REGULATION OF THE HIGH AFFINITY RECEPTOR FOR IGE 
(FCεRI) IN HUMAN NEUTROPHILS

BY

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MASTER’S OF SCIENCE

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<td>Alpha</td>
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<tr>
<td>Antigen Presenting Cell</td>
<td>APC</td>
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<tr>
<td>Atopic Asthmatic</td>
<td>AA</td>
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<tr>
<td>Atopic Non Asthmatic</td>
<td>ANA</td>
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<td>B lymphocyte</td>
<td>B cell</td>
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<tr>
<td>Beta</td>
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<tr>
<td>Bovine Serum Albumin</td>
<td>BSA</td>
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<td>Bronchial Hyper Responsiveness</td>
<td>BHR</td>
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<td>Broncho Alevolar Lavage</td>
<td>BAL</td>
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<tr>
<td>Carbon dioxide</td>
<td>$\text{CO}_2$</td>
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<td>Celsius</td>
<td>C</td>
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<tr>
<td>Delta</td>
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<td>Double distilled water</td>
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<td>Digoxigenin</td>
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<td>Deoxyribonucleic acid</td>
<td>DNA</td>
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<td>Epsilon</td>
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<tr>
<td>Endoplasmic Reticulum</td>
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<td>Et cetera</td>
<td>Etc</td>
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<td>Fluorescein Isothiocynate</td>
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<tr>
<td>Forced expired volume of air</td>
<td>FEV1</td>
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<tr>
<td>Gamma</td>
<td>$\gamma$</td>
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<td>Grams</td>
<td>Gms</td>
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<td>Gata binding protein – 3</td>
<td>GATA-3</td>
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<tr>
<td>Granulocyte Maturation Colony Stimulating factor</td>
<td>GM-CSF</td>
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<tr>
<td>Hours</td>
<td>Hrs</td>
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<tr>
<td>House Dust Mite</td>
<td>HDM</td>
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<tr>
<td>Term</td>
<td>Abbreviation</td>
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<td>Human lymphocyte Antigen</td>
<td>HLA</td>
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<td>Immunoglobulin (D,M,G,E,A)</td>
<td>Ig (D,M,G,E,A)</td>
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<td>Immunoreceptor Tyrosine based Activation motifs</td>
<td>ITAMs</td>
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<td>Interferon</td>
<td>IFN</td>
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<tr>
<td>Interleukin (1-13)</td>
<td>IL-(1-13)</td>
</tr>
<tr>
<td>Kilo Dalton</td>
<td>KDa</td>
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<tr>
<td>Liter</td>
<td>l</td>
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<td>Lipopolysacharide</td>
<td>LPS</td>
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<tr>
<td>Matrix Metalloproteinases</td>
<td>MMPs</td>
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<tr>
<td>Mast Cell- Tryptase</td>
<td>MCₜ</td>
</tr>
<tr>
<td>Mast Cell- Tryptase and Chymase</td>
<td>MCₜₗC</td>
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<tr>
<td>Major Histocompatibility</td>
<td>MHC</td>
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<td>Messenger Ribonucleic Acid</td>
<td>mRNA</td>
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<tr>
<td>Microgram</td>
<td>μg</td>
</tr>
<tr>
<td>Millilitre</td>
<td>ml</td>
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<tr>
<td>Minutes</td>
<td>min</td>
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<tr>
<td>Moles</td>
<td>m⁻¹</td>
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<tr>
<td>Nanogram</td>
<td>ng</td>
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<tr>
<td>Natural Killer Cells</td>
<td>NK cells</td>
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<tr>
<td>Nuclear Factor</td>
<td>NF</td>
</tr>
<tr>
<td>Percentage</td>
<td>%</td>
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<tr>
<td>Phosphate buffer saline</td>
<td>PBS</td>
</tr>
<tr>
<td>Picogram</td>
<td>pg</td>
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<tr>
<td>Polymorphonuclear leukocytes</td>
<td>PMNs</td>
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<tr>
<td>Red Blood Cells</td>
<td>RBC</td>
</tr>
<tr>
<td>Ribonucleic Acid</td>
<td>RNA</td>
</tr>
<tr>
<td>Rotations per minute</td>
<td>RPM</td>
</tr>
<tr>
<td>Signal Transducing Activator of Transcription (1-6)</td>
<td>STAT-(1-6)</td>
</tr>
<tr>
<td>Single nucleotide polymorphism</td>
<td>SNP</td>
</tr>
<tr>
<td>Skin Prick Test (+/-)</td>
<td>SPT (+/-)</td>
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Sodium Azide  \( \text{NaN}_3 \)
Sodium chloride  \( \text{NaCl} \)
Standard Error of the Mean  SEM
T cell receptor  TCR
T Helper Cell (0-3)  Th
T lymphocyte  T cell
T Regulatory cells 1  Tr1
T box expressed in T cells  T-bet
Transforming Growth Factor  TGF
Tumor necrosis factor  TNF
Units  U
ACKNOWLEDGEMENTS

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**ABSTRACT:**

Polymorphonuclear neutrophils (PMNs) are important effector cells in host defense and the inflammatory response to antigen. The involvement of PMNs in inflammation is mainly mediated by the Fc receptor family, including IgE receptors. Recently, we have shown that human PMNs from allergic asthmatic subjects express the high affinity receptor, FcεRI. In this study, we have examined the regulation of FcεRI by human PMNs in vitro and in vivo during the allergic pollen season.

First we studied the pattern of expression of FcεRI in PMNs during the pollen allergic and outside the pollen season. Peripheral blood neutrophils were isolated from adult atopic asthmatics (AA) (n=17), allergic non asthmatics (ANA) (n=15) and healthy donors (n=16) by dextran, ficoll gradient centrifugation and magnetic cell sorting (MACS). Surface, total protein and mRNA expression of FcεRI were investigated in the three groups by FACS, immunocytochemistry (ICC) and fluorescent in situ hybridization (FISH) respectively. Secondly, we investigated the effect of Th-2 cytokines which are known to regulate IgE receptor expression. PMNs from atopic asthmatic subjects were stimulated in vitro with Th-2 cytokines (IL-4, IL-9, GM-CSF) and Th-1 cytokine IFN-γ. Finally we determined whether the expression of FcεRIβ chain correlated with the surface expression of FcεRIα chain in PMNs.

Irrespective of the season, PMNs from atopic asthmatic subjects showed increased expression of FcεRIα chain in surface, total protein and mRNA compared to atopic non asthmatics and healthy donors (n=20). Interestingly, FcεRIα chain surface and mRNA expression increased significantly during pollen season compared to non
pollen season (P=0.001) in PMNs isolated from AA (n=9) in contrast to healthy donors and ANA (n=8). Furthermore similar pattern of FcεRI expression were observed in vitro when PMNs were stimulated with Th2 cytokines. IL-4, IL-9 and GM-CSF showed increased protein and mRNA expression of FcεRIα chain at 6 and 18hrs (n=6) whereas IFN-γ down regulated the mRNA expression of FcεRIα chain at 6hrs. Also, irrespective of season AA (n=11) subjects showed increased expression of FcεRI β chain when compared to ANA (n=10) and healthy donors (n=9). Western blot analysis showed increased FcεRI β protein in atopic asthmatic subjects (n=4). Interestingly irrespective of the groups, there was a positive correlation r = 0.8054 between total protein expression of β chain with surface expression of α chain of FcεRI in neutrophils.

Our data suggest that the expression of FcεRI in neutrophils of atopic asthmatic patients is highly regulated. Our in vitro studies provide evidence that Th-2 cytokines such as IL-9, IL-4 and GM-CSF up-regulate the expression of FcεRI. Furthermore we show evidence of increased expression of FcεRIβ chain in neutrophils of atopic asthmatic subjects. Collectively these results suggest that FcεRI mediated neutrophil dependent activation may play a key role in allergic diseases.
1. INTRODUCTION:

1.1 **Allergic Diseases - Disorders of immune system:**

The immune system is a balance between increased protection from unwanted invasion and decreased over-reactions to harmful events. In allergic diseases this balance is disturbed leading to adverse reactions (1). The term “allergy” was coined by Von Pirquet in 1906, describing the role of antigens in protective immune responses (favorable effect) and hypersensitivity reactions (harmful effect) (2).

Allergic diseases such as asthma, rhinitis, eczema and food allergies are increasing in recent years in developing and developed countries (3). Studies of worldwide prevalence of asthma by the International Study of Asthma and Allergies in Childhood (ISSAC) and European Community Respiratory Health Survey (ECRHS) clearly shows the prevalence of asthma to be higher in developed countries (4-6). Asthma is one of the most prevalent conditions affecting Canadians; over 2.4 million Canadians are diagnosed with asthma. It is the leading cause of absenteeism from school and third leading cause of work loss. An estimated 10% of children and 5% of adults have active asthma and the prevalence of asthma among adults has been shown to increase over the last 20 years. Direct costs of asthma in Canada are estimated at $600 million per year and the costs of public health due to allergic disease are growing every year (7-9). Due to their impact, major research efforts have been made to understand the complex processes and reactions in allergic diseases.
1.2. **Asthma: a heterogenous disease**:

Asthma is a complex disorder, with a number of features characterizing its complexity. Asthma is defined by the National Asthma Control Task force as “A disorder of the airways characterized by paroxysmal or persistent symptoms (dyspnea, chest tightness, wheeze and cough) with variable airflow limitation [and] airway hyperresponsiveness to a variety of stimuli” (10). The symptoms vary with different patients and may include shortness of breath, cough, chest tightness, sputum, and wheezing or noisy respirations (11). Activators of asthma (e.g.: viral infections, occupational agents, allergens etc.,) and the clinical manifestation of the disease are closely related, further they determine the severity of asthma (12).

"Atopy has previously been used as a poorly defined term to refer to allergic conditions which tend to cluster in families, including hay fever (allergic rhinitis), asthma, eczema, and other specific and non-specific allergic states. More recently, atopy has been characterized by the production of specific IgE in response to common environmental allergens" (13). Allergy (atopy) is known to be a major risk factor for asthma. The important link between atopy and asthma is in the development of persistent inflammation in the airway wall leading to functional and structural changes in local tissues responsible for the various symptoms of the disease (14). When atopic asthmatics are challenged with a specific allergen they have a biphasic response including acute and late-phase reactions separated by 2-8hrs time (Fig 1.2.1). Various cellular and molecular mechanisms underlie these reactions which are discussed in the following sections.
1.3. Mechanisms of allergic diseases:

The process of allergic diseases is complex and diverse. This section will give an overview of some of the mechanisms and hypothesis that are involved in allergic diseases.

**Fig 1.2.1: Biphasic response including acute and late-phase reactions.** “An asthmatic response in the lungs can be measured as a fall in the forced expired volume of air in one second (FEV$_1$). The immediate response peaks within minutes after antigen inhalation and then subsides. Approximately 8 hours after antigen challenge, there is a late-phase response that also results in a fall in the FEV$_1$. The immediate response is caused by the direct effects on blood vessels and smooth muscle of rapidly metabolized mediators such as histamine released by mast cells. The late-phase response is caused by the effects of an influx of inflammatory leukocytes attracted by chemokines and other mediators released by mast cells during and after the immediate response.” Adapted from (15).
1.3.1. **Immunoglobulin E (IgE) mediated mechanisms:**

IgE is one of the major mediators of allergic reactions such as urticaria, seasonal allergy, asthma and anaphylaxis (16). In allergic conditions, IgE is overproduced to environmental allergens such as pollen, house dust mites and animal danders. Allergens crosslink by binding to IgE molecules bound to the high-affinity receptor for IgE (FcεRI) on the surface of mast cells and basophils. This leads to the aggregation of FcεRI and its subsequent activation of the cells. Ultimately this activation leads to mast cell degranulation, synthesis of pro-inflammatory lipid mediators, synthesis and secretion of cytokines and chemokines. These reactions take place rapidly once the immune system encounters allergens; know as “Immediate hypersensitivity” or “type I hypersensitivity” (2, 17).

In the case of asthma, the acute-phase component of allergy is typical of immediate hypersensitivity reactions triggered through IgE cross linking. The results of effector functions of immediate reactions such as cytokines and other mediators set the stage for late phase reactions, which commences several hours later with influx of various cells such as eosinophils, T-cells, monocytes, basophils, neutrophils and platelets (18, 19).

1.3.2. **Th1/Th2 hypothesis:**

T helper cells (CD4+ cells) can be differentiated into Th1 or Th2 subsets based on the cytokines they produce following stimulation. Th1 cells produce cytokines such as IL-2, IL-12, Tumor necrosis factor (TNF)-β, Interferon (IFN)-γ which are involved in elimination of intracellular pathogens. Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-
13, GM-CSF that promote allergic inflammation and diseases mediated by IgE antibodies (20, 21). Previously it was thought that the different subsets were derived from two cellular lineages, but studies have shown that they are from the same cellular lineage and different subsets are produced by environmental and genetic factors (22). Cytokines produced by each subset seems to inhibit the development and functions of the other. The cytokines produced by Th1 subset (e.g.: IFN-γ) inhibit the development and production of Th2 subset cells (21).

In the differentiation process of Th1 cells the cytokine IL-12 (produced by macrophages, dendritic cells) acts through the signal transducing activator of transcription – 4 (STAT-4) signaling pathway. It leads to increased levels of T box expression in T cells (T-bet), which in turn controls the expression of Th1 cytokine IFN-γ. IFN γ also acts through STAT-1 to upregulate the expression of T-bet. Furthermore IFN-γ produced by T cells, NK cells act in a positive feedback mechanism in regulating the production of IL-12 by macrophages (23-25). In the process of Th2 differentiation IL-4 acts via STAT-6 signaling pathway leading to the binding of transcription factor GATA-3. The expression of GATA-3 is necessary for the production of Th2 cytokines (26).

The expansion of Th2 cells play a central role in development of allergic response and presence of Th2 cytokines modulate the balance of Th1/Th2 cells (21). Asthma is generally accepted to be Th2 dominant. A working hypothesis has been shown that Th2 cytokines contribute significantly for asthma pathology through their ability to increase epithelial damage and Bronchial Hyper Responsiveness (BHR) by
promoting IgE synthesis, maturation and activation of mast cells, basophils and other cells (27, 28).

1.3.3. Other factors – Environment/ Genetics:

1.3.3a. Genetic factors:

Allergic diseases have long been known to run in families indicating the role of genetic factors. Since the phenotype of allergy and asthma is heterogeneous and do not follow simple Mendelian patterns, study of the genetics has been complex (29).

Several candidate genes are linked with the development of allergic diseases. Chromosome 5 is linked to total serum IgE concentration and several candidate genes that encode various cytokines (IL-4, IL-5, IL-9, IL-13, CD14 and GM-CSF) are found to be associated (30). Furthermore polymorphisms on Chromosome 11q13 has been linked to the β chain of the high affinity IgE receptor, which is related to atopy, asthma, bronchial hyperresponsiveness and severe atopic dermatitis (31-35). A polymorphism within the FceRI-β gene has been shown to be associated with levels of IgE (36). More recently, Single Nucleotide Polymorphisms (SNPs) in the ADAM33 gene has been shown to be associated with the function of airway smooth muscle cells or structural cells, linking its role in BHR and remodeling in asthma. ADAM 33 belongs to ADAM (a disintegrin and metalloprotease), a family of proteins having varied function in the cell adhesion and other regulatory roles in cells (37). Thus several genes on different chromosomes may contribute to the pathogenesis of allergy and asthma (38, 39).
Even though genetic factors play a significant role in the pathogenesis of allergic diseases, environmental factors are thought to play an important and major role in affecting the development of allergic diseases.

1.3.3b. Environmental factors:

Around 40% of the clinical expressions of allergic diseases are accounted for genetic factors; but there is an absolute requirement for interactions with environmental factors (40). Several hypotheses have been proposed to explain the interaction of environmental factors in the increase of allergic diseases; one of the widely discussed is the “Hygiene Hypothesis”. The hypothesis suggests that westernized lifestyle characterized by higher standard of household, declining family size and personal cleanliness has contributed to the rise in allergic diseases. Infections in early childhood reduce the risk of developing allergic disease (41).

The involvement of environmental factors and complex genetic factors are combined together to contribute to the development of allergic diseases.

1.4. Cellular components of IgE mediated allergic reactions:

There are many cellular components involved in allergic reaction. This section focuses on important cells and their functions in allergic responses through IgE mediated mechanisms.
1.4.1. Mast Cells:

Mast cells are derived from CD34+ hematopoietic progenitor cells. “Cytokines that might influence mast cells proliferation, maturation, activation and survival includes IL-4, IL-5, IL-9 and IFN-γ ” (42). In humans there are two types of mast cells based on their secretory protease content namely (i) MCᵦ, which contains tryptase alone and is located mainly in the mucosa of the lung and small intestine and (ii) MCᵦC which has tryptase and chymase and predominantly found in skin, gastrointestinal submucosa and blood vessels (43).

Mast cells are the initial cells to respond to an allergen. It propagates its response through IgE antibodies attached to FcεRI. If FcεRI is not expressed, IgE mediated allergic reactions is less effective and other mechanisms have not been shown to compensate for its absence (44). IgE and FcεRI cross-linking lead to the activation of mast cells. Activation culminates in immediate allergic response, eventually releasing mediators through degranulation (2, 45, 46).

1.4.2. Basophils:

Like mast cells, basophils develop from CD34+ stem cells. The half life of basophils in circulation is hours to days. These cells have equal sensitivity like mast cells when allergens cross-link IgE and FcεRI complexes. Further basophils upon activation release preformed mediators like histamine, lipid mediators, produce cytokines and chemokines. Although these cells do not reside in peripheral tissues, they are recruited after mast cell activation (2).
1.4.3. **Eosinophils:**

Eosinophils differentiate from CD34+ pluripotent stem cells. IL-5 is important in differentiation and maturation of eosinophils. Immature CD34+ cells may be recruited to sites of allergic inflammation where differentiation into eosinophils is enabled by local IL-5 cytokine production (47).

FcεRI receptor is shown to be expressed in eosinophils in hypereosinophilic syndrome and allergic asthmatic subjects (48, 49). Studies have shown that cross-linking of IgE and FcεRI by anti-IgE causes degranulation in subjects who have hypereosinophilic disease. Eosinophil activation leads to release of preformed, highly basic mediators stored in granules. The granules contain major basic protein, neurotoxin, eosinophilic cationic protein and other mediators which are toxic to respiratory epithelial cells (allergic inflammation) and parasites (43).

1.4.4. **Antigen Presenting Cells (APCs):**

Dendritic cells, Langerhans cells and macrophages are antigen presenting cells that play a role in IgE mediated allergic responses. The expression of FcεRI is variable in these cells (50).

An immature APC is thought to internalize a specific allergen via IgE – FcεRI receptor recognition (51). The internalization enables the APCs, especially dendritic cells to migrate to lymphatic tissues. Subsequently in lymphatic tissues they prime T cells into effector and memory cells. Also APCs and Th2 cells play an important role in B cell maturation and differentiation into cells producing allergen specific IgE (52). Further, mature APCs can be recruited to the sites of inflammation by changes in their
receptor expression and upregulating chemokine receptors like CXCR4 and CCR4 (50). Mature APCs may have increased numbers of FcεRI, which can bind to IgE with different specificities, thereby enhancing cross-linking by a defined allergen at the surface. It is thought that APCs contribute to the chronic allergic disease in skin and respiratory tract (50, 53).

1.4.5. **Lymphocytes:**

T helper (CD4+) lymphocytes differentiate into either Th1 (pro-inflammatory) or Th2 (pro-allergic) cells depending on the regulatory cytokines present in the stimulation process. Th2 cells provide signals such as IgE receptor expression and mucus production which are important in the pathogenesis of inflammation. In the production of IgE, interaction between B cells and T cells via CD40 and CD40L is an important signal. “IL-4 and CD40L, the two signals required for IgE production, are provided by CD4+ Th2 cells present in respiratory mucosa and regional lymphoid tissues of individuals with asthma” (54). Furthermore Th-2 derived IL-4 and IL-13 have been shown to drive the germline transcription of Cε exons promoting the isotype switching of IgE.

Th2 cells also play a critical role in the maintenance of allergic bronchial inflammation (typical of asthma) and allergen induced hyperresponsiveness. In addition Th2 cells produce IL-5 and GM-CSF promoting eosinophilopoiesis, thus enhancing allergic inflammation (54).
B lymphocytes act as the source of IgE. Binding of IgE to the IgE receptors sets the stage for allergic reactions. Binding of IgE to FcεRII has two consequences either inhibition or amplification of IgE production (16).

1.4.6. Neutrophils:

Neutrophils are polymorphonuclear leukocytes (PMNs) which play an essential role in the immune system. It is the first line of defense against microorganisms and acts as a critical effector cell in both innate and humoral immunity (55). The process of elimination of microbial infection by PMNs is beyond the scope of this project. However neutrophil mediated inflammatory response and their role in asthma is discussed.

The inflammatory response mediated by PMNs is a multi-step process involving the initial adhesion of circulating PMNs to activated vascular endothelium. This leads to subsequent extravasations and migration towards the inflammatory site, where they exert their actions depending on the in situ milieu. Most of the stages in the process depend on the mobilization of cytoplasmic granules and secretory vesicles. These components are contained within PMNs granules, and they constitute of antimicrobial proteins, proteases, components of the respiratory burst oxidase, membrane bound receptors, extracellular matrix proteins and soluble mediators of inflammation (56).
1.5. **Neutrophils in Asthma:**

The contributions of various cells such as mast cells and eosinophils have been recognized in asthma pathogenesis. However, a recent review reported that only about 50% of asthma cases are associated with eosinophilic inflammation and the remaining subjects had increased neutrophils (PMNs) and IL-8 in the airways (57). PMNs have only recently been targeted with considerable interest. Earlier the role of PMNs in inflammation was thought to be restricted to phagocytosis, release of cytotoxic agents and enzymes. Now it is clear that they release various other mediators such as cytokines and Matrix Metalloproteinases (MMPs) that could have a significant effect on the airways in disease states such as asthma (58).

Recently, PMNs have been reported to be increased in bronchial biopsies, especially in subjects with severe asthmatics (59). Sputum analysis from the patients showed that PMNs were a prominent cell type, even in milder asthmatics (59). Under basal conditions increased PMNs have been observed in subjects with persistent asthma compared with controls, especially subjects with low eosinophil counts (60). Similar observations have been reported for Bronchoalveolar lavage (BAL) where severe asthmatics show increased number of PMNs when compared to normal subjects (61-63). These data suggest that the underlying type of asthma may have an effect on neutrophil numbers.

1.5.1. **Neutrophil products:**

There is a wide range of inflammatory products produced by PMNs including cytokines (IL-1β, IL-6, IL-8, TNFα, TGFβ), lipids (LTB4, LTA4, PAF, TXA2),...
proteases (elastase, collagenase, MMP-9), microbial products (lactoferrin, MPO, Lysozyme), reactive oxygen intermediates (superoxide, H₂O₂, OH⁻), and nitric oxide (64).

Although almost all of the products have been implicated in asthma, some of the important products are cytokine IL-8, protease elastase, lipid mediator LTB₄ and matrix metalloproteinase MMP-9. Interleukin – 8 is a potent chemoattractant for PMNs has been reported to be increased in asthmatic patients (65). Recently TGF-β, a growth factor has been demonstrated to be produced by airways and peripheral blood PMNs which is thought to be important in airway remodeling (66).

The protease elastase has a wide range of actions. It breaks down collagen III and promotes the formation of Collagen IV, drives proteolysis of surfactant proteins, and activates matrix metalloproteases (MMPs). Elastase acts on the epithelium, enhancing mucus secretion, reducing ciliary beat frequency and may lead to secondary release of leukotrienes from the epithelium (64). It may also release IL-8 and other cytokines from airway epithelium (64). Elastase damages the endothelium by increasing the permeability, promoting apoptosis and activates eosinophils and mast cells (64).

PMNs produce large amounts of lipid mediator LTB₄ and also its precursor LTA₄. LTB₄ is a potent chemoattractant for PMNs, eosinophils, monocytes and fibroblasts (67). It also activates nuclear factor NF-κB, promotes the synthesis of IL-5, IL-6 and IL-8 and enhances the synthesis of IgE in B cells (68-70).

MMPs consist of over 20 different proteases which are stored as inactive proforms which undergo proteolytic activation. This enables various abilities such as
cleavage of components of the extracellular matrix, enhancing cell trafficking, and activating other enzymes (71). MMPs play an important role in the degradation of vascular basement membranes and interstitial structures during neutrophil extravasation and migration (56). MMP-9, a gelatinase - is thought to play an important role in asthma (72). It has been shown to be increased in chronic asthma and in status asthmatics. Recently studies suggest that MMP-9 in asthmatic airways might influence tissue injury and repair processes (72).

1.5.2. **Receptors expressed by PMNs:**

PMNs not only produce a number of diverse substances but also express various receptors that have been shown to have a role in inflammatory reactions including allergy. PMNs express various Th1 and Th2 cytokine receptors including IL-4, IL-6, IL-9, IFN-γ and GM-CSF (73-76). Human PMNs express IgE receptors such as high affinity IgE receptors (FceRI), low affinity IgE receptors (FceRII/CD23) and Galactin 3 (Mac-2/εBP) (77-79).

1.5.3. **Potential roles of PMNs in asthma:**

When PMNs are associated with asthma they are likely to be involved in the chronic inflammation, wound repair and remodeling processes. It is known that PMNs play an important role in remodeling processes in skin, eyes and blood vessels (80). It is a possibility that PMNs may contribute in the fibrotic processes associated with asthma, especially the thickening of sub basement membrane due to the production of TGF-β and MMP-9. Studies have shown TGF-β to be present in BAL fluid of
asthmatic patients with 50% of the production of TGF-β to be produced by PMNs (66, 81). PMNs are also thought to play a role in the mucus production and secretion in severe asthmatics. Even though few studies in humans support the primary role of PMNs in mucus production, animal models have demonstrated removal of PMNs leads to decrease in the mucus production (82).

1.5.4. **Presence of PMNs in various type of asthma:**

PMNs have been shown to be increased with severe asthma exacerbation, nocturnal asthma and viral induced exacerbations of asthma associated with IL-8 production (83-85). In acute severe asthmatic patients PMNs have been shown to be present in sputum, BAL fluid, transbronchial and endobronchial biopsies (61, 86).

PMNs have also been implicated in occupational asthma. Following challenge with toluene diisocyanate, PMNs are increased in association with LTB₄ and IL-8 (87, 88). Studies have demonstrated that young infants who had persistent wheezing had increased numbers of PMNs in the airways without any other infections being observed. The increase had been observed in conjunction with increase in IL-8 and TGF-β (89).

1.6. **IgE and IgE Receptors:**

1.6.1. **Structure of IgE:**

IgE and its receptors are key in IgE mediated mechanisms in allergic diseases.
IgE is one of the five immunoglobulins (Ig) in human immune system. It is a heterotetramer of two heavy (H) and light (L) chains with variable (V) and constant (C) regions. IgE heavy chain is represented as ε. IgE heavy chain consists of one variable region and four constant regions, similar to IgM. In contrast IgA, IgG and IgD consist of three constant regions. The extra domain in IgE is C\textsubscript{ε2}, since C\textsubscript{H2} and C\textsubscript{H3} domains of IgA, IgG and IgD are similar to C\textsubscript{H3} and C\textsubscript{H4} domains of IgE and IgM (Figure 1.6.1). Each of Ig domains contains about 110 amino acids and comprises a β-sheet with three and four β-strands as sandwich in C-type topology (90).

**Fig 1.6.1. Structure of IgE.** Domain structure of IgE; showing the binding sites of high affinity IgE receptor (FceRI) and low affinity IgE receptor (FceRII). Adapted from (91)
The V regions of L- and H- chains form up a pair of identical antigen – binding sites. These together with adjacent CH (Cε1) domain pair form the antigen binding fragment (Fab) region of the antibody. The remaining Ig domains form the Fc (constant) fragment of the antibody. The Fc fragment binds to the cellular receptors. IgE binds to its receptors at the site of Cε3. Similar to other immunoglobulins IgE is also glycosylated and differences in glycosylation affect the interaction of IgE with its receptors (Fig 1.6.1) (2, 52).

IgE is the least abundant antibody in the serum. The concentration of IgE in serum is ~150 ng/ml in the circulation of normal individuals (92). The half life of IgE in serum is three days and much of the IgE is sequestered in tissues (93). In certain parasitic infections serum IgE concentrations may be three times greater than the normal levels without symptoms of allergic disease. IgE concentrations in the circulation may increase ten times the normal levels in allergic individuals. Allergen specific IgE antibody concentrations are tightly correlated with symptoms of the disease (94, 95). In some subjects with asthma or hayfever, IgE antibodies are detectable only in secretions from the target organ, suggesting occurrence of local IgE antibody synthesis (52).

1.6.2. Role of cytokines and transcription factors in the modulation of IgE and IgE mediated allergic reactions:

Immunoglobulin E (IgE) mediated allergic reactions are characterized by various cytokines which influence the modulation of IgE synthesis and allergic inflammation. IgE is an important player in allergic diseases. The process of B cells
producing IgE via isotype class switching is a tightly regulated process. In this process T cells (Th1 and Th2) play a central role in the isotype determination through its interaction with its cognate B cell and by secretion of specific cytokines. The cytokines released by Th2 cells especially IL-4 and IL-13 specify the class switching to IgE by B cells (52).

The two Th cell subsets Th1 and Th2 differentiate from Th0 cells when activated through its T cell receptor. The Th1 differentiation involves various cytokines and transcription factors such as IL-12, IFN-γ, STAT 4, STAT 1 and T-bet (transcription factor expressed in T cells). IL-12 activates STAT 4, which then induces IFN-γ gene expression via T-bet; IFN-γ stimulates T-bet and vice versa forming an auto regulatory network. The IFN-γ produced by Th1 cells directs the B cells to switch to immunoglobulin G (IgGs) and not to IgE. Further IFN-γ also represses the differentiation of Th2 cells from Th0 cells (52, 96, 97).

Th2 cell differentiation involves cytokines such as IL-4 and transcription factors GATA3 and c-maf. The production of GATA3 leads to the transcription of cytokines such as IL-4, IL-5, IL-9, and IL-13. GATA3 is an autoregulator of its own transcription and is thought to control IL-4 gene transcription. The cytokine IL-4 produced by Th2 cells stimulates the B cells to switch to IgE and it suppresses the gene expression of IFN-γ (52).

In mucosal tissues of atopic asthmatics, IL-4 is secreted by mast cells and basophils which might contribute to Th2 cell differentiation of fresh Th0 cells. Further the allergic conditions in these subjects may maintain the Th2 state leading to increased production of IgE (52).
1.6.3. **IgE receptors:**

There are three types of IgE receptors, the high affinity IgE receptor (FcεRI), the low affinity IgE receptors FcεRII/CD23 and Galactin – 3 which are reviewed in this part.

1.6.3.1. **High affinity IgE receptor (FcεRI):**

FcεRI belongs to an immunoglobulin superfamily of proteins which is abundantly present around 200,000 molecules per cell in basophils and mast cell membranes (2).

![Fig 1.6.2 Human FcεRI](image)

**Fig 1.6.2 Human FcεRI.** Tetrameric form (abg2) and trimeric form (ag2), TM represents Transmembrane region. Adapted from (44)
In rodents, FcεRI is expressed in mast cells, basophils and eosinophils. The expression of the receptor is tetrameric consisting of one α chain, one β chain and two identical γ chains in all the cell types (98, 99). The tetrameric form of FcεRI is maintained as a complex in the plasma membrane. Hydrophobic and electrostatic non covalent interactions enable the maintenance of the complex in the membrane (44, 100, 101) (Figure 1.6.2).

The FcεRIα chain is a type I integral membrane protein. It consists of a transmembrane region with a cytoplasmic tail of varied length; cytoplasmic tail (~17 amino acids) does not react with any signaling molecules. The extracellular N terminal region consists of two Ig like domains designated as α1 and α2. The hydrophobic patch formed by these two domains and the interface region form a putative site for binding of Fc portion of IgE. The α chain core protein is ~28kDa in molecular weight. It consists of two extracellular domains and a transmembrane domain with a short cytoplasmic tail. It contains seven N-linked glycosylation sites. The α chain is heavily glycosylated. These glycosylation sites prevent the aggregation of the receptors in the absence of antigen and it plays an important role in the interaction between the α chain and Endoplasmic Reticulum (ER) folding apparatus (2), (99), (102-105). Further the α chain contains a ER retention signal, which is a di-lysine motif located in the cytoplasmic domain; in the absence of other chains the α chain is degraded in the ER. This control mechanism forms an important regulatory role in the expression of FcεRI in the murine system (106).
The β chain consists of four transmembrane domains. The γ subunit is a homo or heterodimer consisting of a transmembrane and cytoplasmic tail. It is a common chain shared among FcγRIII, FcγRI, FcεRI, FcαR and TCR depending on the cellular contexts (44).

In humans, FcεRI is present as tetrameric and trimeric forms. The tetrameric form (αβγ2) is constitutively expressed on effector cells of anaphylaxis such as mast cells and basophils. The trimeric form (αγ2) is variably expressed on antigen presenting cells such as langerhan cells, monocytes, dendritic cells. The table 1.6.1 gives an overview of the FcεRI types expressed by various cell types. FcεRI αβ and γ mRNA has been shown to be present in PMNs, eosinophils and human platelets (53).

Table 1.6.1: FcεRI types expressed by various cell types and their known cell functions

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Cell function</th>
<th>FcεRI type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mast cells, basophils</td>
<td>Cell activation and degranulation</td>
<td>Tetrameric</td>
</tr>
<tr>
<td>Monocytes, Langerhan cells, Blood Dendritic cells</td>
<td>Antigen presenting cells and modulation of cell differentiation</td>
<td>Trimeric</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Allergic diseases</td>
<td>Tetrameric*</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>Defense against parasites</td>
<td>Unknown</td>
</tr>
<tr>
<td>Platelets</td>
<td>Inflammation</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Source: adapted from (53). * see our work.
The β chain increases the stability and signaling capacity of FcεRI. The γ chain which is shared by other Fc receptors is important for signaling (53). Both the chains contain Immunoreceptor Tyrosine based Activation Motifs (ITAMs) in their cytoplasmic tails. However there is minor difference in the ITAMs of the β and γ chains, leading to their functional differences (Fig 1.6.2) (2).

1.6.3.2. Low affinity IgE receptors:

1.6.3.2a. FcεRII/ CD23:

The low affinity IgE receptor which is known as FcεRII or CD23 is a type II integral membrane protein belonging to the C-type lectin superfamily. It has a C-type (Calcium dependent) lectin domain at the distal, C terminal end of the extracellular sequence (107). CD23 is found on sub populations of neutrophils, B and T lymphocytes, monocytes, macrophages, NK cells, eosinophils, Langerhans cells and platelets. In humans two isoforms of CD23 are generated namely CD23a and CD23b due to different mRNA splicing patterns and transcription initiation sites (2). These two isoforms differ by six to seven amino acid sequences in their cytoplasmic N-termini. CD23a is expressed in antigen-activated B cells whereas CD23b is induced by IL-4 in various types of cells (52), whereas both can be expressed on eosinophils (108).

The lectin domains of CD23 are separated from the cell membrane by a three stranded coiled-coil stalk, which is known as “Leucine Zipper”. These lectin domains provide the binding sites for IgE and complement receptors. CD23 exists as a 45-kDa
monomer and a trimer on the cell surface in an equilibrium mixture. The trimer has a ten fold higher affinity for IgE (109). Membrane bound metalloproteases cleaves the 45-kDa CD23 stalk to a 37-kDa soluble fragment (sCD23). Other proteases act on the soluble fragment CD23 (sCD23) at specific sites to produce 16-kDa fragment (110). CD23 and 16-kDa sCD23 have been shown to have opposite effects on the regulation of IgE synthesis (52, 111).

1.6.3.2b. Functions of CD23:

CD23 has been shown to be important for antigen presentation in human B lymphocytes. It is bound in the membrane of human B cells to HLA –DR, which enables it to undergo endocytosis and recycling (112). CD23a have been shown to facilitate antigen presentation in murine B cells in vivo and human B cells in vitro.

CD23 fragments which are more than 25-kDa stimulate IgE synthesis in B cells on incubation with suboptimal concentrations of IL-4 and anti-CD40 (113, 114). It has also been shown to have a negative feedback regulation on IgE synthesis (115, 116).

25kDA soluble CD23 (sCD23) promotes growth and differentiation of promyelocytes into basophils and prothymocytes into T cells and differentiation of Germinal Centre B cells into plasma cells in vitro (117).

1.6.3.2c. Galactin-3/Mac-2/ε-BP:

Galactin 3 also known as Mac-2 or ε-Binding protein (ε-BP) belong to S –type lectins with the ability to bind IgE through carbohydrate recognition domains
Mac-2 is endogenous soluble lectins and can be expressed by various cell types, including PMNs. Mac-2 has a restricted recognition pattern for IgE, they are recognized by specific glycoforms of IgE (79, 119-121).

Galactin 3 has been shown to play a role in leukocyte recruitment, during *Streptococcal pneumonia* infections it has a direct function in mediating adhesion of neutrophils to endothelium and induce extravasation into the lungs (122). It promotes adhesion of neutrophils and other cell types to laminin in an integrin independent manner (123-125). Furthermore Galactin 3 is chemotactic for human monocytes and macrophages which are thought to be more potent than chemokine monocyte chemoattractant protein-1 (MCP-1). Macrophages which lack receptor for MCP-1 has been shown to respond readily to Galactin 3 (126). Also recently a role for Galactin 3 in phagocytosis was shown in the studies of macrophages from null mutant mice (127, 128).

1.6.4. **IgE binding with its receptors:**

The binding of IgE with its high affinity receptors is bimolecular and is characterized by an association constant $K_a$ of $10^{10}$ M$^{-1}$ (129, 130). The slow dissociation rate of IgE and its receptors with a half life of 20 hrs may be one of the reasons for the high affinity of IgE to FcεRI (131).

The binding of IgE to its low affinity IgE receptors have a $K_a$ of $2-7 \times 10^{6-7}$ M$^{-1}$ (52). Two molecules of 16-kDa CD23 fragment binds to Ce3-4 fragment of IgE, where one IgE molecule crosslinks two molecules of the membrane bound CD23 trimers and several IgE molecules crosslinks to form linear or cyclic oligomers (132).
1.7. Regulation of surface expression of FcεRI:

Various studies have shown IgE as an important regulator of surface expression of FcεRI. The density of basophil surface IgE and FcεRIα expression have been shown to correlate with serum levels of IgE regardless of the disease state (133). Experiments in IgE deficient mice system shows that mast cells which lack FcεRI receptors in vivo, when incubated with IgE in vitro or injected in vivo upregulate the expression of FcεRI (134). This upregulation is also observed in human mast cells and basophils (135), suggesting that IgE ligand-mediated upregulation may be a general mechanism in rodents and humans. “The strong correlation between total IgE receptors and serum IgE was an unexpected finding that suggests that either the two are associated by primary genetic programming, or that the presence of one induces or modulates the other” (136). Another hypothesized mechanism of IgE upregulation is hypothesized that it could upregulate FcεRI by protecting it from degradation (44). Also recent studies favor the hypothesis showing that the stabilization of the receptor at the cell surface enables the use of preformed receptor pool and basal level expression of the receptor (137).

Other important factors that play a crucial role in the regulation of FcεRI are cytokines. Experiments from animal models and cell lines suggest that the basal level expression of FcεRI could be under the control of cytokines (138-140). Bone marrow cultured mouse mast cells and human basophils precursors, cultured in the presence of IL-3 express FcεRI very early during differentiation before granules are detected
IL-4 and IL-9 have been reported to induce FcεRI α chain expression in mast cells, eosinophils and monocytes and fetal liver cells (139, 140, 142, 143).

The intracellular assembly of α and γ chains of FcεRI is an important regulation step in the surface expression of the receptors. This interaction between the α and γ chain leads to masking of a retention signal and export of the receptor complex from the ER to the cell surface. Also the assembly of α and γ is mandatory for their transfer into the Golgi compartment where terminal glycosylation takes place. In this process, glucosidase trimming of terminal glucose residues in the ER is thought to be a quality control step in the export of immature FcεRIα (144).

The mRNA transcripts of FcεRI subunits namely α, β and γ are not equally abundant. The γ transcripts are the most abundant of the three, whereas β transcripts are less abundant than α transcripts, suggesting that the subunits are not coordinately synthesized (44).

1.7.1. Role of β chain in the regulation of FcεRI:

The β chain (FcRβ) has been implicated in the pathogenesis of allergy by a number of genetic studies. Genetic linkage with atopy has been demonstrated on the chromosome 11q12-13 (145-147). Further the association was found between the atopic phenotypes and polymorphisms in the coding region of FcRβ gene (148), but their significance in contributions to these allergic phenotypes is yet to be established (149).
The β chain and the γ chain of the FcεRI complex contain Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) in their cytoplasmic tail. ITAMs act as acceptors of high energy phosphates and also provide docking sites for other signaling proteins. Mutational studies show that the ITAM of the FcRβ has a different function than the ITAM of FcRγ. The FcRγ ITAM is directly responsible for signal transduction whereas the FcRβ ITAM does not itself signal but rather amplifies the FcRγ signal (150). Lyn and Syk are two tyrosine kinases that are associated with FcεRI. Lyn preferentially binds to β chain ITAM, whereas Syk can bind to both β and γ chains with more affinity to γ chain (2). Signaling capacity of two complexes αβγ2 and αγ2 has been analyzed in vitro. The αβγ2 complexes induce lyn-dependent tyrosine phosphorylation of FcRγ chains, activation of syk tyrosine kinase and calcium mobilization at a much higher levels than αγ2 complexes (150, 151).

The β chain enhances maturation and assembly processes of FcεRI; eventually increasing the surface expression of the receptor (152). It amplifies activation signals mediated within the cells (153). *In vivo* analysis of FcRβ has shown to be involved in early signaling events and also in late responses such as cell degranulation and cytokine release, suggesting its role in modulating allergic responses due to its function as an amplifier of effector cell responses (149). Therefore atopy-associated isoforms of FcRβ may contribute to the manifestation of atopy through some modulation of FcRβ’s ability to promote effector responses in vivo and functional polymorphisms in FcRβ gene is a potential immunologic dysregulator that could lead to the development of atopy (149).
1.8. Project Summary:

Previously, it was thought that FcεRI was expressed only on mast cells and basophils. However studies have shown that they are expressed on monocytes, eosinophils and various other cell types (48, 154, 155). Recently our work showed that FcεRI is expressed on human PMNs in atopic asthmatic subjects (77). The expression of the receptor was heterogeneous in atopic asthmatics and its absence in freshly isolated PMNs from healthy individuals suggest that their expression could be regulated (77). Therefore in the present study we proposed to investigate the factors that influence the regulation of FcεRI expression in peripheral blood PMNs from atopic asthmatics, atopic non asthmatics and healthy individuals.

There is growing evidence of the role of PMNs role in allergic diseases. Various studies have shown seasonal changes influence the activities of PMNs in the allergic condition. In pollen-induced asthma, seasonal antigenic exposure increases the number of PMNs in bronchial biopsies (156). Further studies have shown that neutrophil chemotactic activity in BAL fluid is significantly increased in pollen season (157). Investigating the expression of FcεRI in PMNs during and out of pollen season might provide an insight into the in vivo fine tuning of neutrophil functions in asthmatic conditions.

It is known that allergic asthmatic individuals are influenced by seasonal changes which are associated with elevated Th2 cytokines. Furthermore, experiments from animal models and cell lines suggest that the basal level of FcεRI expression is under the control of cytokines. IL-4 and IL-9 have been reported to induce FcεRI α chain expression in mast cells, eosinophils and T cell line (138-140). GM-CSF have
been reported to induce CD23 in human PMNs (78). Furthermore GM-CSF has a pleiotropic activity on the functions of PMNs such as survival, release of chemokines such as IL-8 (76). Therefore investigating the role of Th2 cytokines may describe its role in the regulation of FcεRI in PMNs.

*We hypothesize that the high affinity IgE receptor (FcεRI) in human PMNs is regulated in vivo; likely by Th-2 cytokines which are highly expressed in allergic asthma.*

We used freshly isolated human peripheral blood neutrophils and *in vitro* cultured PMNs (with Th-2 cytokines) to investigate the expression of FcεRI at both protein and mRNA levels. The expression was studied during pollen season and non pollen season. We were able to study the (i) Extracellular surface expression of FcεRI (ii) Total protein expression levels of FcεRI and (iii) mRNA expression levels of FcεRI in freshly isolated and Th-2 cytokines stimulated PMNs.

To test the hypothesis, we proposed two specific aims: (i) To characterize the expression of FcεRI during the pollen allergic season and non pollen season, and (ii) To determine separately the effects of various Th-2 cytokines such as IL-4, IL-9 and GM-CSF on FcεRI expression *in vitro.*
2. Materials and Methods:

2.1. Subjects:

Peripheral human blood was obtained from recruited adult donors. The subjects were grouped into healthy, atopic non asthmatic (ANA) and atopic asthmatics (AA) subjects. Clinical diagnosis of allergy (atopic) and asthma were determined by: (i) Previous history of allergy and/or asthma, (ii) positive wheal and flare reaction to one or more common allergens such as grass pollen or a history of allergic rhinitis and grass pollen, (iii) a substantial improvement in spirometry test post-Ventolin administration (asthma). Normal subjects had no prior history of allergic diseases or asthma, further they did not have any positive epicutaneous allergen tests nor significantly improved spirometry post-bronchodilator. ANA subjects were grouped to have either allergic rhinitis and/or grass pollen allergy who did not have asthma. AA subjects were grouped to have either allergic rhinitis and/or grass pollen allergy with asthma. Three days prior to blood donation the donors were refrained from using anti-inflammatory medications, such as $\beta_2$ agonists, anti-histamines or corticosteroids. Further written and informed consent was obtained from each donor. The study is approved by the University of Manitoba Faculty Committee on the Use of Human Subjects in Research.

2.2. Isolation of neutrophils:

50ml of peripheral blood was collected by venipuncture and collected into 2ml of 2.7% EDTA (Sigma, St.Louis, MO, USA). 10ml of 0.85% saline was added to
every 20ml of the blood collected. The saline diluted blood (every 10ml) was layered on 3.5ml of Ficoll-Paque gradient (Histopaque 1077, Sigma, St. Louis, MO, USA) without disturbing the Ficoll. The tubes were then centrifuged (Eppendorf Centrifuge 5810 R) at 1600 rpm for 30 minutes at room temperature (RT). This gradient centrifugation enables the separation of cells into layers based on weight. After centrifugation, the plasma layer was collected. The granulocyte-rich fraction and red blood fraction was mixed well with 6ml of Dextran (Amersham Pharmacia Biotech, Piscataway, NJ, USA)-saline solution (3%Dextran and 0.9% saline) and allowed to sediment for 20-30min at RT. The supernatant was recovered into 50ml centrifuge tube without disturbing the dark red bottom layer. The tube containing the supernatant was filled up to 45ml mark with 0.85% saline and centrifuged at 1000rpm at 6°C for 7min to recover the granulocytes. The cell pellet was treated with hypotonic solution (12.5ml of 0.2% saline for 30 seconds followed by 1.6% saline to stabilize the cells) to lyse the residual RBCs. The tube is filled up to 45ml mark with 0.85% saline and centrifuged at 1000rpm at 6°C for 7min; occasionally this step was repeated once or twice depending on the lysis of RBCs. Further the cells were washed twice with 0.85% saline with 2% plasma, which was centrifuged as mentioned above. The cells were then re-suspended in 10ml of complete media consisting of RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS) and 1% 2-mercaptoethanol (2-ME). The concentration of the cells and the viability of the cells (>98%) was assessed by 4% trypan blue dye (Fisher Scientific, Pittsburgh, Pa) exclusion using hemocytometer counts. The purity of the cells (>95%) was determined by staining cytospin slides (ThermoShandon) with Diff-Quick (Fisher Scientific, Pittsburgh, Pa) staining. The
cells which were <95% pure were purified using autoMACS (Miltenyi Biotech, Auburn, CA, USA).

2.2.1. **Purification of neutrophils by autoMACS:**

The cells to be purified were concentrated to 5x10^7/50μl with running buffer (PBS with 2mM EDTA and 0.5% BSA). Equal volume of anti-CD16-coated microbeads (Miltenyi Biotech, Auburn, CA, USA) was mixed with the concentrated cells for 30min. on ice. The concentrated cells were diluted with 1ml/50μl concentrated cell suspension with running buffer. The CD-16 positive neutrophils were positively selected using Possel’s program in autoMACS (Miltenyi Biotech, Auburn, CA). The concentration and purity of the separated neutrophils was estimated by trypan blue dye exclusion using hemocytometer counts and Diff-Quick staining respectively.

2.3 **Neutrophils cell culture:**

Using complete media, isolated neutrophils were adjusted to give a maximum concentration 5x10^6 cells/ml. The cells were then treated with the cytokines as tabulated in 2.3.1. Stimulated cells were cultured at 37°C in humidified 5% CO₂ for 6 and 18 hrs. Subsequently, stimulated cells were used for Fluorescence Activated Cell Sorting (FACS) and the remaining cells were further stored at -80°C for protein and mRNA isolation.
Table 2.3.1: Cytokines for cell culture

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Company</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhIL-9</td>
<td>Peprotech Canada Inc</td>
<td>10ng/ml</td>
</tr>
<tr>
<td>rhIL-4</td>
<td>Peprotech Canada Inc</td>
<td>10ng/ml</td>
</tr>
<tr>
<td>rhGM-CSF</td>
<td>Peprotech Canada Inc</td>
<td>10ng/ml</td>
</tr>
<tr>
<td>rhIFN-γ</td>
<td>Peprotech Canada Inc</td>
<td>10ng/ml</td>
</tr>
</tbody>
</table>

2.4. Flow Analytical Cytometry Analysis (FACS):

Freshly isolated or stimulated neutrophils (2x10^5 cells/ml) were stained for different surface markers. All steps are carried out on ice. The cells were incubated with different primary antibodies in FACS tubes (Beckman Coulter, Inc., Fullerton, CA, USA) as tabulated in Table 2.4.1.

The cells were then incubated for 30 minutes at room temperature with constant shaking. After the incubation the cells were washed twice with 3ml of 1x Phosphate Buffered Saline (PBS) (0.25g sodium phosphate monobasic dehydrate, 1.42g dibasic sodium phosphate, 8.77g sodium chloride with pH 7.4 for 1L) which was centrifuged at 1200 rpm at 4°C for 7min. Supernatants was discarded and the cells were incubated with 1:100 dilution of FITC conjugated affinipure Rat Anti Mouse IgG (H+L) (Jackson Immuno Research Laboratories, West Grove, PA, USA) following incubation in dark for 30 minutes with constant shaking on a shaker.
Table 2.4.1: **Primary antibodies for FACS**

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Company</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC-conjugated Monoclonal Anti-Human CD16</td>
<td>Sigma, MO, USA</td>
<td>100μg/ml</td>
</tr>
<tr>
<td>FITC-conjugated Mouse IgG1, Kappa (MOPC21)</td>
<td>Sigma, MO, USA</td>
<td>100μg/ml</td>
</tr>
<tr>
<td>Mouse Monoclonal Anti-human FceRIα (CRA1)</td>
<td>-</td>
<td>100μg/ml</td>
</tr>
<tr>
<td>Mouse IgG2a</td>
<td>Sigma, MO, USA</td>
<td>100μg/ml</td>
</tr>
</tbody>
</table>

*P.S: CRA1 antibody binds to the stalk region (other than IgE binding site) of FceRIα*

After the incubation stained cells were washed twice with 3ml of 1x PBS. The supernatant was discarded and the cells were re suspended with 200μl of 1x PBS and analyzed on Beckman Coulter Flow Cytometry. (Beckman-Coulter, Inc., Fullerton, CA, USA). Acquisition and analysis was performed on Cell Quest PRO (Beckman Coulter).

**2.5. Fluorescent Immunocytochemistry (ICC):**

**2.5.1. Cytospin slide preparation:**

Freshly isolated neutrophils (1x10⁵ cells/ml) were added into cytospin funnels added onto filter covered slides. Slides were then spun in Cytospin3 (ThermoShandon, Cheshire, UK) at 450rpm for 2min. Slides were then dried and a circle was marked
with a DAKO pen (DAKO Diagnostics Canada Inc., Mississauga, ON) around the adhered cells. The slides were then fixed with 4% paraformaldehyde for 20min at RT in a chemical hood. Following fixation the slides were then washed thrice for 5min with 0.05M Tris-HCl buffered isotonic saline pH.7.6 (TBS). The slides were then dried at 37°C; after drying the slides were stored at -20°C before ICC and ISH.

2.5.2. Immunocytochemistry for FcεRIα chain:

Cytospin prepared slides for ICC was taken out from -20°C and allowed for 10-15min to bring the slides to RT. The fixed cell spot was blocked with Universal Blocker (DAKO Diagnostics Canada Inc., Mississauga, ON) for 20min at RT. The blocking solution was removed by shaking off the slides. Meanwhile the primary antibody CRA1 (final concentration 10μg/ml) was prepared diluting in Antibody Diluting Buffer (DAKO Diagnostics Canada Inc.,) which was added to each slide (50 - 100μl for each spot). Simultaneously isotype control Mouse IgG2a (10μg/ml) was added to the respective control slides. All the slides were incubated overnight at 4°C.

Following the incubation the slides were washed thrice for 5min in TBS. In obscure light the slides were incubated with 1:100 dilution of FITC conjugated affinipure Rat Anti Mouse IgG (H+L) (Jackson Immuno Research Laboratories, West Grove, PA, USA) for 2hrs in dark at RT. The incubated slides were washed in obscure light thrice for 5min in TBS. To stain the nucleus, slides were counterstained with (1:10 dilution in TBS) Propidium Iodide (PI) (Molecular Probes, Burlington, ON, Canada) for 2min in dark. To remove excess staining the slides were copiously washed with TBS. Following the staining the slides were mounted with mountant Moviol and the slides
were stored at -20°C till acquisition in Olympus FluoVIEW confocal laser scanning system (Olympus America Inc., Melville, NY, USA). FluoVIEW 2.0 (Olympus America Inc.,) was used to acquire and analyze the confocal images.

2.5.3. Immunocytochemistry for FcεRIβ chain:

Fluorescent immunocytochemistry for FcεRIβ chain was performed similar to FcεRIα chain with changes as tabulated in Table 2.5.1. Following the staining the slides were mounted with ProLong® antifade (Molecular Probes, Burlington, ON, Canada) and the slides were stored at -20°C till acquisition in Axiophot2 (Carl Zeiss Canada Ltd., Ontario, Canada) confocal laser scanning system. AxioVision 3 (Carl Zeiss Canada Ltd., Ontario, Canada) was used to acquire and analyze the confocal images.

2.6. Immunoprecipitation and Western blot analysis:

2.6.1. Preparation of protein from neutrophils:

Stimulated and stored neutrophils from -80°C was taken out and 200ul of complete NP-40 (USB Corporation, OH, USA) lysis buffer [50mM Tris-HCl pH8.0, 150mM NaCl, 1% NP-40, before adding to cells -10X protease inhibitor cocktail (Roche Mississauga, Ontario, Canada), 2mM PMSF (Sigma, MO, USA)] was added. The mixture was kept on ice for 10-20min. After the incubation the lysed cells were centrifuged at 12000rpm for 10min at 4°C. The supernatant containing the protein phase was carefully collected avoiding the pellet.
Table 2.5.1: **Reagents for FcεRIβ chain Immunocytochemistry**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Composition/purpose</th>
<th>Company</th>
<th>Working Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking</td>
<td>TBS – 85%</td>
<td>Goat and Donkey Serum –</td>
<td>~100μl</td>
</tr>
<tr>
<td></td>
<td>Normal human serum – 5%</td>
<td>Sigma, MO, USA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat Serum – 5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Donkey Serum – 5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-FcεRI, β subunit</td>
<td>(Rabbit antiserum)</td>
<td>Upstate Biotechnology, NY, USA</td>
<td>10μg/ml</td>
</tr>
<tr>
<td></td>
<td>Diluent - 2% Goat Serum in TBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Diluent - 2% Goat Serum in TBS</td>
<td>Sigma, MO, USA</td>
<td>10μg/ml</td>
</tr>
<tr>
<td>Alexaflour 488®</td>
<td>Secondary antibody</td>
<td>Molecular Probes, ON, Canada</td>
<td>1:200</td>
</tr>
<tr>
<td>Donkey Anti-Rabbit IgG</td>
<td>Diluent – 1% Goat Serum in TBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibodies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAPI</td>
<td>Nuclear counter stain</td>
<td>Molecular Probes, ON, Canada</td>
<td>1μg/ml</td>
</tr>
</tbody>
</table>

**2.6.2. Estimation of protein by Lowry’s Method:**

Isolated protein samples (10μl) were taken in new 1.5ml eppendorf tubes. Protein standards (BSA-2mg/ml) were prepared by serial dilution up to 1:5. Also working reagent was prepared by adding 20μl of reagent S (surfactant solution, Bio-Rad) and 1ml of reagent A (alkaline copper tartrate solution, Bio-Rad). The working reagent (50μl) was added to all the samples and standard. The Reagent B (dilute Folin reagent, Bio-Rad) was added (400μl) to the samples and standards. All the samples and standards were incubated for 15min at RT after quick vortex. Each sample and standards (76μl) were taken in duplicates in a microtitre plate and the protein
concentration was measured using Softmax Pro version 3.1.2 software in SpectraMax 190 microplate spectrophotometer (Molecular Devices, Sunnyville, CA, USA) at 750nm.

2.6.3. Immunoprecipitation of FcεRIα chain:

To 200μg of protein samples, purified human IgE - 2μg/ml (Diatec.com, Oslo, Norway) was added and incubated at 4°C overnight on a shaker. Simultaneously 20μl of Protein G Sepharose (beads) for each sample was washed thrice with 1ml NP-40 lysis buffer, to remove traces of ethanol from the beads by centrifuging at 12000rpm for 5min at 4°C. After the washes the beads were reconstituted with 400μl of NP-40 lysis buffer. To this washed beads, purified anti-human IgE – 2μg/ml (PharMingen, San Diego, CA, USA) was added and incubated at 4°C. The following day the incubated protein and beads were mixed together and incubated for 2 hours at 4°C. Further the incubated samples were centrifuged at 14000rpm for 10min at 4°C to pellet the immunoprecipitate complex. The residual proteins were washed six times with NP-40 lysis buffer and centrifuged as above and reconstituted with 13.5μl of NP-40 lysis buffer.

2.6.4. Immunoprecipitation of FcεRIβ chain:

To 20μl of TrueBlot™ anti-rabbit IgG beads (eBioscience, San Diego, CA, USA) 200μl of the cell lysate was added and incubated in ice for 30min, for preclearing the cell lysate. The incubated cells were centrifuged at 12000rpm for 3min.
To the supernatant rabbit anti-human FcεRIβ - 2μg/ml (Upstate biotechnology, Lake Placid, NY, USA) was added and incubated for 1 hour on a shaker. Further the incubated samples were centrifuged at 14000rpm for 10min at 4°C to pellet the immunoprecipitate complex. The immunoprecipitate was processed as mentioned above.

2.6.5. **Western Blot for FcεRIα chain:**

Sodium dodecyl sulphate – Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) was performed for separation of proteins. 13% separating gel (3ml - ddH2O, 4.3ml - 30% Bis-Acrylamide, 2.5ml – 1.5M Tris-HCl pH 8.8, 100μl – 10% SDS, 100μl – freshly prepared 10% APS, 4μl – TEMED) was prepared with a 4% stacking gel (6.1ml - ddH2O, 1.33ml - 30% Bis-Acrylamide, 2.5ml – 0.5M Tris-HCl pH 6.8, 100μl – 10% SDS, 100μl – freshly prepared 10% APS, 5μl – TEMED). Meantime the protein samples were prepared by adding 1.5μl of 2X loading dye (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 125mM Tris-HCl pH 6.8, 0.02% bromophenol blue, 200mM DTT) to the samples and boiled at 100°C for 5min. The samples were then centrifuged to pellet and loaded in the gel placed in 1X Running buffer [100ml – 10X running buffer (0.25M Tris-base, 1.9M Glycine), 890ml-H2O, 10ml – 10% SDS] fixed in the apparatus. The gel was run at 110V and 90mAmps. Meantime PVDF membrane was cut to the size of the separating gel and was rinsed in methanol in the chemical hood. It was then washed in distilled water for 5min and placed in transfer buffer (100ml – 10X Running buffer, 700ml – H2O and 200ml – Methanol) for 10min at 4°C.
After the run, the gel was removed from the apparatus and rinsed with distilled water. The stacking gel was removed with the plastic scraper and carefully the gel was transferred onto the membrane. The gel and membrane was placed on the transfer unit sandwiched between presoaked pads in transfer buffer. Using a 15ml tube as a rolling pin, air bubbles were removed from the sandwich. After securing the lid, the proteins were transferred onto the membrane overnight at 30V and 90mAmps. The following day the membrane was rinsed and washed twice in TBST (100ml – 10X TBS, 1ml – Tween-20 and 1l – ddH2O) for 5min at RT on a shaker. Further the membrane was blocked with 5% Blotto, non – fat dry milk (Santa Cruz Biotechnology, CA, USA) for 2 hrs at RT. Meanwhile the primary antibody CRA1 (1μg/ml) was prepared diluting in 1% Blotto, non-fat dry milk. The membrane was incubated with the primary antibody overnight at 4°C. Following the incubation the membrane was rinsed and washed twice with TBST for 15min at RT. The membrane was incubated with secondary antibody HRP-goat anti-mouse IgG (H+L) prepared in TBST (1:5000) for 1 hr at RT on a shaker. In a clean container the membrane was rinsed and washed as mentioned above. The membrane was developed using ECL- detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.6.6. Western Blot for FcεRIβ chain:

The western blot analysis for FcεRIβ chain was performed as mentioned above with 15% separating gel (2.3ml - ddH2O, 5ml - 30% Bis-Acrylamide, 2.5ml – 1.5M Tris-HCl pH 8.8, 100μl – 10% SDS, 100μl – freshly prepared 10% APS, 4μl – TEMED) was performed with a 4% stacking gel (6.1ml - ddH2O, 1.33ml - 30% Bis-
Acrylamide, 2.5ml – 0.5M Tris-HCl pH 6.8, 100μl – 10% SDS, 100μl – freshly prepared 10% APS, 5μl – TEMED Anti-FcεRI, β subunit (1:500) antibody was used as the primary antibody. The secondary antibody HRP- donkey anti rabbit IgG (H+L) was used with ECL detection system.

2.7. In-situ hybridization analysis:

2.7.1. Digoxigenin (DIG) FcεRIα probe preparation:

Previously subcloned pBluescript vector (Stratagene, La Jolla, Calif., USA) with FcεRIα cDNA (within coding region prepared by RT-PCR using specific primers α1: 5’TACAGTAATGTTGAGGGGCTCAG3’ and α2: 5’CTGTTCTTCGCTCCAGATGGCGTT3’) was used for probe preparation (77). The plasmid was linearized with Xho1 and Bam H1 overnight (18hrs) at RT for preparing sense and anti-sense probe respectively. The probe was precipitated by adding 20μl – 3M sodium acetate and 300μl of 100% ethanol into the linearized tube. The tubes were incubated for 2hrs at -20°C and centrifuged at 14000 rpm for 15min. The precipitate was washed twice by adding 500μl of 75% ethanol and centrifuged as above. After the washes the DNA was dissolved in 50μl (1μg/ml) of sterilized ddH2O. The linearization was confirmed on 2% agarose gel electrophoresis (AGE) before probe labeling. The DIG RNA probe labeling was prepared by adding 1μl (1μg) of the prepared antisense or sense DNA with DIG-NTP mixture (Roche Mississauga, Ontario, Canada) transcribed by T7 or T3 RNA polymerase (Promega, Madison WI,
USA). The reaction was incubated for 2hrs at 37ºC. After the incubation DNase I (Sigma, MO, USA) was added and incubated for 15min at 37ºC. The reaction was stopped by adding 2µl of 0.2mM EDTA stop solution. The DIG - RNA probes were confirmed by Agarose Gel Electrophoresis (AGE); following precipitation as mentioned above the probes concentration were measured using Softmax Pro version 3.1.2 software in SpectraMax 190 microplate spectrophotometer (Molecular Devices). The probes were stored at -80ºC until use.

2.7.2. ISH staining of FcεRIα:

The ISH staining was performed on cytospin slides which were prepared as mentioned above. All the steps were performed in RNase Free condition. The slides were taken out from -20ºC and left in RT for 20-30min to bring it to RT. The slides were washed twice with 2X SSC (prepared from 20X SSC – 175.3gm/l – NaCl, 88.2 – gm/l Sodium Citrate pH 7) at RT for 5min. The slides were then prehybridized with hybridization buffer (50% Formamide, 5X SSC, 100µg/ml Heparin, 1X- Denhardt’s solution, 0.1% Tween 20, 0.1% CHAPS, 5mM EDTA and 0.3mg/ml yeast tRNA) for 3hrs at 42ºC. Meantime the DIG labeled sense and anti-sense probe (1µg/ml) was diluted in hybridization buffer which was added to the slides and incubated overnight at 42ºC.

The slides were then washed with 0.2X SSC for 15min at 65ºC and consequently twice with 0.2X SSC for 30min at 65ºC. The slides were again washed twice with PBT (0.1% Triton X 100, 2mg/ml BSA, PBS) for 20min at RT. The endogenous peroxide activity was blocked by adding freshly prepared 1% hydrogen
peroxide in methanol for 15min at RT. The slides were then washed thrice in TNT (0.1M Tris HCl pH7.5, 0.15M NaCl, 0.05% tween 20) for 5min each at RT, followed by incubation with mouse monoclonal anti-digoxiginin antibody (Roche Mississauga, Ontario, Canada) (1:100) for 30min at RT.

The detection was performed using Tyramide Signal Amplification (TSA) Kit (Molecular Probes, Burlington, ON, Canada) as per the instructions. The slides were blocked with a blocking reagent at RT for 30min and incubated with HRP anti-mouse IgG (1:100) for 30min at RT; washed thrice with PBS for 5min at 37°C. Meantime tyramide solution was prepared in amplification buffer and was added to the slides. After incubation for 5-10min at RT the slides were again washed with PBS for 5min at 37°C. The slides were counterstained with Propidium Iodide (Molecular Probes, Burlington, ON, Canada) (1:10) for 1min. After washing copiously with PBS, the slides were mounted with ProLong® antifade (Molecular Probes, Burlington, ON, Canada) and the slides were stored at -20°C till acquisition in Olympus FluoVIEW confocal laser scanning system (Olympus America Inc., Melville, NY, USA). FluoVIEW 2.0 (Olympus America Inc.,) was used to acquire and analyze the confocal images.

2.8. **Real time PCR analysis:**

2.8.1. **Total RNA isolation from neutrophils:**

Total RNA was isolated from stimulated neutrophils using TRIzol® Reagent (Invitrogen, Burlington, ON, Canada). The procedure was performed as per the
instructions of the product. 1ml of TRIzol® Reagent was added to the pellet of cells and homogenized well with a pipette. The homogenized samples were incubated for 5min at RT for complete dissociation of nucleoprotein complexes. To the incubated samples 200μl of chloroform was added and vigorously shaken and incubated at RT for 3min. The samples were then centrifuged at 12000rpm for 15min at 4°C. Following the incubation the aqueous phase containing the RNA was collected in a fresh eppendorf tubes. RNA was then precipitated by adding 500μl of isopropyl alcohol and incubating for 10min at RT and centrifuged as above. The supernatant was removed and the pellet was washed with 75% ethanol followed by centrifugation at 7500rpm for 5min at 4°C. The pellet was air dried and dissolved in RNase free water. The prepared total RNA was estimated using Softmax Pro version 3.1.2 software in SpectraMax 190 microplate spectrophotometer (Molecular Devices) with Absorbance 260/280.

2.8.2. **First complementary strand DNA (cDNA) synthesis:**

cDNA was synthesized by Reverse Transcriptase (RT). 100ng/ml RNA was taken in PCR tubes with 1μl of Oligo (dT) 12-18 Primer (Invitrogen). The reaction was made up to 11μl with PCR grade ddH2O and the mix was incubated at 65°C for 5min; quick chilled on ice with brief centrifugation. Meantime the reaction mix was prepared [5X First strand buffer, dNTPs (10mM), 0.1M DTT, RNase OUT (40units/μl) and Superscript II (Invitrogen)] to make up to 9μl. Both were mixed and the reaction was set up for overnight at 37°C.
2.8.3. Real time PCR amplification:

The quantification of mRNA of FcεRIα chain expressed by neutrophils at various conditions was estimated using Real time PCR analysis. The cDNA synthesized were used as template for the quantification. The Light Cycler™ - Primer set (LC Search, GmbH Heidelberg, Germany) was used as a ready to use amplification primer mix for FcεRIα chain. The procedure was followed as per the instructions of the primer kit. The cDNA were serially diluted in 1: 2 and 1:4 dilutions in PCR grade water. Similarly known standards were serially diluted in 1:10, 1:100 and 1:1000 dilutions. Meantime reaction mix was prepared by adding 6μl H2O, 2μl Light Cycler™ primer set, 2μl Light Cycler™ Fast Strand DNA Master Sybr® Green I (premixed) (Roche Mississauga, Ontario, Canada) for each reaction. This 10μl of reaction mix was added to the pre-cooled Light Cycler™ capillaries. Similarly 10μl of the serially diluted standards and samples were added to the capillaries. The capillary tubes were sealed with the stopper and placed in the adaptors, into the centrifuge and was spun at 2000rpm for 30sec. The capillaries were then placed in the rotor of Light Cycler™ II and the PCR cycle was run for 35 cycles with the settings provided by the kit.

Similarly real time PCR analysis were carried out for G3PDH with similar conditions and dilutions for control reactions and the quantification of mRNA expression of FcεRIα was carried out normalizing to G3PDH. All the data acquisition was performed by LightCycler™ Software.
2.9. Statistical analysis:

All the statistical analysis was carried out using GraphPad Prism Version 3.02 for Windows (GraphPad Software, San Diego, CA, USA). The association between expression of FcεRI expression during and out of allergic season was determined by Wilcoxon signed rank test (paired, non parametric). Association between the expression, in the subgroups was studied using Mann-Whitney U test (unpaired non parametric). Statistical significance: p<0.05. The correlation analysis between extracellular expression of FcεRIα chain and its respective FcεRIβ chain protein expression was studied using Spearman Rank correlation analysis.
3. RESULTS:

3.1. Introduction:

The main focus of this thesis was to study the regulation of FcεRI expression in human PMNs. Previously we showed that FcεRI is expressed on human PMNs in atopic asthmatic subjects (77). Further the expression of the receptor was found to be heterogeneous in atopic asthmatic subjects, while being totally absent in freshly isolated PMNs from healthy individuals, suggesting that its expression could be regulated (77).

There is growing evidence of PMN role in allergic diseases. Various studies have shown seasonal changes influence the PMN activity in allergic conditions. Furthermore, it is known that allergic reactions are influenced by seasonal changes, leading to elevated Th2 cytokines. Also experiments from animal models and cell lines suggest that the basal levels of FcεRI expression are under the control of cytokines (138-140).

Our study cohort was divided into three groups of subjects namely Atopic Asthmatics (AA), Atopic Non Asthmatics (ANA) and healthy individuals. We have studied the expression of FcεRI in PMNs at both protein and mRNA levels. To test our hypothesis that the high affinity IgE receptor (FcεRI) in human PMNs is regulated in vivo and Th-2 cytokines which are highly expressed in allergic asthma regulate its expression; we analyzed the in vivo expression of the receptor, by studying its expression during and out of allergic season. Also we tested the in vitro effect of Th-2 cytokines such as IL-4, IL-9 and GM-CSF separately on the expression of FcεRI at
both protein and mRNA levels. Finally we investigated the role of the β chain expression in FcεRI receptor regulation.

3.2. Expression of FcεRI in AA, ANA and normal subjects irrespective of season:

Previously, studies have shown that neutrophils from atopic asthmatic subjects express FcεRI (77). Since the expression of receptor mRNA and its subsequent protein translation of the receptor may not necessarily be the same, we compared the expression of FcεRI by human PMNs in AA, ANA and normal subjects at the levels of membrane bound receptor, total cellular receptor and mRNA.

We used fluorescent In Situ Hybridization (ISH), fluorescent Immunocytochemistry (ICC), and Flow Analytical Cell Sorting (FACS) to investigate the mRNA, total protein and surface receptor expression respectively in PMNs of AA, ANA and healthy subjects.

ISH is a powerful technique used to detect the expression of mRNA at the cellular level. In the course of this work, we improved the detection step of ISH by using fluorescent dyes (tyramide) which increased the sensitivity of our assay by amplifying the riboprobe signal.

To detect the total protein expression of FcεRI, we used fluorescent ICC which enabled us to determine the intracellular and extracellular (total) protein expression of FcεRI. Membrane bound receptor expression was analyzed using FACS, which is a widely used technique for detection of membrane bound receptors. We used mouse monoclonal anti-human FcεRIα (CRA1) directed against the stalk region (non binding
site of IgE) of FcεRI unlike mAb15 or CRA2 which recognize the IgE binding site. This antibody enabled to determine the presence of membrane bound FcεRIα even if it was bound to IgE. The receptor FcγRIII/CD16 (FcγRIIIb) is constitutively expressed in human PMNs (158) and (CD16) was used as a positive marker for PMNs.

As demonstrated by fluorescent ISH, percentage of FcεRIα mRNA positive PMNs were increased in AA subjects (mean value ± SEM = 63.21% ± 10.21; n=19) when compared with ANA (22.89% ± 8.08; n=18) and healthy subjects (10.14% ± 6.25; n=21) as shown in Fig 1 and summarized in Fig 2. Similarly FcεRIα protein immuno positive PMNs were increased in AA subjects (mean value ± SEM = 86.92% ± 9.03; n=10) when compared with ANA (28.82% ± 13.47; n=11) and healthy subjects (0.4793% ± 0.4793; n=11). The representative confocal images of total protein expression are shown in Fig 3 and summarized in Fig 4. Similarly, FACS analysis (Fig 5 and 6) showed an increased surface expression in AA subjects with an average positivity of 48% (mean value ± SEM = 47.67 ± 5.46; Average Mean Fluorescent Intensity (MFI) = 49.5 ± 12.37; n=17) compared with the ANA group, which had an average positivity of 8% (7.74 ± 4.41; MFI = 3.54 ± 2.3; n=15) and healthy individuals with a mean positivity of 6% (6.23 ± 3.39; MFI = 0.65 ± 0.3; n=16).

These data suggest that AA subjects show an increase in both extracellular surface expression and total protein expression of FcεRI, when compared with ANA and healthy subjects. Interestingly, the average percentage of mRNA and total protein expression in AA subjects is increased compared with FACS (surface) expression, suggesting the regulation in extracellular surface expression of FcεRI. All together
these data suggest that *in vivo*, PMNs of AA subjects have increased expression of FcεRI. This suggests those individuals may be more prone to respond to IgE mediated activation in comparison with those from the ANA group and healthy individuals.
Figure 1. Representative confocal images showing the sense control (left panels) and FceRIα chain (right panels) mRNA expressing PMNs by Fluorescent ISH. A. Cytoxin prepared neutrophils from normal subject were stained for digoxigenin labeled sense and FceRIα chain. B & C. PMNs from ANA and AA subjects represents the digoxigenin labeled sense and FceRIα chain staining respectively. The slides are positively stained with TSA- FITC and PI for staining the nuclei.
Figure 2. FcεRIα mRNA expression by human neutrophils of the three subjects estimated by ISH. Expression of FcεRI α chain mRNA at 0hrs by normal (n=21), ANA (n=18) and AA (n=19) subjects. The positive and negative cells were scored with eye. Expressed on y axis are values of percentage FcεRI α mRNA of positive neutrophils. *P=0.0022, #P=0.0149.  

P values were calculated using Mann Whitney U test.
Figure 3. Representative confocal images showing the isotype control (left panels) and FcεRIα (right panels) total protein expressing PMNs by Fluorescent Immunocytometry. A. Cytopin prepared neutrophils from normal subject were stained for IgG2a and CRA1. B & C. PMNs from ANA and AA subjects represents the isotype control and CRA1 staining respectively. The slides are positively stained with FITC and PI for staining the nuclei.
Figure 4. Total protein expression of FcεRI by human neutrophils of the three subjects estimated by ICC. Expression of FcεRI α chain receptor at 0hrs by normal (n=11), ANA (n=11) and AA (n=10) subjects. Expressed on y axis are values of percentage of mAb CRA-1 positive neutrophils. *P<0.0001, #P=0.0121. P values were calculated using Mann Whitney U test
Figure 5. Representative histograms showing the surface expression of CD16 (left panels) and FcεRIα (right panels) positive cells by FACS. A. Freshly isolated neutrophils from normal subject were stained for CD-16 and CRA1. B & C. PMNs from ANA and AA subjects show the CD-16 and CRA1 positive staining by FACS respectively. In the histograms, the green line shows the isotype control and the blue shaded block represents the receptor expression.
Figure 6. Extracellular expression of FcεRIα by neutrophils of the three subjects (Normal, ANA and AA) estimated by FACS. The surface expression at 0 hrs of normal (n=16), ANA (n=15) and AA (n=17), on y axis are values of percentage of mAb CRA-1 positive neutrophils. The percentage of positive cells was calculated by subtracting the isotype control IgG2a from specific signal. *#P<0.0001, P values were calculated using Mann Whitney U test.
3.3. Expression of FcεRI in and out of allergic season:

Many factors influence the severity of allergic diseases. Environmental factors influenced by seasonal changes are important inducers of allergic diseases, whereby seasonal changes modulate the immune response (156, 159). Due to sensitization by allergens (which are increased in the allergic season), the initial response to an allergen is dominated by products such as histamine and prostaglandin D2 (PGD$_2$) which is released by activated mast cells. Within hours of the initial response, inflammatory cells such as T cells, neutrophils, eosinophils, basophils and monocytes are recruited from the circulation to the airways (160). Furthermore, seasonal changes also alter the expression of various receptors in these cells (161).

Various studies have showed that seasonal changes influence the activities of PMNs in allergic conditions (156). In pollen-induced asthma, seasonal antigenic exposure increases the number of PMNs in bronchial biopsies (156). Further studies have showed that neutrophil chemotactic activity in BAL fluid is significantly increased in pollen season (157). The expression of FcεRI in AA subjects clearly indicates an increased although heterogeneous expression of the receptor when compared with the other two groups. Since we had the opportunity to recruit the same patients in and out of the allergen pollen season, we investigated whether changes in these environmental factors could influence FcεRI expression in PMNs. Ultimately, this study has provided us with new insight into the in vivo modulation of neutrophil functions in asthmatic conditions.
3.3.1. **Protein expression of FcεRI during and out of allergic season:**

Seasonal influence in the expression of FcεRI in PMNs from AA, ANA and healthy subjects was studied by analyzing the expression patterns during and out of pollen allergic season. Extracellular expression of FcεRIα was studied by FACS. The surface expression of CD16 (representative histogram shown in Fig.7A, B, – left panel) and FcεRIα (representative histogram shown in Fig.7A, B, – right panel) during and out of the season of an AA is shown. The percentage of PMNs expressing surface receptors (CD16 and FcεRIα) during and out of season for all the three groups is shown in Fig 8. During the allergic season there is a greater increase in the surface expression of FcεRI in the AA subjects (mean value ± SEM = 57.37 ± 6.632; P=0.002; n=9) compared with the other two groups (11.24 ± 5.81; n=8 and 0.0 ± 0.0; n=8 for ANA and healthy subjects respectively). This increase in the expression of FcεRI during pollen allergic season which is reduced out of season suggests that the expression is regulated *in vivo*.

Seasonal changes are influenced by changing patterns of air pollution, which in turn is associated with a rise in asthma prevalence (162). Also it is known that AA individuals are influenced by seasonal changes leading to elevated Th2 cytokines. Furthermore, Th2 cytokines such as IL-4 and IL-9 have been shown to induce FcεRIα chain expression in various cell types (138-140). Due to this elevated Th2 environment during the allergic season, it is possible that the same cytokines might increase the expression of FcεRI in PMNs as observed during allergic season.
To further confirm whether this effect is specific for FcεRI or whether other neutrophil surface receptors may also be modulated, we analyzed the changes in the expression of FcγRIII/CD16 in these subjects. As shown in Fig 8B, no significant change was detected in FcγRIII/CD16 surface expression during and out of allergic season (Fig 8B), indicating that the seasonal increase in the expression of PMN FcεRI was specific.

The total protein expression of FcεRIα was studied by fluorescent ICC for which cytospin prepared slides of PMNs from AA subjects were used. Isotype control IgG2a (representative confocal images are shown in Fig 9A) and CRA-1 was used for detection of FcεRIα (representative confocal images shown in Fig.9B – out of season and 9C – during season). The difference in the total protein expression of FcεRIα during and out of the season of the three groups is shown in Fig 9D. Similar to extracellular expression, a statistically significant increase in the fraction of positively stained cells, was observed within AA subjects (mean value ± SEM = 64% ± 11.30; P=0.0313; n=8) where as similar significance was not observed in the other two groups (43.56% ± 19.16; n=7 and 3.757% ± 2.68; n=9 for ANA and healthy individuals respectively).

3.3.2. Messenger RNA expression of FcεRI during and out of allergic season:

mRNA expression of FcεRIα during and out of the allergic season was estimated by fluorescent ISH. Digoxigenin labeled FcεRIα sense probe were used as control (Fig 10A, B – left panel). An anti-sense probe which could detect FcεRI
mRNA was used to detect FcεRI α mRNA (Fig 10 A, B, C – right panel). The expression of FcεRIα mRNA in and out of season is shown for the three groups in Fig 10C. Similar to protein expression, a statistically significant mRNA increase was observed within AA subjects group during the allergic season (mean value ± SEM = 91.60 ± 4.01; P=0.031; n=10) where as it was not significant in the other two groups (34.13 ± 14.65; n=9 and 2.10 ± 1.05; n=10 for ANA and healthy subjects respectively).

Our results demonstrate that during the allergic season, FcεRI expression in PMNs increases both at the protein (surface and intracellular) and mRNA levels. This suggests that allergen exposure may directly or indirectly influence neutrophil functions through FcεRI mediated mechanisms.

Interestingly, in PMNs from ANA subjects, 3 of 9 patients showed an increase of FcεRI mRNA and surface expression in the allergic season (Fig 10c and 8a). This data suggests that FcεRI regulation in human PMNs might depend on the severity of the allergic condition. Further, FcεRI expression studies in a larger population of ANA subjects might provide insight into the seasonal influence in this group.
Figure 7. Representative histograms of extracellular expression of FcεRI during and out of allergic season by FACS. 

A. Representative histograms showing expression of CD16 positive cells (left) and FcεRIα (right) of an AA subject, out of allergic season. 

B. Representative histograms showing expression of CD16 positive cells (left) and FcεRIα (right) of the same AA subject during allergic season. In the histograms, the green line shows the isotype control and the blue shaded block represents the receptor expression.
Figure 8. Extracellular expression of FcεRI of human neutrophils during and out of allergic season by FACS. 

A. Extracellular expression of FcεRIα at 0 hrs of normal (n=8), ANA (n=8) and AA (n=9) during and out of allergic season. *P=0.002. 

B. Extracellular expression of CD16 of the same normal, ANA and AA subjects during and out of allergic season. # Mean is ~ equal. On y axis are values of percentage of mAb CRA-1 positive neutrophils. The percentage of positive cells was calculated by subtracting the isotype control IgG2a from specific signal. P values were calculated using Wilcoxon signed rank test.
Figure 9. Immunocytochemistry for total protein expression of FcεRI during and out of allergic season by human neutrophils. A. Representative confocal images showing isotype control of AA subject. B. Representative confocal images and FcεRIα of AA subject out of allergic season. C. Representative confocal images and FcεRIα of AA subject during the season, stained with FITC and PI. D. Expression of FcεRI α chain receptor at 0hrs by normal (n=9), ANA (n=7) and AA (n=8) subjects during and out of allergic season. *P=0.0313. On y axis are values of percentage of mAb CRA-1 positiveneutrophils. P values were calculated using Wilcoxon signed rank test.
Figure 10. In Situ hybridization (fluorescent) for mRNA expression of FcεRI α by human neutrophils during and out of allergic season. A. Representative confocal images showing sense strand control (left) and FcεRIα chain mRNA (right) for AA subject out of allergic season. B. Representative confocal images showing showing sense strand control (left) and FcεRIα chain mRNA (right) for AA subject during allergic season stained with TSA (Tyramide Signal Amplification-FITC) and PI. C. Expression of FcεRI α chain mRNA at 0hrs by normal (n=10), ANA (n=9) and AA (n=10) subjects during and out of allergic season. Expressed on y axis are values of percentage of mRNA positive neutrophils. *P=0.0391. P values were calculated using Wilcoxon signed rank test.
3.4. **Effect of Th-2 cytokines on the expression of FcεRI in atopic asthmatic subjects:**

A Th2 cytokine profile is observed in asthma and also in allergic conditions such as allergic rhinitis and atopic dermatitis (160). In AA individuals, cytokines such as IL-3, IL-4, IL-5 and GM-CSF are significantly upregulated after allergen challenge in asthmatic airways and their receptors were identified on the surface of inflammatory cells (163). These Th2 cytokines have been well shown to be associated with pathological changes of asthma (163). Of note, Th2 cytokine IL-9 has been shown to be associated with airway hyperresponsiveness, mucus hypersecretion, eosinophil function, IgE regulation and up regulation of calcium-activated chloride channel (160, 164-166).

The increased expression of FcεRI, especially in the AA subjects during the allergic season suggests tight regulation of FcεRI expression. To investigate the effect of Th-2 cytokines on FcεRI expression, PMNs from AA subjects were stimulated *in vitro* with graded doses of IL-9, IL-4 and GM-CSF for 6hrs and 18hrs. The effect of IFN-γ on the expression of FcεRI in PMNs was also determined, since this Th-1 cytokine has been shown to upregulate FcεRIγ chain in mouse mast cells (167, 168). Upon stimulation, FcεRI expression at surface, total protein and mRNA levels was determined by FACS, ICC and real time RT-PCR respectively.
3.4.1. **Effect of Th-2 cytokines on mRNA expression of FcεRIα in AA subjects:**

To investigate the effects of Th2 cytokines on the expression of FcεRIα mRNA in human PMNs, we performed real time RT-PCR to quantify the message levels of FcεRI α chain. Further, to provide more evidence for the involvement of Th2 cytokines in FcεRI expression at mRNA level we performed fluorescent ISH, as shown in Fig 11.

Real time PCR analysis shows GM-CSF followed by IL-9 and IL-4 increases the expression of FcεRIα mRNA when compared with media and IFN-γ, as shown in Fig 12. These data suggest that Th-2 cytokines IL-9, IL-4 and GM-CSF increase the expression of FcεRI in vitro in AA subjects at mRNA levels (which is shown by ISH and real time PCR analysis). These data suggest that Th2 cytokines play a significant role in upregulating the expression of FcεRI at transcription levels at 6hrs of incubation. We further studied the effect of Th2 cytokines on expression of FcεRI protein was studied by FACS and Western blot analysis.

3.4.2. **Effect of Th2 cytokines on protein expression of FcεRIα in AA subjects:**

Extracellular expression of FcεRI in Th2 cytokine stimulated neutrophils was studied by FACS as is shown in Fig 13. CD-16 positive PMNs from AA subjects (Fig 13A.) were stimulated with IL-9 and the effect on surface expression (n>3) of the receptor (Fig 13C and D) was determined at 6hrs. Th2 cytokine IL-9 increases the surface expression of FcεRIα when compared with media at 6hrs.
In our earlier observations we noted that AA subjects have increased FcεRIα mRNA expression (mean value ± SEM = 63.21% ± 10.21 - Fig2) when compared with surface expression (mean value ± SEM = 47.67% ± 5.46 – Fig6). Also total protein expression as determined by ICC is increased (mean value ± SEM = 86.92% ± 9.03 – Fig 4) when compared with FACS results (surface expression), suggesting that FcεRI is regulated at the translational and or protein transport level.

The expression of FcεRIα protein in Th2 cytokines treated cells was studied by IP and Western blot analysis (n=3). Protein concentration was estimated in Th-2 cytokines and IFN-γ stimulated PMNs at 6 hrs and 18hrs. The protein was immunoprecipitated (IP) with IgE and αhIgE (2μg/ml) and the western blot was performed using CRA-1 (mouse αh FcεRI α). The protein expression of FcεRIα at 6hrs and 18hrs is shown in Fig 14A and Fig 14B respectively. The increase in the expression of FcεRIα is observed in Th-2 stimulated cells when compared with media, suggesting that Th-2 cytokines increase the protein expression of FcεRI. Interestingly, IFN-γ increases FcεRIα protein expression at 6hrs which is lost at 18hrs (Fig14). It is a possibility that IFN-γ may first stimulate and later inhibit the expression of FcεRI during the course of incubation. The inhibition of FcεRI mRNA by IFN-γ at 6hrs (Fig12A) supports this possibility. Further studies at early time points (before 6hrs) may provide an insight into the effects and possible role of IFN-γ on the expression of FcεRI in PMNs.
Figure 11. Effect of Th-2 cytokines on mRNA expression of FcεRI in neutrophils, studied by fluorescent ISH. PMNs were stimulated with IL-9, GM-CSF, IL-4 and IFN-γ (10ng/ml) and mRNA expression is studied at 6 hrs for its expression of FcεRIα chain by fluorescent ISH stained with TSA (Tyramide Signal Amplification-FITC) and PI. A. Representative confocal images showing sense strand control in IL-9 stimulated slide at 6hrs. B. Representative confocal images showing FcεRIα mRNA expression in media at 6hrs. C. IL-9 at 6hrs D. IL-4 at 6hrs E. GM-CSF at 6hrs F. IFN-γ stimulated slides at 6hrs.
Figure 12. Quantitative analysis of FcεRIα mRNA expression of Th-2 stimulated neutrophils. PMNs were stimulated with GM-CSF, IL-9, IL-4 and IFN-γ (10ng/ml) and studied at 6 hrs for mRNA expression of FcεRIα by Real Time PCR analysis. A. Quantitative expression of FcεRIα mRNA to Th-2 stimulated PMNs normalized to G3PDH estimated at 6hrs (n=3). B. Agarose gel showing the Real time PCR amplified products of expression of FcεRIα by Th-2 stimulated PMNs (upper lane) and its respective Real time PCR amplified G3PDH. Lane1→14: Marker, Standards 1, Standard 2, Standard 3, Media (1:2 dil), (1:4 dil), IL-9 (1:2 dil), (1:4 dil), IL-4 (1:2 dil), (1:4 dil), GM-CSF (1:2 dil), (1:4 dil), IFN - γ (1:2 dil), (1:4 dil) respectively.
Figure 13. Effect of IL-9 on extracellular expression of FcεRI by human neutrophils. PMNs were stimulated with IL-9 (10ng/ml) and studied at 6 hrs for its expression of FcεRI α chain by FACS. A. Histograms representing the CD-16 positive cells. B. Histogram showing the absence of FcεRI α chain in media at 0hr C. Histogram showing the absence of FcεRI α chain in media at 6hrs. D. Increased expression of FcεRI receptor when stimulated with IL-9 at 6 hrs. Similar results were observed for GM-CSF and IL-4 (data not shown). In the histograms, the green line shows the isotype control and the blue shaded block represents the receptor expression.
Figure 14. Effect of Th-2 cytokines on FceRIα protein expression of neutrophils, estimated by Western blot analysis. PMNs were stimulated with Th-2 cytokines GM-CSF, IL-9, IL-4 and IFN-γ (10ng/ml) and studied at 6 hrs and 18 hrs for protein expression of FceRIα by Immunoprecipitation and Western blot analysis. KU812 cells were used for positive and negative control. IP: IgE and αhIgE (2μg/ml). WB: CRA-1 (mouse ah FceRI α), where FceRIα is a 46Kd protein and the 52Kd represents IgG heavy chain. A. Expression of FceRIα chain at 6hrs in Th-2 stimulated PMNs. Lane1: Positive control, Lane 2: Media, Lane 3: IL-9, Lane 4: IL-4, Lane 5: GM-CSF, Lane 6: IFN-γ, Lane 7: Negative control. B. Expression of FceRIα chain at 18hrs in Th-2 stimulated PMNs. Lane1: Positive control, Lane 2: Negative control, Lane 3: Media, Lane 4: IL-9, Lane 5: IL-4, Lane 6: GM-CSF, Lane 7: IFN-γ.
3.5. **Expression of FcεRIβ chain in neutrophils:**

As mentioned earlier in the introduction, FcεRIβ chain plays an important role in the pathogenesis of allergic diseases. The β chain (FcRβ) has been implicated in the pathogenesis of allergy by a number of genetic studies. Furthermore, at the cellular level, over expression of FcRβ enhances maturation and the assembly processes of FcεRI and increases its surface expression. Upon FcεRI cross-linking, the β chain amplifies activation signals of early signaling events and also late responses such as cell degranulation and cytokine release. Taken together β chain seems to play a role in allergic response modulation by its function as an amplifier of effectors cell responses (149, 152, 153).

The expression of β chain has been shown to be present in cells such as mast cells and basophils whereas in PMNs it has not been well established; even though the mRNA of β chain of FcεRI has been shown to be expressed in AA subjects (77). Since the β chain plays a significant role in the regulation of FcεRI, it led us to investigate the expression of β chain in PMNs isolated from all three group of subjects.

The total protein expression of β chain was estimated by fluorescent ICC and IP/ Western blot analysis. The protein isolated from the three groups of subjects was both immunoprecipitated and western blotted with anti-FcεRI, β subunit (n=2). Fig 15 shows the β chain expression in PMNs from the three groups (AA, ANA and healthy). AA subjects showed increased β chain expression (Fig 15 Lanes:1 ->4) when
compared with the ANA subjects (Fig 15 Lanes:5 ->7). Healthy individuals (Fig 15 Lanes: 8 ->9) did not express the β chain.

Similarly, in ICC cytospin prepared PMNs from the three group of subjects were stained for isotype control (rabbit IgG) (representative confocal image shown in Fig 16A) and polyclonal rabbit αhFceRIβ was used (representative confocal image shown in Fig 16B, C and D) for detection of total protein expression of β chain. The fraction of PMNs showing staining for the β chain in all three groups is shown in Fig 17. The data clearly shows increased expression of the β chain in AA subjects (mean value ± SEM = 81.82% ± 12.20; P = 0.0029 and P = 0.04; n=11) when compared with the other two groups (30.0% ± 15.28; n=10 and 1.33% ± 1.33 n=9 for ANA and healthy subjects respectively).

When the expression of β chain is compared with the surface expression of FceRIα, it is interesting to note that there is increased β protein expression in subjects which show surface expression of the receptor (FceRIα) especially in AA subjects, as shown in Fig 18A. Further correlation analysis (Fig 18B) shows increasing linear relationship (r = 0.8054) between the total protein expression of β chain with the surface expression of α chain of FceRI in human PMNs.

Our results show that AA subjects show increased protein expression of β chain when compared with the other two groups both by ICC and Western blot analysis. Further there is a linear relationship between the expression of β chain and surface expression of FceRI in human PMNs.
In general our results show that the expression of FcεRI in PMNs of AA patients is increased when compared with ANA and healthy individuals. Supporting our hypothesis, the expression of FcεRI is regulated \textit{in vivo} in AA subjects. Th2 cytokines such as IL-9, IL-4 and GM-CSF upregulate the expression of FcεRI in human PMNs \textit{in vitro}. Further the evidence that β chain is expressed in PMNs of AA subjects suggests that FcεRI is expressed in a tetrameric form in PMNs and the expression of β chain might be one of the mechanisms that are involved in the regulation of surface expression of FcεRI in PMNs.
Figure 15. FcεRIβ protein expression of neutrophils from the three subjects, estimated by Western blot analysis. Protein from PMNs of the three subjects at T-0 is analyzed for protein expression of FcεRIβ by Immunoprecipitation and Western blot analysis. KU812 cells were used for positive and negative control. IP: rabbit anti-human FcεRIβ (2μg/ml). WB: Anti-FcεRI, β subunit (1:500), where FcεRIβ is a ~29Kd protein and the 52Kd represents IgG heavy chain. Lane1-4: AA subjects, Lane 5 -7: ANA subjects, Lane 8 -9: Normal subjects, Lane 10: Positive control, Lane 11: Negative control (rabbit IgG).
Figure 16. Representative confocal images showing the total protein expression of FcεRIβ expressing PMNs by Fluorescent ICC. Cytopin prepared neutrophils from the three subjects were stained for isotype control (rabbit IgG) and polyclonal rabbit αhFcεRIβ. A. Representative confocal image of isotype control. B. PMNs from normal subject showing the expression of FcεRIβ protein. C. PMNs from ANA subject representing FcεRIβ staining. D. PMNs from AA subject representing FcεRIβ staining. The slides are positively stained with FITC and DAPI for staining the nuclei.
Figure 17. Total protein expression of FcεRIβ by human neutrophils of the three subjects estimated by ICC. Expression of FcεRI β chain receptor at 0hrs by normal (n=9), ANA (n=10) and AA (n=11) subjects. Expressed on y axis are values of percentage of mAb CRA-1 positive neutrophils. * P = 0.0029, # P = 0.04. P values were calculated using Mann Whitney U test.
Figure 18. Comparative analysis of extracellular expression of FcεRIα chain and its respective FcεRIβ chain protein expression. A. The extracellular expression of FcεRIα estimated by FACS is compared with its respective β chain expression estimated by fluorescent ICC at T=0 of the three subjects. B. Correlation analysis between total protein expression of beta chain and surface expression of alpha chain shows positive correlation Spearman $r = 0.8054$, $P<0.0001$ by Spearman Rank Correlation test.
4. DISCUSSION:

4.1. Introduction:

The role of PMNs has been largely neglected despite evidence that they have the capacity to stimulate and produce cytokines and to release chemoattractants such as LTB4 and IL-8 (74, 77, 169). The high affinity IgE receptor is a central molecule in the initiation of allergic diseases (150) and the functional expression of FcεRI in PMNs from atopic asthmatic subjects illustrates its importance in asthma (77).

In an initial study (77), great heterogeneity in the surface expression of FcεRI in PMNs from atopic asthmatics was observed; this led us to investigate its regulation. In our study we first compared FcεRI expression in PMNs from atopic asthmatics, atopic non asthmatics and healthy donors. Secondly, we investigated how pollen exposure affects the receptor expression in order to obtain an overview of the in vivo regulation of the receptor in PMNs. Further we stimulated PMNs with various Th-2 cytokines such as GM-CSF, IL-9 and IL-4 to study the effects in vitro. Eventually detecting the protein expression of β chain provided some information on the mechanisms involved in the regulation of FcεRI in human neutrophils.

4.2. Expression of FcεRI in human neutrophils:

Previously, the expression of high affinity IgE receptors in PMNs was observed in atopic asthmatic subjects (77). Here we investigated the expression of
FcεRI in PMNs from AA, ANA and healthy subjects at both mRNA and protein level. The protein expression of FcεRI was measured by FACS (surface expression) and ICC (total protein). mRNA expression was determined by ISH, which enabled us to rule out the possibility of contamination and facilitated the colocalization of mRNA of FcεRIα chain at the cellular level.

Human FcεRI expression on the cell surface requires at least α and γ chains of FcεRI (99, 170). However the extracellular domain of the α chain of FcεRI contains sufficient molecular information to provide the receptor with high affinity binding (44). In our FACS analysis we used CRA-1 (which is a non IgE binding site antibody) to determine the surface expression of FcεRI. It enabled us to determine the presence of FcεRIα surface expression, even if the receptors were bound to IgE. Previously CRA-1 has been used and shown to bind extracellular FcεRIα receptors in transfectants and peripheral blood eosinophils (171).

One of the limitations of using CRA-1 may have been the detection of receptors on the membrane bound to soluble receptors. In transfection experiments it has been shown that soluble α chain is present in human truncated constructs (172). Functionally, these recombinant soluble (rs)FcεRIα chains were shown to completely inhibit IgE binding to the cell surface, resulting in abrogation of the chemical mediator release from RBL-2H3 cells (173). Possibilities of these soluble receptors binding in vivo to its receptors on the membrane cannot be ruled out.

Antigen aggregation of IgE occupied FcεRI causes phosphorylation of the immunoreceptor tyrosine based activation motifs (ITAMs) found within the β and γ
chains of the multisubunit FcεRI. Further Lyn and Syk kinases are recruited to β and γ chains. Downstream multiple adaptor proteins or proteins containing adaptor like regions (eg: LAT) have been identified in mast cells. It is thought that these adaptor molecules might play a critical role in organizing and coordinating receptor-stimulated responses such as degranulation (174).

Total FcεRI protein expression was estimated by ICC. Significant differences were observed between the levels of surface expression and FcεRIα immunopositive PMNs detected by ICC, which detects both the surface and intracellular stored protein. These data suggest that the surface expression of FcεRI may be under regulatory control. Transfection experiments suggest that the α chain contains an endoplasmic reticulum (ER) retention signal, which is a di-lysine motif located in its cytoplasmic domain (105). In the absence of the other chains especially the γ chain, the α chain remains and is degraded in ER. However, γ chain masks the ER signal and enables the complex to be exported to the Golgi apparatus and to the plasma membrane (44).

4.3. In vivo expression of FcεRI in AA subjects:

Seasonal changes influence the activities of PMNs (156, 157); which in turn may affect the expression of FcεRI. Investigating the expression of FcεRI during and out of pollen season, would provide an insight into the in vivo regulation of the receptor in human PMNs.
Extracellular surface expression of FcεRI in AA subjects during the pollen season is clearly increased when compared to the non pollen season, whereas a similar pattern of surface expression is not observed in ANA or normal subjects. CD-16 expression during and out of the pollen season remains unaltered in all three groups (Fig 8B) suggesting that the increase in the expression of FcεRI in AA subjects during the allergic season is specific.

Atopic or allergic conditions, such as seasonal allergy, urticaria and eczema are characterized by type I hypersensitivity reactions. More serious conditions such as asthma and anaphylaxis are believed to share a similar pathogenesis (16). However, activators of asthma, such as allergens and viral infections are linked closely to the clinical manifestation of the disease and they determine the severity of the disease to a large extent (12). Activators of asthma such as pollen are increasingly present in the environment during the allergic season. Studies with house dust mite (HDM) in allergic asthmatic patients, suggest increased airway hyperresponsiveness during autumn, depending on sensitization to HDM and an increase of exposure to HDM allergen (175). Our data indicate in vivo regulation of the expression of the FcεRI receptor in AA subjects. Even though not statistically significant, ANA subjects show similar expression patterns in total protein and mRNA levels suggesting that PMNs might play an important role by IgE mediated mechanisms.

IgE affects a positive feed back mechanism that enhances immediate hypersensitivity reactions. Asthma, allergic rhinitis and atopic dermatitis are almost invariably accompanied by elevated levels of IgE (54). Studies have shown a strong correlation between total IgE receptors and serum IgE in basophils (136).
Furthermore, IgE binding to FcεRI in the absence of specific antigen has been shown to up-regulate its surface expression in mast cells and basophils (134, 176, 177). In a variety of studies in both mouse and man, a relationship between IgE and FcεRI is clearly shown both in vivo and in vitro (51, 134, 135, 176). During allergic season there are increased amounts of IgE in atopic individuals, suggesting its role in the increased expression of FcεRI during pollen season when compared with non pollen season. Further correlation analysis of serum IgE concentrations and its receptor expression in these ANA and AA subjects would provide more information in the in vivo regulation of FcεRI in human PMNs.

4.4. Role of Th-2 cytokines in the expression of FcεRI in neutrophils:

In vivo and in vitro studies have shown a modulatory effect of cytokines on neutrophil receptor expression. In particular, receptors of the immunoglobulins and their subunits have been shown to be regulated by both Th-1 and Th-2 cytokines (178, 179). Cytokines especially Th-2 cytokines such as IL-4 and GM-CSF have been shown to induce surface expression of CD23 and CD18 on human neutrophils (78, 180). IL-4 and IL-9 have been shown to up regulate the FcεRIα chain expression in human eosinophils, mast cells and T cell line (138-140).

IL-9 up-regulated the surface expression of FcεRI at 6hrs in AA subjects (Fig 13). Similarly previous experiments in our lab have shown cytokines such as IL-4 and GM-CSF up regulate the surface expression of FcεRI at 6hrs (data not shown).
IFN-γ is a Th-1 cytokine which has been shown to regulate many surface receptors in neutrophils such as CD64/FcγRII (167). At mRNA levels, IFN-γ a Th-1 cytokine seems to down regulate the expression of FcεRIα at 6hrs (Fig 11 and 12). At the protein level expression Th-2 cytokines such as IL-9, IL-4 and GM-CSF up regulate the expression of FcεRI at both 6hrs and 18hrs. It is interesting to note that IFN-γ up regulates the protein expression of FcεRIα at 6hrs whereas at 18hrs it is reduced, correlating with its mRNA expression at 6hrs (Fig 15). Studies have demonstrated that IFN-γ acts to down regulate the expression of IFN-γ receptors on the surface of PMNs by internalization of the receptors (181, 182). Further studies have demonstrated that IFN-γ priming of PMNs prepares the cells for increased oxidative metabolism, surface receptor expression, degranulation and various other functions (183, 184). It can be speculated that when stimulated with INF-γ, it may up regulate the expression of FcεRI early on which may be shut down or down regulated on further incubation by inhibiting the mRNA synthesis of FcεRI (as seen at 6hrs by quantitative PCR analysis). Also it is a possibility that IFN-γ may internalize its receptors as a regulatory mechanism to prevent PMNs from causing damage during the resolution of inflammation (184) and as a result might also down regulate the expression of FcεRI.

Th-2 cytokines such as IL-4, IL-9 and GM-CSF are secreted in increasing amounts during atopic conditions, which in vitro up regulates the expression of FcεRI at both mRNA and protein levels suggesting a role in regulating the expression of FcεRI in neutrophils derived from atopic asthmatics.
4.5. **Role of β chain in the expression of FcεRI in neutrophils:**

FcεRI is expressed in tetrameric or trimeric forms in humans. The tetrameric form is expressed in mast cells and basophils, whereas the trimeric form is variably expressed in monocytes and various other cell types, such as Langerhans cells. mRNA expression of α, β and γ chains has been shown in PMNs (77). β chain expression plays a significant role in the regulation of FcεRI expression. It enhances maturation and the assembly of FcεRI, increasing surface expression of the receptor. It has also been shown to be involved in early signaling events and also in late responses such as cell degranulation and cytokine release, suggesting its role in modulating allergic responses due to its function as an amplifier of effector cell responses (149, 152, 154).

Here we have demonstrated the protein expression of β chain in PMNs. Atopic asthmatic subjects display increased expression of β chain when compared with ANA and healthy subjects (Fig 16 and 17). These results suggest that β chain, expressed in atopic asthmatic subjects might play an important role in the regulation of FcεRI expression and provides further evidence for tetrameric form of expression of FcεRI in human PMNs. Interestingly the surface expression of FcεRIα in atopic asthmatic subjects is also increased suggesting the importance of β chain in the receptor surface expression. Further correlation analysis (Fig 18B) shows an increasing linear relationship (r = 0.8054) between the total protein expression of β chain with the surface expression of α chain of FcεRI in human PMNs.
4.6. Global Summary:

As previously observed PMNs from AA subjects display increased expression of FcεRI when compared with ANA and healthy subjects at both protein and mRNA levels. This expression of FcεRI in AA subjects is regulated *in vivo*, with an increased expression of the receptor during the pollen season compared with the non pollen season, suggesting that PMNs might play an important role in asthma by IgE mediated mechanisms.

Th-2 cytokines such as GM-CSF, IL-9 and IL-4 up regulate the expression of FcεRI in neutrophils at both mRNA and protein levels *in vitro*. It is well established that these cytokines are secreted in large quantities in allergic diseases, which might influence the expression of the FcεRI in PMNs. Also, the β chain which is shown to be associated with atopy might be an important regulator in the expression of the high affinity IgE receptor in PMNs. This study presents evidence for protein expression of β chain in PMNs, suggesting its role in the regulation of FcεRI, especially in AA subjects.

PMNs may be significantly involved in immunoregulatory functions such as allergen presentation, production of cytokines, chemokines and release of various reactive oxygen species (ROS) thereby contributing to allergic diseases by increasing airway inflammation and bronchial hyperresponsiveness. These findings suggest that neutrophil mediated FcεRI dependent activation may play a key role in allergic diseases.
5. References:


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