CD23KO mice were sensitized with 100 mg of ova plus 4 mg alum given i/p on day zero. On day 21, the mice were challenged with 1 % aerosol ova for 15 minutes and 24 hours later BAL fluids were collected. (**A**). Total leukocytes were counted using hemocytomer. (**B**)-(**C**). Flow cytometric analysis of siglec F +, CD11c +, CD11c+ SiglecF+ macrophages, CD11clow/- SiglecF+ eosinophils. (**D**). IL4 present in BAL were counted by ELISA.

DISCUSSION

IgE mediated allergic diseases is more common and increasing in prevalence in developed country (1, 202). Allergic diseases involve Th2 mediated immune response with production of cytokines like IL-4, IL-13, allergen specific IgE, etc. (202, 233). The first step in the development of allergic disease is the process of sensitization to a particular allergen to elicit Th2 mediated immune response. Subsequent production of allergen specific IgE binds to the surface expressed FcεRI on mast cells and basophils. The development of allergic inflammations can be divided into two phases. The early phase in the development of allergic inflammation involves the inhalation of air borne allergen, followed by cross-linking of allergen specific IgE on mast cells and basophils results in degranulation and release of various mediators promoting inflammatory reactions (1, 202, 243). The late phase reactions involve synthesis and secretion of various cytokines and chemokines which are able to recruit a variety of immune cells including leukocytes, eosinophils, monocytes, etc. (1, 202). The airway epithelium acts as a major barrier for the inhaled allergen to engage with and activate immune cells in the lamina propria. Several mechanisms for airway allergen crossing epithelial barrier have been proposed, for example, the intrinsic nature of some allergens having proteolytic activity or the luminal allergens sampled by dendritic extensions between the epithelial cells from dendritic cells (244). Previously, we have showed that CD23 expressed in the human airway epithelial cells acts as a transepithelial transporter for IgE and IgE-derived immune complex (238). Here we further demonstrated the expression of CD23 in a mouse airway epithelium and it is capable of transporting IgE and IgE based immune complex across the airway epithelial barrier in vivo.

Mouse CD23 is expressed in airway epithelial cells isolated from the mice. We used various approaches to demonstrate the expression of CD23 at mRNA and protein level and immunohistochemistry analysis verified this expression. Similar to human CD23 expression pattern in airway epithelial cells (224), mouse lung epithelial cells LA4 also expressed CD23b but not CD23a mRNA. This result is in agreement with previous studies that CD23b is only expressed in mouse intestinal epithelial cells like IEC-4, enterocytes isolated from mouse jejunum (142). However, in situ hybridization may be useful to further demonstrate the expression patterns of CD23 mRNA in tissue levels of mice. Exposure of epithelial cells to Th2 cytokine IL-4 increases the CD23 mRNA expression (141, 238). Exposing the murine lung epithelial cell line LA4 to IL-4 also augmented the expression of CD23. Most importantly, sensitization of mice with antigen OVA significantly enhances CD23 expression in the airway epithelial cells of lung and tracheal (Figure 3.3). Immunohistochemistry analysis of mouse lung and trachea further verified the expression of CD23 protein in the epithelial cells by co-localizing with epithelial marker cytokeratin in vivo (Figure 1 & 3). Immunohistochemistry staining analysis of CD23 expression was also performed with anti-CD23 mAb B3B4 confirming the expression of CD23 in the lung and tracheal epithelial cells (data not shown). These data unequivocally suggest CD23 is expressed in mouse airway epithelial cells and may play a role in airway allergic inflammation.

CD23 has been shown to have multiple functions, which is involved in IgE-specific antigen processing and presentation (117-120), homeostasis of IgE (63, 125, 126, 128), and survival and growth of B cells, T-cells and myeloid precursors (131, 133-135). CD23 also mediates the bidirectional transcytosis of IgE in human respiratory epithelial

cells in vitro and in mouse intestinal epithelial cells (144, 145, 238). Here, we further showed CD23 is capable of transporting IgE across the epithelial barrier in mouse. This conclusion is supported by several evidences that CD23 co-localizes with mouse IgE in the airway epithelial cells and the sera of wild-type mice have significant amount of IgE after mouse was intranasally inoculated with IgE. There was a time-and dose-dependent increases in the amount of IgE transported across the airway epithelium (Figure 3.5). To show the specificity of IgE transcytosis by CD23, experiments performed with CD23 KO mice demonstrated the limited amount of IgE transported across the epithelial barrier in both directions (Figure 3.5). The functional significance of transepithelial transport of IgE across the airway epithelial barrier may allow IgE released into the airway lumen, subsequently IgE binds to allergen and leads to formation of the immune complex. Expression of CD23 allows the immune complex transported back to the lamina propria. This CD23-mediated transport mechanism may result in the release of intact allergen molecule by the epithelial cell (145), further allowing allergens to interact with immune cells underlying epithelial cells.

Previous studies have demonstrated that CD23 was capable of delivering the intact antigen in the form of IgE immune complex across human respiratory epithelial cells in vitro and in mouse intestinal epithelial cells in vivo (145, 238). Moreover, animal sensitization with HRP specifically increases the transepithelial transport of HRP across the tracheal epithelium (230) or the intestinal epithelium in the sensitized animals (140-142). Our data further showed that CD23 is capable of transporting the OVA/IgE immune complexes across the airway epithelium from apical to basolateral direction and the intact OVA can be detected in the serum. CD23 is shown to be involved in this specific

transport mechanism by two ways. First, antigen alone was not transported in either wild type or CD23 KO mice and second, CD23 KO mice did not transport OVA/IgE immune complex (Figure 3.6). Moreover, CD23 expressed on B cells is involved in the transport of IgE derived immune complex and enhance the antigen presentation (213, 227).

The roles of CD23 in airway allergy development remain unknown and information in the previous studies is mixed; studies with sensitization and challenge in CD23KO mice showed a significant increase in the allergic responses (170, 172, 173) and severe impairment of antigen specific IgE mediated immune response (122). However, CD23-transgenic mice exhibited suppression in IgE immune response (123). Sensitization and challenge with allergen for multiple times may compromise, damage and remodel the airway epithelium. Hence we reduced the sensitization and challenge to a single dose. Results from our experiments showed there was no significant difference exhibited between wild type and CD23KO mice in the development of allergy (Figure 3.7). This may be complexed by CD23 expression in a variety of cell types. To exclude the role of CD23 expressed in the hematopoietic cells which are important in regulating the allergic inflammation, we created chimeric CD23KO mice in which CD23 was not expressed in the airway epithelium but were positive in the hematopoietic cells. We generated chimeric knock out mice repopulated with wild-type derived bone marrow cells, showed the expression of CD23 in the chimeric CD23 KO spleen B cells similar to wild-type spleenic B cells, demonstrating that CD23 was negative in epithelial cells but expressed in hematopoietic cells (Figure 3.8). Sensitization and challenge of these mice have showed the involvement of CD23 in the early stages of allergy development. Although wild-type mice showed increased siglec-F+ cells, eosinophils, macrophages

and IL-4 in the BAL (Figure 3.9), however, we did not find significant difference in total and OVA specific IgE in the BAL. It is possible that a mild sensitization and challenge procedure in our study may be a contributing factor. Altogether, we showed that CD23 is expressed in the airway epithelium and responsible for the transport of IgE and antigen. Expression of CD23 in airway epithelial cells may be responsible in the early stages of allergy development, although more experiments and evidences are needed.

CHAPTER 4: CD23-MEDIATED IMMUNOTHERAPY TO AIRWAY INFLAMMATION

ABSTRACT

Allergic airway inflammations, such as asthma, are an increasingly important disease caused by bronchial inflammation and characterized by bronchial hyper-responsiveness and intermittent airway obstruction with an underlying Th2 cell-biased inflammatory response in the airways. Targeting of the functions of Th2 cells and their products have been proposed as an effective strategy for the development of potential stand-alone treatments for allergic asthma. The reduction or elimination of allergen-specific Th2 cells in early disease development is expected to reduce the consequences of repeated allergic inflammation. Hence, efficient delivery of immunotherapeutic proteins into the airway tract could effectively and directly interfere with allergen-specific Th2 cell activation in its earliest phase of function. However, the polarized epithelial monolayer lining the airway forms mucosal barrier which is impervious to macromolecule diffusion. This barrier poses a major difficulty for an efficient delivery of immunotherapeutic proteins to access and cross-talk with underlying immune effector cells, such as Th2 cells, in the airway. Our recent studies have shown that human CD23 receptor is functionally capable of transporting IgE antibody across lung and bronchial epithelial cells. In this study, we further propose to examine the feasibility of CD23 to deliver the immunotherapeutic proteins, CTLA4-Fc (IgE), which are targeted to interfere with CD4+ Th2 cell function, across airway mucosal barrier in a mouse allergy model.

The effect of this fusion protein on the development of allergic inflammation is being fully investigated in wild-type, CD23-KO, and chimeric mouse model.

INTRODUCTION

Epithelial monolayers lining the respiratory tracts are impervious to macromolecule diffusion in the absence of inflammation, due to the presence of intercellular tight junctions at the apical poles. Polarized airway epithelial cells are divided into apical and basolateral domains by tight junctions (148, 149). These domains further form mucosal barriers that allow for the selective exchange of macromolecules between the lumen and submucosal tissue under physiological conditions. Therefore, soluble macromolecules, such as immunotherapeutic proteins, across the mucosal epithelium are generally blocked by mucosal barrier. However, crossing of the macromolecules over the mucosal barrier can be mediated by a transcellular transport pathway, or transcytosis. Specifically, the transcellular pathway involves endocytic uptake of macromolecules, specifically by receptor-mediated and/or fluid-phase endocytosis, at the apical or basolateral membrane (148, 150). The molecules are then transported through the cell in endocytic vesicles to the opposite membrane surface, where they are released into the lumen or submucosal space. Beneath the epithelial lining, lymphocytes, mast cells, basophils, eosinophils, and dendritic cells accumulate in either a loosely-infiltrated fashion or an organized fashion forming the organized bronchus associated lymphoid tissue (BALT) (1, 148, 149). Therefore, the transcellular pathway is a major route in moving soluble macromolecules across the airway epithelial barrier and initiates a cross-talk between immunotherapeutic agents and the immune cells.

IgE is present in airway secretions; its level can be enhanced in persons who have allergic rhinitis or bronchial asthma (1, 206, 207, 234, 236). For many years, the presence of IgE in airway secretions has been simply believed to be transduced passively from the serum. The complete paucity of mechanistic studies of IgE transport in the airway has seriously hampered our understanding of allergic inflammation in the lung. Our recent study has found that human and murine respiratory epithelial cells express CD23, a receptor for the Fc portion of IgE. In comparison with high affinity IgE receptor FcεRI, CD23 was considered as a low affinity receptor for a long time. However, recent studies show that CD23 forms a trimer that allows its lectin domains to come within close proximity of each other and to cooperate in binding one IgE molecule, consequently resulting in CD23's high affinity binding to IgE antibody ($K_a \approx 10^8$ -10^9 M^{-1}) (1, 63, 64). Our recent studies have shown that CD23 is expressed in human airway epithelial cells (238). Most importantly, CD23 was functionally capable of transporting IgE antibody across human lung and bronchial epithelial cell lines or primary bronchial epithelial cells (238). Furthermore, we have demonstrated that mouse IgE was transported across airway mucosal barrier in wild-type, but not in CD23 knockout mice, when OVA allergen specific IgE molecules were intranasally administrated.

Airway inflammation and dysfunction is one of the most important human diseases in the developed world (202). The disease is currently treated with bronchodilators or anti-inflammatory drugs such as corticosteroids, leukotriene modifiers, and anti-IgE therapy, etc (2). However, the current treatments are not curative and some patients do not respond well to intense anti-inflammatory therapies. Additionally, the use of long-term steroids may result in many undesired side effects. For this reason, novel

and more effective intervening strategies are greatly needed and explored. Airway inflammation is characterized by activation of Th2 type CD4+ T cells secreting cytokines like IL-4, IL-5, granulocyte-macrophage colony-stimulating factor and eosinophil accumulation (233). The Th2 type cells contribute to IgE class switch and development of allergen specific IgE secreting plasma cells (236, 237). Thus, inhibiting or eliminating Th2 cells is a beneficial strategy for treating asthma as long as a generalized immunosuppression is avoided. Since airway epithelial cells are the first cell layer to come into contact with inhaled immunotherapeutic proteins, evidences of CD23-mediated IgE transport in polarized airway epithelial cells may lead to the possibility of CD23 transporting an immune therapeutic proteins, if fused to an IgE Fc fragment, across the airway barrier for serving as an immunotherapeutic purpose, consequently modulating or dampening the inflammations and hypersensitivity responses in the airway.

Cytotoxic T-lymphocyte antigen 4 (CTLA4) is a homodimer and expressed by activated, memory and regulatory T cells (245-248). CTLA4 binds to its ligand CD80 (also known as B7-1) and CD86 (also known as B7-2) with high affinity on the antigen presenting cells (APC) (249-251). Hence, CTLA4 is a negative regulator of T-cell activation, and its inhibitory effects can be accomplished by competition with CD28 for binding to CD80 or CD86 with a much high affinity on APCs, consequently causes inhibition of T cell activation and prevents the development of inflammatory diseases (252, 253). Mouse CTLA4-IgG Fc fusion protein immunotherapy inhibits airway eosinophilia, reduces airway hyperresponsiveness and prevents mild form of airway allergy in a murine model of allergic asthma (254, 255). In this study we generated the

mouse CTLA4-IgE Fc fusion protein. The impact of the fusion protein transport by CD23 on airway inflammation and hyper-reactivity was assessed in an ovalbumin (OVA)-based murine asthma model. CD23-mediated transport of immunotherapeutic proteins may be an important part of intervening strategy for the allergic inflammation. This study may offer effective treatment options for patients suffering with allergic diseases, such as asthma and chronic obstructive pulmonary disease.

MATERIALS AND METHODS

Antibodies, cells and reagents. Chinese hamster ovary (CHO) cell line was purchased from the American Type Culture Collection (ATCC, Manassas, Virginia). The RBL-2H3 cell line was a gift from Dr. Juan Rivera (NIH, Bethesda, MD). The CHO-mB7-1 and CHO-mB7-2 stable cell lines were obtained from Dr. Gordon Freeman (Harvard Medical School, Boston, MA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 15 % FBS, 1% L-glutamine, non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin. CHO cells harbouring the protein expression plasmid was also grown in complete DMEM supplemented with G418 (400 μg/ml). The cells were maintained in a humidified atmosphere at 37ºC containing 5% CO_2. Hamster anti-mouse CTLA4 mAb, clone UC10-4F10-11, was a kind gift from Dr. Kenneth Frauwirth, University of Maryland, College Park, MD and affinity purified mouse anti-ovalbumin (OVA) IgE was purchased from Bio X-cell (West Lebanon, NH). Rat anti-mouse IgE mAb, B1E3 was a gift from Dr. Daniel Conrad (Virginia Commonwealth University School of Medicine, VA). Proteinase inhibitor cocktail was purchased from Calbiochem (San Diego, CA). FITC-conjugated goat anti-mouse IgE polyclonal antibody

was obtained from Novus Biologicals (Littleton, CO). FITC-conjugated goat anti-hamster IgG was obtained from Rockland (Gilbertsville, PA). HRP conjugated bovine anti-goat IgG antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Biotinylation of mouse anti-OVA IgE antibody was performed with EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) following manufacturer's instructions.

Construction, expression, and purification of mouse CTLA4-IgE Fc fusion protein. The cDNA encoding the extracellular domain of mouse CTLA4 cDNA was amplified from total RNA isolated from mouse spleen using the primer pair (5'-CCGCGGATCCGCGATTTG CCCCCCAGCCATGGCTTGTCTTGGACTCCGGAGGTACAAAGC–'3 & 5'-AGATC CCGAGCCACCTCCTCCGGACCCACCCCCGCCTGATCCAGAATCCGGGCATGGTT C–'3). The antisense primer encodes a glycine and serine rich linker sequences for 14 codons (GSGGGGSGGGGSGS). The cDNA of mouse IgE Fc fragment coding the CH2, CH3 and CH4 domain was amplified from the total RNA extracted from SPE-7 hybridoma (kind gift from Zelig Eshhar, Weizmann Institute of Science, Rehovot, Israel) using the primer pair (5'- GGATCAGGCGGGGGTGGGTCCGGAGGAGGTGGCTCGGGA TCTATCACTGAGCCCACCTTG –'3 & 5'-ATCTCCGCTCGAGCGGCAGCTACAT GGAGGCCTAGGAGGGACGGAGGGAGGTGTT-'3). Here the forward primer for mouse IgE Fc has the complementary sequence for glycine and serine rich linker sequence. Fusions of mouse CTLA4 and mouse IgE Fc cDNA were performed with PCR-based gene assembly method. This fused DNA fragments was ligated into the pCDNA3 vector and the sequences were verified by DNA sequencing. The pCDNA3 plasmid containing the mouse CTLA4-IgE Fc cDNA fusion gene was transfected into the CHO cells and G418-resistant

colonies were selected and cloned for the secretion of mouse CTLA4-IgE Fc fusion proteins. SDS-PAGE and Western blotting, immunofluoresence and ELISA were used to verify the expression and secretion of fusion proteins. Cells secreting the highest amount of fusion proteins were cloned and purified by affinity chromatography using anti-mouse CTLA4 antibody 4F10-Sepharose beads or anti-mouse IgE antibody B1E3-Sepharose beads. Either 4F10 or B1E3 mAb was conjugated to the Cyanogen bromide-activated-Sepharose 4B beads following the manufacturer's instructions (Sigma, St. Louis, MO).

SDS-PAGE and Western blotting. SDS-PAGE and Western blot were performed as described previously (238). Protein concentrations of cell lysates were determined by Bradford method. The purified proteins or cell lysates were resolved on 12 % SDS-PAGE gel electrophoresis under reducing or non-reducing conditions. Proteins were electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell) and the membrane was blocked with 5% skim milk in PBS. The membrane was probed with goat anti-mouse IgE polyclonal antibody for 1 hr at room temperature or overnight at 4°C and then incubated with HRP-conjugated bovine anti-goat antibody. All blocking, incubation, and washing procedures were performed in PBST solution (PBS and 0.05% Tween 20). Proteins were visualized using enhanced chemiluminescence (ECL) method (Pierce).

Flow cytometry. Binding of mouse CTLA4-IgE Fc fusion proteins to mouse B7-1 (CD80), B7-2 (CD86) or FcεRI was analyzed by flow cytometry. For flow staining, 1 X 10^6 CHO, CHO-mB7-1, CHO-mB7-2 or RBL-2H3 cells were washed with washing buffer (2% FBS in PBS) and blocked with 3 % normal goat sera (Rockland,

Gilbertsville, PA) in PBS on ice for 30 min. The cell suspensions were incubated with mouse IgE or purified mouse CTLA4-IgE Fc fusion protein (2 μg/ml) for 30 min at 4°C, then washed and further incubated with FITC-conjugated goat anti- mouse IgE Ab (1:500) for 30 min at 4°C. Cells were then washed and analyzed using a FACSAria II and the software FlowJo.

Immunocytochemistry. Immunocytochemistry was performed as previously described (238). The control CHO and recombinant CHO cells were grown on coverslips to 80% confluence overnight. The cells were washed with cold PBS and fixed in 4% paraformaldehyde in PBS for 20 min, then washed and quenched with 100 mM glycine for 10 min. Then the cells were permeabilized with 0.1% Triton X-100 for 30 min and blocked with 3% normal goat serum. After washing, cells were incubated with goat anti-mouse IgE polyclonal antibody or affinity purified hamster anti-mouse CTLA4, 4F10 antibody (1 μg/ml) in 3 % normal goat serum in PBS for 1 hr and further incubated with FITC-conjugated goat anti-mouse IgE antibody or FITC conjugated goat anti-hamster IgG antibody (1:500) for 30 min. After each incubation, cells were washed with PBST (0.1% Tween 20 in PBS). The prolong antifade reagent (Molecular Probes) was used to mount the coverslips and the images were taken using Zesis fluorescent microscope (Zeiss Microimaging Inc., NY). Images were processed by Zen 2007 software and Adobe Photoshop.

Enzyme-linked immunosorbent assay (ELISA). The mouse CTLA4-IgE Fc fusion protein was detected with ELISA. ELISA plates (Nunc) were coated with hamster anti-

mouse CTLA4 antibody 4F10 (10 μg/ml) overnight at 4ºC. Plates were then washed three times with PBST (0.05% Tween-20 in PBS) and blocked with 10 % FBS in PBS for 1 hr at room temperature. Serially two-fold diluted fusion protein or CHO cell culture supernatant diluted in 10% FBS in PBS were added and incubated for 2 hr at room temperature. Goat anti-mouse IgE (1:1000, Novus Biologicals) and HRP-conjugated bovine anti-goat antibody (1:10,000, Santa cruz) were added and further incubated for 1 hr at room temperature. After each step, plates were completely washed with PBST (0.05 % Tween 20 in PBS). A colorimetric assay was done with substrate tetra methyl benzidine (TMB) and hydrogen peroxide and a Victor III microplate reader (Perkin Elmer).

Degranulation assay. Degranulation assay in RBL-2H3 cells was performed as previously described with minor modifications (238). The RBL-2H3 (2 X 10^4/well) cells were grown overnight in 96 well plate and sensitized with different concentrations of biotinylated IgE (0-500 ng/ml) and mouse CTLA4-IgE Fc fusion protein (0- 2000 ng/ml). For measuring spontaneous release, control wells were set without addition of biotinylated IgE or fusion protein. The cells were washed twice with HEPES-Tyrode buffer (130 mM NaCl, 5.6 mM glucose, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM MgCl2, 10 mM HEPES, 0.1 % BSA, pH 7.4) and samples were collected for measuring the baseline degranulation. Degranulation was stimulated by addition of streptavidin (500 ng/ml) in HEPES-Tyrode buffer and supernatant collected at 10, 20 and 30 min, followed by addition of 0.1 % Triton X-100 and supernatant collected for measuring total enzyme content. β-Hexosaminidase in the supernatant (50 μl) was measured as a degranulation

marker using 3.3 mM *p*-nitrophenyl-*N*-acetyl-β-*d*-glucosaminide (Sigma-Aldrich) dissolved in 0.1 M sodium citrate buffer (pH 4.5) as substrate (50 μl) by incubation at 37°C for 1 h. The reaction was terminated with a 2 M glycine solution (pH 10.7). Quantification of the substrate cleavage was measured by reading absorbance at 405 nM and the results were expressed in the percentage of the total cellular concentration.

Statistics. The statistical difference between groups was tested by Student *t* test. A *P* value of less than .05 was considered significant. Data are expressed as mean ± SD.

RESULTS AND DISCUSSION

Expression of mouse CTLA4-IgE Fc fusion protein. Mouse CTLA4 and IgE Fc cDNA was amplified, fused with PCR based gene assembly and ligated into pCDNA3 vector (Figure 4.1A). The recombinant plasmid was transfected into CHO cells. Fusion protein expression was analyzed in a western blot assay (Fig. 4.1B, lane 3). Mouse IgE (Fig. 4.1B, lane 1) and normal CHO cell lystate (Fig. 4.1B, lane 2) were used as positive or negative control. Fusion proteins from the cell culture medium was purified using affinity column from Sepharose beads conjugated with either anti-mouse CTLA4, 4F10 mAb or anti-mouse IgE, B1E3 mAb. As shown in Figure 4.1C, purified mouse CTLA4-IgE Fc fusion protein forms a monomer under reducing condition (Fig. 4.1C, lane 1) and forms a dimer under non-reducing conditions (Fig. 4.1C, Lane 2). The predicted molecular weight under reducing condition as a monomer is the expected size, however, under non-reducing condition it was exhibited over 250 kDa protein, which is higher than expected.

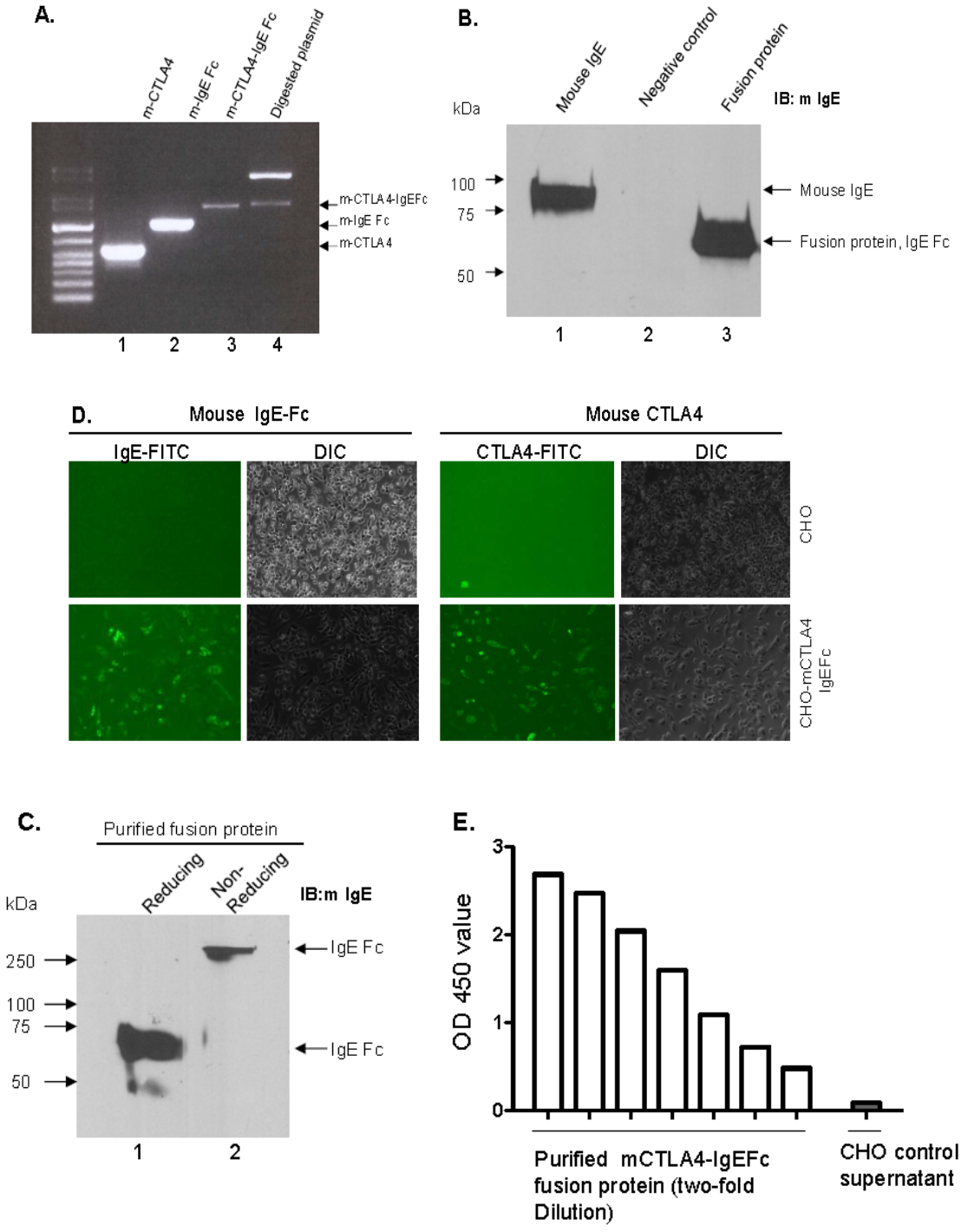

A.
m-CTLA4
m-IgE Fc
m-CTLA4-IgE Fc
Digested plasmid
m-CTLA4-IgEFc
m-IgE Fc
m-CTLA4
1 2 3 4
B.
Mouse IgE
Negative control
Fusion protein
IB: m IgE
kDa
100
75
50
Mouse IgE
Fusion protein, IgE Fc
1 2 3
D.
Mouse IgE-Fc
Mouse CTLA4
IgE-FITC
DIC
CTLA4-FITC
DIC
CHO
CHO-mCTLA4 IgEFc
C.
Purified fusion protein
Reducing
Non-Reducing
IB:m IgE
kDa
250
100
75
50
IgE Fc
IgE Fc
1 2
E.
OD 450 value
3
2
1
0
Purified mCTLA4-IgEFc fusion protein (two-fold Dilution)
CHO control supernatant

Figure 4.1. Construction, purification and expression of mCTLA4-IgE Fc fusion protein. (A) Construction of mCTLA4-IgE Fc plasmid. The mRNA for mCTLA4 (lane 1) and IgE Fc (lane 2) fused together using PCR based assembly (lane 3) and double enzyme digested pCDNA3 plasmid containing mCTLA4-IgE Fc (lane 4).
(B) Expression in CHO cells. The cell lysates (50 μg) from normal CHO cells (lane 2), transfected CHO cell lysates (lane 3) and positive control mouse IgE (lane 1) were gel were gel electrophoresed and separated on 12 % SDS-PAGE gel under reducing condition. The separated proteins were transferred on nitrocellulose membrane, blocked and blotted with goat anti mouse IgE antibody. The blots were washed and further incubated with HRP conjugated bovine anti goat IgG antibody and the protein bands were visualized by ECL method. The arrow indicates the location of mouse IgE and mouse IgE Fc.
(C) Purification of fusion protein. The fusion protein purified using either anti mouse IgE B1E3 or anti mouse CTLA4 UC10-4F10-11 conjugated sepharose 4B beads were gel were gel electrophoresed and separated on 12 % SDS-PAGE gel under reducing condition (lane 1) or under non-reducing condition (lane 2). The separated proteins were transferred on nitrocellulose membrane, blocked and blotted with goat anti mouse IgE antibody. The blots were washed and further incubated with HRP conjugated bovine anti goat IgG antibody and the protein bands were visualized by ECL method. The arrow indicates the location of mouse IgE Fc.
(D) Immunofluorescence detection of fusion protein. The normal or transfected CHO cells were grown on glass coverslips, fixed with 4% *para*-formaldehyde and permeabilized in 0.1% Triton X-100. Subsequently, the cells were incubated with goat serum for blocking and affinity-purified hamster anti-mouse CTLA4 mAb, followed by staining with an FITC conjugated goat Armenian hamster IgG and another set of cells were incubated with FITC conjugated anti mouse IgE Ab. The images were photographed using Zesis fluorescent microscope. Samples were viewed using the same contrast and brightness settings.
(E) Detection of fusion protein using ELISA. The purified fusion protein was detected by ELISA. Normal CHO cell culture supernatant was used as control.

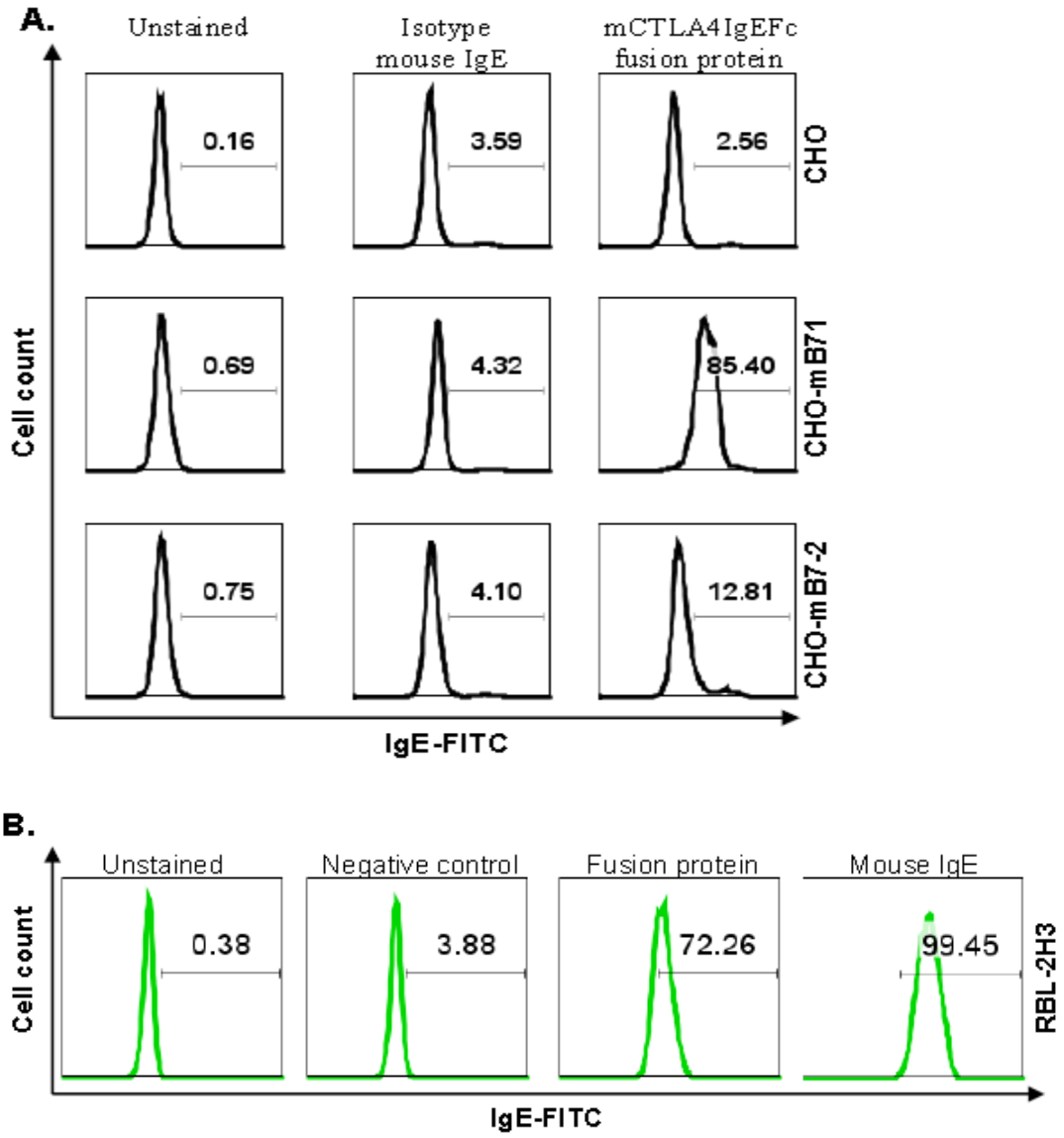
This may be caused by non-reducing conditions in electrophoresis. Immunofluoresence staining of transfected CHO cells, but not normal CHO cells, by anti-mouse CTLA4 or IgE antibody revealed the expression of the fusion protein (Fig. 4.1D). In a two-fold serial dilution, ELISA detected a decrease in OD450 value for fusion protein but not for normal CHO cell culture supernatant (Fig. 4.1E).

Characterization of the CTLA4-Fc fusion protein. Mouse CTLA4 is a negative regulator of peripheral T cell immune response by competing with CD28 for binding to mouse B7-1 or B7-2. The ability of fusion protein binding to mouse B7-1 or B7-2 expressed on CHO cells was characterized by flow cytometry. The CTLA4-Fc fusion protein alone, but not mouse IgE, bound efficiently to CHO cells expressing B7-1 or B7-2. The ability for binding to B7-1 CHO cells was much stronger in comparison with that of B7-2 CHO cells (Fig. 4.2A). The reduced binding to B7-2 in CHO cells may be due to the reduced expression of B7-2 on cell surface or some CHO cells have lost expression of B7-2. As a negative control, untransfected CHO cells showed a background staining to either mouse IgE or to fusion protein (Fig. 4.2A).

Mouse IgE Fc portion of the CTLA4-Fc fusion protein was characterized by its binding ability to FcεRI expressed on RBL-2H3 mast cell line. As shown in Fig. 4.2B, fusion protein bound well to FcεRI receptor and 72 % of RBL-2H3 cells stained positive while 99 % of the cells stained positive for mouse IgE binding (Fig. 4.2B). We further analyzed whether CTLA4-Fc fusion proteins can block degranulation of RBL-2H3 cells. RBL-2H3 cells were incubated overnight with biotinylated IgE antibody together with the fusion proteins in different concentrations. Cells were then cross-linked by

streptavidin for measuring β-Hexosaminidase release. The level of β-Hexosaminidase in RBL-2H3 cells that were incubated with biotinylated IgE alone were increased in a time dependent manner; interestingly the level of β-Hexosaminidase in the presence of CTLA4-Fc fusion proteins was decreased to the background level of mast cells (Fig. 4.2C). Overall, these data strongly demonstrated that the CTLA4-Fc fusion proteins possess their capability to bind to both CD86/CD80 and FcεRI, further suggesting their functional integrity.

Future experiments. We will further show whether mouse CD23 can transport CTLA4-IgE Fc fusion protein across the polarized epithelial cells both in vitro and in vivo. Mouse CTLA4-IgE Fc fusion protein will be further tested for their capability to inhibit airway eosinophilia and hyperresponsiveness in a murine allergy model. We will intranasally administer the affinity purified fusion proteins into the airway tract of wild-type and CD23-KO mice and thus analyze its specific transport. The effect of these fusion proteins on the development of allergic inflammation will be fully investigated. The impact of fusion protein transport on airway inflammation and hyper-reactivity will be assessed in an ovalbumin (OVA)-based murine asthma model. A chimeric mouse created between wild-type and CD23 KO mice will be used to show the specific transport function of epithelial CD23 in blocking inflammation initiation and development. The subsequent allergic inflammation in the lung will be evaluated by immunological parameters and histopathology.


A.
Unstained
Isotype mouse IgE
mCTLA4IgEFc fusion protein
0.16
3.59
2.56
CHO
0.69
4.32
85.40
CHO-mB71
0.75
4.10
12.81
CHO-mB7-2
Cell count
IgE-FITC
B.
Unstained
Negative control
Fusion protein
Mouse IgE
0.38
3.88
72.26
99.45
RBL-2H3
Cell count
IgE-FITC

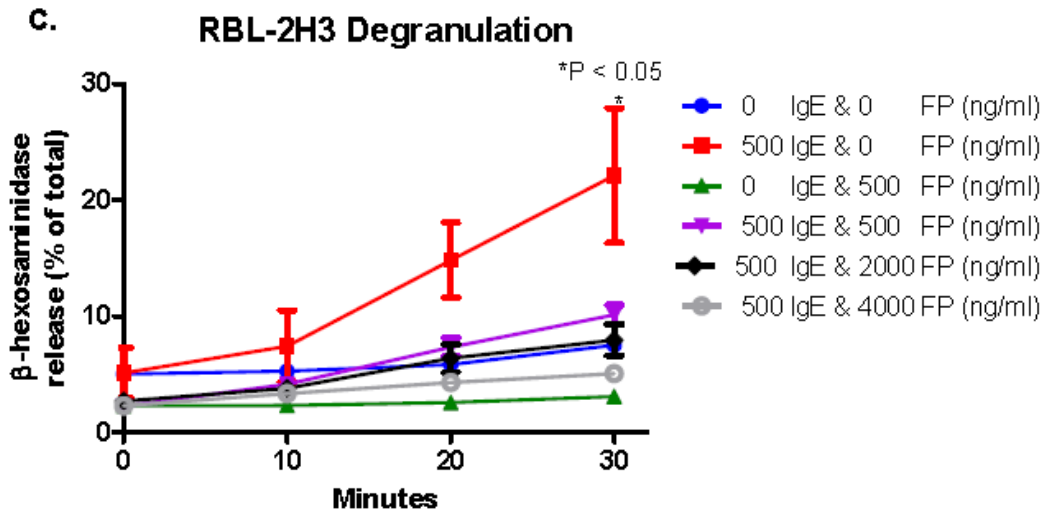


Figure 4.2. Characterization of mCTLA4-IgE Fc fusion protein.
(A) Fusion protein binding to mouse B7-1 and B7-2. Fusion protein mCTLA4-IgE Fc binding affinity to mouse B7-1 and B7-2 expressed on CHO cells was analyzed by flow cytometry. Cells were stained as described in Materials and Methods. Results are expressed as histograms of fluorescence intensity (log scale). Values in each rectangle correspond to the proportion of cells binds to fusion protein. Mouse IgE was used as isotype control and binding of fusion protein to normal CHO cells represent the background staining.
(B) Fusion protein binding to RBL-2H3 cells. Fusion protein mCTLA4-IgE Fc binding affinity to FcεRI expressed on RBL-2H3 cells was analyzed by flow cytometry. Cells were stained as described in Materials and Methods. Results are expressed as histograms of fluorescence intensity (log scale). Values in rectangle correspond to the proportion of cells binds to fusion protein or positive control mouse IgE. Negative control was performed without the addition of any protein.
(C) Fusion protein blocks degranulation of RBL-2H3 cells. RBL-2H3 cells (2×10^4/well) were grown overnight in 48 well plate. Cells were sensitized with biotinylated IgE in the presence or absence of fusion protein and degranulated by streptavidin. Degranulation of cells was measured by β-Hexosaminidase release in the supernatant and was expressed in the percentage of the total cellular concentration. Spontaneous degranulation was performed without the addition of biotinylated IgE.

CHAPTER 5: CONCLUSION AND PRESPECTIVE

CD23 is a C-type lectin and type II glycoprotein. CD23 has IgE binding activity and its expression level is increased in allergies. CD23 has various functions, such as mediating B cell growth, enhancing IgE mediated antigen presentation, and regulating IgE homeostasis. In the previous study, CD23 has been shown to transport IgE and IgE-derived immune complex across the polarized human and mouse intestinal epithelial cells. IgE antibody is also present in airway secretions and its level can be increased in human patients with allergic rhinitis and bronchial asthma. Little is known about how IgE antibodies arrive in the lumen of the respiratory tract. Although CD23 expression was previously reported on human enterocytes, it remains unknown in its expression and function in airway epithelial cells. In addition, many questions remain to be answered. For example, what are the cellular and molecular mechanisms governing CD23-mediated IgE transport across the polarized epithelial barrier? How does CD23 release IgE at the cell surface following transcytosis? What is the exact role for CD23 in airway inflammation? What kind of roles does CD23 function during viral or bacterial infection-mediated allergic inflammation in the airway? What are all the signals required for CD23 mediated IgE homeostasis?

In this study, in Chapter 2, I am interested in identifying whether CD23 is expressed in the airway epithelium and plays a role in the transport of IgE and immune complex. We utilized a variety of approaches to identify that CD23 was expressed in the airway epithelial cells like Calu-3 and primary airway epithelial cells. Furthermore, we also found that CD23 was capable of transporting IgE bidirectionally and IgE-derived

immune complex from the apical to basolateral direction by mimicking in vivo condition. This observation was demonstrated using multiple approaches. First, CD23 was shown to be expressed by a variety of human airway epithelial cells like Detroit 562, 16HBE140-, Calu-3 and A549 at both mRNA and protein levels. Both immunohistochemistry and flow cytometry were used to demonstrate the cellular location of CD23 protein in the poliarzed airway epithelial cells. This was also verified in the primary airway epithelial cells. Among the two isoforms, CD23b mRNA was found to be expressed at mRNA level. Later we also found that CD23 was responsible for transporting IgE bidirectionally and IgE derived immune complex from apical to basolateral direction. CD23-mediated transcytosis was further confirmed by blocking the transcytosis with either CD23 specific antibody or soluble CD23 protein. Furthermore, IL-4 was found to enhance both CD23 expression as well as CD23 mediated transcytosis of IgE or immune complex across the polarized airway epithelial cells. Finally we also found that the transported immune complex in comparison with antigen or IgE alone was capable of significantly inducing the degranulation in human mast cell line. Taken together, our data demonstrate that CD23 is expressed in the airway epithelium responsible for the transport of IgE and antigen.

However, several intriguing and important questions remain unanswered. First, how does CD23 release transcytosed IgE at the cell surface? Polymeric IgA receptors (pIgR) is responsible for the transport of dimeric IgA from the basolateral to apical direction and releases it as secretory component at the apical cell surface by a protein cleavage mechanism (174,175) and the neonatal Fc receptor for IgG (FcRn) is

responsible for IgG transport and releases IgG by a pH-dependent mechanism (177, 178). In our study, CD23 transports IgE bidirectionally and releases IgE at the cell surface. However, how CD23 releases its ligand at the cell surface needs to be further tested. Second, how does CD23 transport IgE and IgE immune complex across the epithelial barrier at cellular and molecule level? In our study, we found that the bafilomycin inhibiting endosome acidification also reduces IgE transcytosis. Since tubulin is involved in the transport of vesicles, IgE bound to CD23 in the vehicles may be transported with the help of tubulin. This question needs to be further tested. Third, how does CD23 expressed in the airway epithelial cells signal upon binding to IgE or IgE immune complex? It has been shown that CD23 expressed in the human intestinal epithelial cells activates the MAPK signaling pathway when stimulated with the immune complex. This activation subsequently leads to release of inflammatory mediators like IL-8 and CCL20 (146, 147). It will be interesting to know whether CD23 exhibits a similar mechanism or stimulate a different signaling pathway in the airway epithelium. The revealing of this putative signaling mechanism would be important for understanding IgE-mediated allergic inflammation in the airway. Fourth, what is the role of CD23 in allergy and asthma development during bacterial or viral infection? We know that bacterial or viral infections in the early childhood life influence the development of allergy and asthma in the later stages of life (186, 190). It will be interesting to know whether there will be correlation between CD23 mediated viral or bacterial specific IgE transport and asthma development in an animal model.

Many studies have shown that CD23 expressed in the mouse and rat intestinal

epithelial cells was involved in the enhanced transepithelial antigen transport (140-142). However, all these studies did not show a direct correlation between the enhanced antigen transport and the development of food allergy. In Chapter 3, we found that CD23 was expressed in the mouse airway epithelium and transports both IgE and IgE antigen immune complex across the epithelial barrier. Sensitization of mice with OVA antigen enhanced CD23 expression in the airway epithelium. However, many questions also remain to be addressed in this aspect. Although CD23 expressed in the airway epithelium of wild type mice may be involved in the early stages of airway allergy development, substantial evidences are needed to prove this observation. An IgE-dependent allergy model in animals is pivotal to discipher this mechanism.

Previous studies including my work have shown that CD23 was capable of transporting IgE across epithelial barrier (140-142, 238). In Chapter 4, I developed and characterized IgE based fusion protein made of mouse CTLA4-IgE Fc. However, functional characterization of this fusion protein for immunotherapeutic purpose to treat allergy remains completely unknown. This fusion protein's ability to be transported by CD23 on the epithelial cells and its immunotherapeutic properties need to be tested very carefully.

In summary, I have demonstrated the expression of CD23 and its function in transporting IgE in the airway tract (Fig 5.1). CD23 expressed in the airway tract is responsible for the transport of IgE antibody and IgE-allergen immune complexes across the airway barrier, thereby delivering the intact allergen molecules to the immune cells

localized in the lamina propria. Now the immune cells can capture, process and present the allergens to naive T cells. In the presence of IL-4, naive T cells differentiate into Th2 cells. These Th2 cells will secrete cytokines like IL-4, IL-13 and IL-5. Cytokines like IL-4 and IL-13 allow class switching of B cells to secrete allergen specific IgE antibody. Allergens can cross-link the IgE bound to FcεRI expressed at surfaces of mast cells and basophils, trig signal cascade, and subsequebtky lead to the release of allergic mediators such as histamine, cytokines, chemokines etc. Cytokines like IL-5 can recruit and activate eosinophils and all of these finally lead to Th2 mediated allergic inflammation (Fig. 5.1A). CD23 expressed in the airway epithelium also suggests its potential to deliver IgE-Fc based fusion protein such as CTLA4-Fc for immunotherapeutic purpose of airway allergic inflammation (Fig. 5.1B).

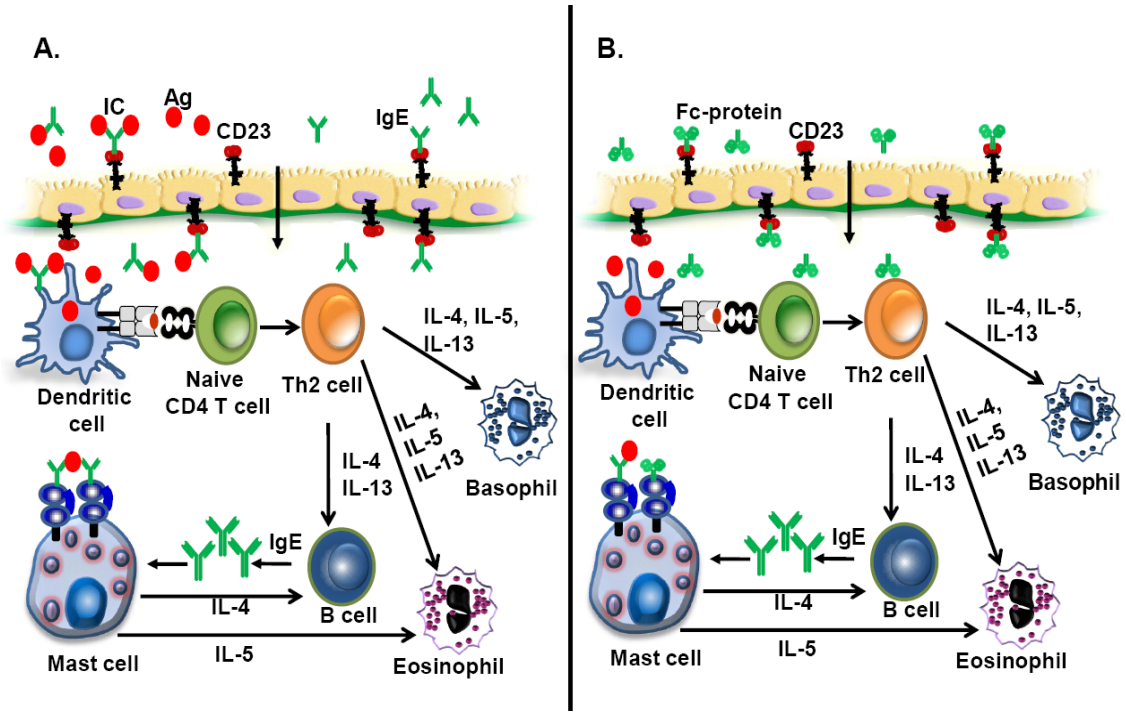


Figure 5.1. Proposed model for CD23 mediated IgE transcytosis and delivery of IgE Fc fusion protein for immunotherapy in airway tract.
(A) CD23 transports IgE and immune complexes. CD23 expressed in the airway epithelium was able to transport IgE bidirectionally and IgE allergen complex across the airway barrier and deliver intact allergen to the lamina propria, where it can be processed and presented by antigen presenting cells to T cells leading to Th2 mediated immune response.
(B) CD23 transports IgE Fc fusion protein. CD23 expressed in airway epithelium suggests its ability to deliver IgE Fc fusion protein across the airway epithelium for immunotherapeutic purpose.

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