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Epigenetic and Purinergic Regulation of Mast Cells Mediator Release

Zahraa Abdulmohsin Mohammed

EPIGENETIC AND PURINERGIC REGULATION OF MAST CELLS MEDIATOR RELEASE

by

Zahraa Abdulmohsin Mohammed

Bachelor of Science
Baghdad University, 2001

Master of Science
Baghdad University, 2005

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Accepted by:

Gregorio Gomez, Major Professor

Jason Kubinak, Major Professor

Traci Testerman, Committee Member

Ioulia Chatzistamou, Committee Member

Daping Fan, Committee Member

Maksymilian Chruszcz, Committee Member

Cheryl L. Addy, Vice Provost and Dean of the Graduate School

DEDICATION

I dedicate this scientific work to the Higher Committee For Education Development in Iraq (HCED) who supported me all the years of my study.

I also dedicate this work to the Ministry Of Higher Education and Scientific Research (MOHESR), to the Al- Mustansiriyah University, college of Medicine, especially the department of microbiology in Iraq as well as to the Iraqi Cultural Attaché in Washington DC.

Finally, I dedicate this work to my father who had passed away from this world, and to my family who have always been my greatest supporters during the good and bad times.

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ABSTRACT

Mast cells (MCs) are well known for their implications in allergic reactions. They are also known to have multiple functions in the innate and adaptive immune system. Their activation plays an essential role in many aspects of physiological and pathological conditions. Allergies are considered chronic conditions that affect more than 60 million people in the U.S. These diseases are driven by the activation of MCs in response to IgE-mediated antigen, rendering these cells as targets for the management of allergies and asthma. Therefore, this research considers the understanding of their activation and the regulation of their response to be an important step for the management of allergies. Previous studies have shown the role of adenosine in the modulation of the activation of mast cells through its interaction with its receptors in allergic asthma. In this study, we investigated the role of the adenosine receptors, and particularly the A_{2A} subtype, on the regulation of allergic mediators from human skin mast cells. We demonstrated that A_{2A} signals inhibit FcεRI–induced proinflammatory cytokines via cAMP mechanism. However, the A_{2A} receptor has no effects on FcεRI-induced degranulation or PGD_2 production. We also showed that FcεRI signaling plays a significant role in the modulation of the expression and the function of adenosine receptors on mast cells. We found that sub-threshold stimulation of FcεRI led to up-regulation of the A_{2A}, and to down-regulation of the A_3 receptors at the mRNA and protein levels. Additionally, we observed that up-regulation of the A_{2A} receptors by sub-threshold of FcεRI led to be more pronounced inhibition of TNF by adenosine, which shifts mast cells into anti-inflammatory phenotype.

Next, we explored the role of miR-155 in MCs function. MiRNAs have been reported to regulate different genes involved in MCs activation, and miRNAs impact the function of MCs in various allergic reactions.

Research indicates miR-155 plays a key regulatory role in the pathogenesis of allergy. In this project, miR-155 expression was induced following IgE-receptor crosslinking on human skin mast cells as well as mast cells derived from bone mouse bone marrow (BMMCs). MiR-155 had no effect on IgE-dependent degranulation or leukotriene C4 secretion. Accordingly, arachidonate 5-lipoxygenase (ALOX5) expression was similar in WT and miR-155 KO BMMCs.

In contrast, FcεRI-induced COX-2 expression was significantly diminished in the absence of miR-155, suggesting that miR-155 plays a critical role in prostaglandin D2 biosynthesis. In addition, FcεRI-induced TNF, IL-6, and IL-13 was significantly diminished in miR-155 KO BMMCs compared to WT. Interestingly, the amount of these cytokines from miR-155 KO BMMCs increased compared to WT following LPS treatment. The phosphorylation of AKT was significantly decreased in miR-155 KO compared with WT following FcεRI crosslinking, whereas p38, and p42/p44 phosphorylation were the same in both types of mast cells. Collectively, these data demonstrate that miR-155 has both a positive and a negative regulatory action on the mast cell mediator release.

Recently, many studies have revealed that the natural polyphenol Resveratrol exhibits different biological and pharmacological properties, including anti- allergic effect. In the current study, experiments were designed to study the role of miRNAs in

Resveratrol-mediated inhibition COX-2 expression in the activated mast cells. We showed that Resveratrol inhibited FcεRI-induced COX-2 production in WT BMMCs and failed to inhibit COX-2 expression in miR-155 KO BMMCs. MiRNA array analysis and Ingenuity Pathway Analysis (IPA) revealed an altered miRNAs profile following Resveratrol treatment. One of the miRNAs that was significantly downregulated after Resveratrol treatment was miR-155. We then validated the miRNA array and IPA analysis by qRT-PCR. According to our results, there was a positive correlation between miR-155 and COX-2 expression in activated SMCs, in which both were downregulated after RSV treatment. However, the ATF3 expression was increased, which suggests that miR-155 could be the target of COX-2 expression. Collectively, Resveratrol inhibits FcεRI-induced COX-2 expression through inhibition miR-155 in mast cells. Therefore targeting miR-155-mediated inhibition of COX-2 by Resveratrol may serve as a new approach for the treatment of the allergic condition.

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LIST OF ABBREVIATIONS

ADA……...Adenosine Deaminase

ADK…….....Adenosine kinase

AND…….Anaphylactic Degranulation

BMMCs.....mouse Bone marrow-derived mast cells

CMCs……Connective Mast Cells

CNT…….. Concentrative Nucleoside Transporter

COX-2……Cyclooxgenase-2

ENT.....Equilibrative Nucleoside Transporter

FcεRI..... High-Affinity IgE Receptor

GPCRs……G-Protein Coupled Receptors

HDC……Histidine Decarboxylase

IgE..... Immunoglobulin E

IL-3……Interleukin-3

IL-4……Interleukin-4

IL-6……Interleukin-6

MAPK..... Mitogen Activating Protein Kinase

MCs..... Mast Cells

MMCs……Mucosal Mast Cells

NP-BSA……4-hydroxy-3-nitrophenyl acetyl-Bovine serum Albumin

PI3K .....Phosphotidyinsitol-3 Phosphate Kinase

PMD……..Piecemeal Degranulation

SCF ..... Stem Cell Factor

SMC ..... Skin Mast cells

TLRs ..... Toll-Like Receptor

CHAPTER 1

INTRODUCTION

1.1 General Biology of Mast Cells

Mast cells (MCs) are highly granulated immune cells of hematopoietic lineage that are found in all mammalian species[1,2]. Mature mast cells are characterized by three main features: (1) expression of FcεRI, the high affinity receptor for Immunoglobulin E, (2) expression of c-kit (CD117), the stem cell factor (SCF) receptor, and (3) expression of cytoplasmic granules containing many different bioactive mediators like histamine, serine proteases, and heparin[3,4]. MCs are ubiquitously distributed in tissues, especially those at the interface with the external environment, such as skin, airways, and gastrointestinal system[5-7]. MCs appear to localize near blood vessels and nerve endings [8]. Based on their strategic location, MCs are among the first cells to initiate defense reactions against invading pathogens, and they play a pivotal role in the innate and acquired immunity[9,10]. Developmentally, MCs, like other immune cells, originate from hematopoietic CD34+ stem cells (HSC) in the bone marrow[11]. At a key point during development, the immature CD34+ stem cells leave the bone marrow and migrate into the peripheral tissue where they further differentiate into mature MCs and acquire their specific phenotype[12,13].

Many factors can influence mast cells' differentiation, maturation, migration, and function, such as tissue microenvironment, activating factors, and cytokines milieu[14-16] . Stem cell factor (SCF), the ligand for c-kit, is produced by many cells in tissue, including

MCs. SCF promotes mast cells' development, survival, adhesion, and proliferation in human and mice[17]. Furthermore, SCF can also regulate the release of mediators from MCs granules after activation[18,19]. The binding of SCF to its surface receptor on MCs induces phosphorylation of tyrosine kinases, leading to the activation of multiple signaling pathways, including phosphatidylinositol-3 phosphate kinase (PI3K), and mitogen activating protein kinase (MAPK), that promote mast cells survival and inhibit apoptosis. These factors make SCF a master regulator for MCs biology [20,21]. Other cytokines such as IL-3 act as driver for MCs growth and differentiation in mice[6]. Interleukin-4 (IL-4) is another factor that plays an important role in the development of MCs in humans. IL-4 does not work alone in human MCs, but it can work synergistically with SCF to enhance mast cells survival, proliferation, and release mediators[22].

Tissue MCs encompass a heterogeneous cell population in mice and humans[11]. The heterogeneity and plasticity is shaped by the intrinsic microenvironment that is found in tissue, which modulates the feature and morphology of MCs[6,23]. Two distinct populations of MCs have been recognized in mice: mucosal mast cells (MMCs) and the connective mast cells (CMCs). The characterizations of these two MCs are based on tissue location, histochemical staining, and protein content[24]. CMCs can be distinguished from MMCs by their expression of tryptase and chymase, and tend to bind to heparin, whereas chymase is the only protease present in MMCs, which tend to bind to chondroitin sulfate. In an analogy to the classification in murine, human MCs can also be divided into two types depending on the storage of proteases in the intracellular granules: MC_T-type contain only tryptase in their cytoplasmic granules and MC_{TC}- type contain both tryptase and chymase in their

granules[25,26]. MC_T cells are present in the airway, and gastrointestinal tract, whereas MC_{TC} cells are found in the skin, as well as in peritoneum, synovium, and perivascular tissue [24].

1.1.1 Activation of mast cells and signaling pathway

Mast cells express a large array of receptors that can be activated in response to immunological or non-immunological triggers resulting in the granule release of many different inflammatory mediators, such as histamine and serine proteases, that are stored in cytoplasmic granules. Activation also results in biosynthesis of lipid mediators, like Prostaglandin D_2 (PGD_2) and Leukotrienes (LTCs), as well as *de novo* synthesis of various cytokines and chemokines[26,27]. The level and nature of MCs response can be affected by growth factors and microenvironmental conditions that impact the expression and functionality of receptors and the signaling pathways they regulate[6,28].The best characterized mechanisms of mast cells activation is the immunoglobulin E (IgE)-associated allergic inflammation mediated by the high affinity Fc receptor for IgE, which are receptors expressed at high levels on the surface of MCs[29].

FcεRI is a heterotetramer comprised of an IgE-binding α subunit, a signal amplification β subunit, and two signal transducing γ chains[30]. Aggregation of FcεRI with multivalent antigen binding to IgE attached to FcεRI leads to phosphorylation-dependent activation of src kinase particularly Lyn and Fyn that regulate signal transduction[31-33]. Phosphorylation of an immunoreceptor tyrosine based activation motif (ITAM) in the g chains by Lyn kinase recruits spleen tyrosine kinase (Syk) and the subsequent formation of a large signaling complex[34-36]. Syk is an essential protein in FcεRI signaling. Syk-deficient mast cells are defective in their ability to degranulate[37]. Once activated, Syk

phosphorylates the protein adaptors LAT1, and LAT2, leading to formation of a large macromolecular complex[36]. FcεRI activates multiple pathways, including the PI3K pathway[31]. Other molecular signaling induced by FcεRI engagement includes MAPK, phospholipase C (PLCγ), which in turn regulates the activation of protein kinase C (PKC) through the generation of secondary messengers (1,2- diacylglycerol inositol-1,4,5 triphosphate, and cytosolic Ca^{2+}). PI3K and PLCγ pathways are important for multiple signaling events such as activation phospholipase D (PLD), PLA2, as well as calcium-dependent PKC isoforms, and for their role in the regulation of the nuclear factor of activated T-cells (NFAT) transcription factors through calcium binding proteins such as calmodulin [6,31]. These ultimately result in two major effector responses: the first response is the immediate degranulation and the second response includes the newly–synthesized products of arachidonic acid metabolism, and various cytokines, chemokines, and growth factors[38].

Interestingly, the level of expression of FcεRI depends on the level of circulating IgE[39]. The existence of FcεRI on the surface of MCs is unstable, and it is affected by the presence of IgE levels[40]. The binding of IgE to FcεRI induces accumulation as well as stabilization of FcεRI, and protects from degradation which leads to upregulation of FcεRI expression[41]. However, Omalizumab, an anti-IgE drug, leads to down-regulation of FcεRI expression[42]. Omalizumab is a recombinant DNA humanized monoclonal antibody that binds specifically to C epsilon on three loci in the same domain of the Fc portion on the heavy chain of free IgE[43]. Omalizumab prevents the binding of free IgE to the FcεRI by attaching itself to the same antigenic epitope on IgE[44].This interaction causes the down-regulation of FcεRI expression and the reduction of mediators released from mast cells,

which in turn reduces or blocks allergic reaction cascade[43,45]. MCs can also be activated through a variety of other receptors[27,46]. For example, IgG also have the ability to activate MCs through binding to FcγRs, and induce signaling events resembling IgE-FcεRI activation that elicits degranulation and *de novo* production because they share a common ITAM- containing γ subunit with FcεRI [20]. These receptors can positively or negatively regulate MCs, and they play a critical role in the protective immune response to pathogens[47].

Another receptor that is found on mast cells is Toll-like receptors (TLRs). These are part of the pattern –recognition receptors family that recognizes pathogen-associated molecular patterns (PAMPs)[6]. MCs have been shown to express multiple TLRs including, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and the activation of these receptors by their ligands elicit different MC responses[48]. For example, lipopolysaccharide (LPS) derived from gram negative bacteria stimulated TLR4 result in cytokines production without induction of degranulation. On the other hand, TLR2 stimulation by peptidoglycan (PG) from gram positive and negative bacteria promotes both degranulation and cytokines production[31,49].

The complement fragments like C3a and C5a can also activate MCs through binding with C3aR and C5aR, and the outcome of activation leads to degranulation and production different cytokines and chemokines[20]. These receptors are members of G-protein coupled receptor (GPCRs) family and the expression of these receptors can be affected by multiple factors, such as location and microenvironment[50]. MCs can also be triggered via inflammatory products such as chemokines, cytokines, adenosine, and sphingosine-1-phosphate (S1P), and lysophosphatidic acid (LPA) through engagement

with their receptors. Thus, mast cells can be activated in many ways, indicating a versatile role of MCs not only in allergies, but also in other diseases[31].

1.1.2 Mast cells Mediators

Mast cells are described as secretory cells that have the ability to secrete and synthesize a broad spectrum of biologically active products in response to allergic or non-allergic triggers[14,51]. The profile of mediators released by functional MCs is enormous, which is reflected in the contribution of these cells in various physiological and pathological processes[22]. Mast cells mediators can be classified into two groups: preformed mediators, and *de novo* lipid and cytokines mediators[1]. The preformed mediators and lipid *de novo* mediators are released within minutes following the mast cells activation and are responsible for the early phase of allergic symptoms such as erythema, edema, increased vascular permeability, and smooth muscles contraction[52]. The preformed mediators are packaged within secretory granules that are rapidly released to the extracellular environment following mast cells activation[53]. Releasing these mediators can be driven either by classic anaphylactic degranulation (AND) or piecemeal degranulation (PMD) depending on the type of stimulus- induced degranulation. Both types of degranulation occur *in- vivo*, *ex- vivo*, and *in- vitro* of different species like humans and mice[51]. AND can be mediated by triggering IgE- signaling pathway, which plays an important role in the pathogenesis of allergic inflammation[54]. AND consists of fusion events between the granule membrane and the plasma membrane or fusion granule to granule, resulting in release of the whole granule contents outside the cells[55]. In contrast to AND, PMD plays an important role in multiple chronic diseases like cancer, cardiovascular disorder and

others. PMD is involved in the partial release of granule contents without granule to granule or granule to plasma membrane combination, leading to selective contents discharge[48,51].

One of the most important pre-formed mediators is histamine, a short-lived biogenic amine, that is synthesized by decarboxylation of histidine through the action of histidine decarboxylase (HDC)[56]. MCs express four histamine receptors designated as H_1, H_2 , H_3 and H_4 receptors that belong to G protein coupled receptors[57]. The binding of histamine to its receptor can produce both pro-inflammatory and anti-inflammatory effects, which depend on the histamine receptor subtype and the cells stimulated type[58]. H_1-mediated MCs activation can cause bronchoconstriction, vasodilatation, mucous secretion, edema, and inflammatory response which contributes to the symptoms of allergic disease[59]. On the other hand, the H_2 receptors regulate gastric secretion and vascular dilatation[60]. The H_3 receptors are found mainly in the central nervous system (CNS) and play a significant role in neuroinflammation[61], whereas the H_4 receptors have been shown to contribute to allergic responses. Histamine interaction with H_4 receptors on mast cells causes the release of different inflammatory mediators like various cytokines and chemokines which mediate chemotaxis[57]. Another important constituent of MC granules are proteoglycans, such as heparin, and chondroitin sulfate. These negatively charged, highly sulfated structures play a pivotal role for granules organization and storage of protease[62].

Mast cell granules also contain many types of lysosomal enzymes, such as β-hexosaminidase, that present in mast cell granules of all subtypes and species[63]. Thus, the release of this enzyme can be used to quantify mast cell degranulation[51,64]. It is worth noting that MCs granules contain proteases, including tryptase, chymase, and carboxypeptidase, which make up 30-50 % of the total protein contents of MCs granules[65,66]. MCs proteases

are positively charged molecules that combine with the negatively charged proteoglycans in an interaction that stabilizes these enzymes and regulates their function[31, 66]. Numerous studies have revealed the detrimental and protective role of MC proteases. These enzymes have been implicated in numerous conditions, such as arthritis, allergic inflammation, and tissue remodeling[66]. However, they also play a protective role against various pathogens[65].

The *de novo* lipid mediators, or eicosanoids, are produced within minutes after MCs activation, such as PGD_2, leukotrienes B_4 (LTB_4) and (LTC_4)[48]. The bioactive eicosanoids are involved in allergic and other inflammatory conditions[67]. These mediators are derived from the release of arachidonic acid from the membrane phospholipids by the action of cytosolic phospholipase A_2 ($cPLA_2$) through two different pathways[68]. Prostaglandins are produced by the conversion of arachidonic acid with the action of cyclooxygenase (COX). There are two isomerase forms of this enzyme that are expressed on mast cells. The constitutive (COX-1) and the inducible (COX-2) forms[69]. These enzymes catalyze the formation of the precursor of prostaglandin H_2 (PGH_2), which represents the common precursor for all prostanoids forms, such as PGD_2, PGE_2, and PGF_2[70]. Similar to prostaglandins, the generation of leukotrienes occurs through the conversion of arachidonic acid by 5-lipoxygenase (5-LOX) to 5 *S*-hydroperoxy-6, 8- trans-11, 14- cis-eicosatetraenoic acid. The product of this conversion is then converted to leukotriene and its products, such as LTA_2, LTB_4, and LTC_4[71]. Collectively, these lipid mediators that are produced by MCs can provoke bronchoconstriction, increased vascular permeability, smooth muscles contraction, mucus secretion, cellular infiltration and immunosuppression[72].

Additionally, MCs also synthesize a wide spectrum of cytokines and chemokines in the hours following mast cells activation[73,74]. Some of these cytokines are stored in secretory granules, such as TNF, and IL-4, and are immediately released upon activation[75]. Many others, such as IL-1, IL-2, IL-6, IL-13, CCL5, and CCL8, are newly synthesized after the transcriptional activation. These inflammatory products are responsible for the late phase-response of allergic reaction. This response is characterized by infiltration of tissue with further cells recruitment such as eosinophils, neutrophils and lymphocytes[6,76].

1.2 Allergic Disease

Allergic responses are common chronic conditions that cause a significant negative effect in the quality of patients' lives worldwide. These conditions, including asthma, food allergy, allergic rhinitis, and atopic dermatitis, affect people of all ages[77]. The global prevalence and the complexity of allergic responses have been continuously increasing due to environmental changes, such as industrialization, urbanization, improvements in hygiene, and developments in technology[78]. Additionally, multiple susceptibility factors contribute to the development of these illnesses, such as genetic predisposition and the amount of allergen exposure[79]. The World Health Organization estimates that asthma affects approximately more than 300 million people globally and 8.4% of the U.S population[80,81]. Allergic rhinitis affects more than 400 million people worldwide and more than 15% of the U.S population. Moreover, 2-4% of people in the United States suffer from food allergy[82,83].

To date, the options for the treatment for allergic inflammation remains inadequate and new approaches are in demand[84]. Basically, an allergic reaction is characterized by an

inappropriate immune response toward an inherently harmless antigen, known as an allergen, which results in different forms of allergic disease[85]. The clinical manifestations of allergic reactions varies depending on the organ affected[86,87]. For example, individuals with asthma suffer from airway hyperreactivity, reversible airflow obstruction, and bronchospasm[88], while individuals with allergic rhinitis suffer from itching, sneezing, and local mucosal edema, which all together lead to blockage and irritation of the nasal passages[89]. Importantly, MCs play a primary role in the pathogenesis of the IgE- mediated type –I allergic reactions in the respiratory airways, skin, and gastrointestinal tract[90].

The classical allergic response can be divided into three phases: sensitization, early phase, and late phase reaction. The sensitization stage of the allergic reaction starts with the production of specific IgE due to exposure to exogenous allergens – like pollen, mites, and others– that pass into the body via different routes such as inhalation, ingestion, and skin contact[52]. This allergen is processed into small fragments by dendritic cells (antigen-presenting cells) that present these fragments to T helper-2 (Th_2), leading to the production of cytokines such as IL-4, IL-3, and IL-5, which cause B- cells to switch class to synthesize IgE[22]. Once synthesized, IgE circulates in the blood and sensitizes tissue MCs by binding to FcεRI.

The early phase response of an allergic reaction occurs within minutes of the re-exposure to the same allergen binding to the IgE attached to FcεRI, which causes the crosslinking of the receptor and activates MCs[44]. The activation of MCs leads to degranulation and the release of histamine, tryptase, lipid mediators, and platelet activating factor. These mediators can have an immediate effect on epithelial, smooth muscles, endothelial, and nerves cells. Therefore, early phase symptoms can produce a variety of

effects, including increased epithelial permeability, mucous production, smooth muscles contraction, vasodilation, and neurogenic signal[27]. The late phase reaction occurs several hours following the antigen challenge, and promotes inflammatory responses with the production of mediators, such as chemokines and cytokines production, as well as the attraction and infiltration of leukocytes[91].

1.3 Adenosine

Adenosine is an endogenous purine nucleoside that has a fundamental role in many biological functions, such as the biosynthesis of nucleic acids, cellular energy and metabolism, in addition to its role in the regulation of inflammation and the function of the immune system[92-95]. Adenosine is ubiquitously found in every tissue and organ of the human body and is formed both intracellularly and extracellularly in response to cellular challenge, tissue injury, and inflammation[96,97]. The formation of adenosine involves mainly dephosphorylation of adenosine triphosphate (ATP) through the action of specific enzymes such as ectoenzyme apyrase (CD39), and 5`-nucleotidase (CD73). Subsequently, ATP and its degradation precursors adenine nucleotides (like ADP) breaks down to adenosine monophosphate (AMP) by the action of CD3, and then converts to adenosine by the action of CD73[98-100]. Alternatively, adenosine is also produced from other sources like hydrolysis of S adenosyl homocysteine[101]. Once formed, adenosine can further undergo multiple metabolic pathways; it can be converted to AMP by adenosine kinase (ADK), or it can be converted to inosine and hypoxanthine by ecto-adenosine – deaminase (ADA) and then broken down to uric acid by xanthine oxidase[102-104]. This pathway of adenosine metabolism helps the body to keep the physiological level of adenosine low under homeostatic

conditions[105-107]. Adenosine levels can also stay in equilibrium through the diffusion of adenosine back into the cells by efficient nucleoside transporters (NTs)[108].

These transporters are divided into two categories based on energy requirements as well as molecular and functional properties: equilibrative nucleoside transporter (ENT, SLC29 gene family), and concentrative nucleoside transporter (CNT, SLC28 gene family)[99,109]. These transporters play a pivotal role in the regulation of exogenous adenosine levels at receptor sites and are useful in the treatment of certain diseases such as cardiovascular, cancer, and others[108,110]. CNTs are classified into CNT1, CNT2, and CNT3, which mediate unidirectional sodium dependent nucleoside transport across cell membranes[111]. These transporters can be found mainly in the epithelial cells of many organs, like the kidney, liver, intestine, as well as in the immune cells[112]. ENTs are classified into ENT1, ENT2, ENT3, and ENT4, and they transport nucleosides molecules via bidirectional sodium- independent[110]. These types of transporters are broadly expressed in different tissues, like vascular endothelial, skeletal muscles, heart, brain as well as in the immune cells[113]. ENT1 is considered one of the most efficient membrane transporters in controlling adenosine levels. ENT1 can be functionally distinguished from other ENTs as it is more sensitive to classic inhibitors such as nitrobenzylmercaptopurine ribonucleotide (NBMPR) and dipyridamole. These types of inhibitors are increased the extracellular concentration of adenosine and can be clinically used to treat specific diseases, such as cardiovascular disease, cancer, and others[114,115].

Adenosine exerts its effect in physiological and pathological conditions through binding to transmembrane adenosine receptors of which four exists ($A_{1A}R$, $A_{2A}R$, $A_{2B}R$,

and $A_{3A}R$. All these receptors are coupled to different GTP-binding proteins and play differential effect on target cells[116].

1.3.1 Adenosine Receptors

To date, there are four subtypes of adenosine receptors namely, A_1, A_{2A}, A_{2B}, and A_3 receptors, according to their order of discovery[117-119]. These receptors are widely expressed on diverse cells in a variety of tissues such as brain, heart, lung, skin and immune system and their signaling is involved in the regulation of multiple processes such as circulation, homeostasis, immune system and inflammation[116]. Each adenosine receptor (ARs) is characterized by pharmacological profile response, tissue specific distribution, and in their ability to couple to trimeric-G proteins[120]. ARs have been cloned in humans and many other species, and exhibit the greatest homology between the A_{1A} and $A_{3A}R$ (49% sequence identity) and the A_{2A} and A_{2B} receptors (59% sequence similarity)[121-123]. Numerous studies have indicated adenosine and its receptors as a therapeutic target in treating a variety of diseases, such as neurodegenerative diseases, cardiovascular diseases, diabetes, and cancer[124].

Structurally, ARs belong to the class A (rhodopsin-like) G protein-coupled receptor (GPCR) family that is well conserved among vertebrates[125]. These receptors share many structural features, including an extracellular amino terminus (N-terminus), an intracellular carboxyl terminus (C-terminus), and a conserved transmembrane structure comprising seven α helices. Each helix consists of 20-27 amino-acids, and connects by three intracellular loops and three extracellular loops[120]. Both N-terminus and C-terminus have posttranslational modifications such as glycosylation sites that are found in the

extracellular N-terminus, which play an important role in maintaining the ligand binding without changing its properties[120,126].

On the other hand, the cytosolic C-terminus of these receptors contains phosphorylation at the serine and threonine residues and palmitoylation sites that are important for protein kinases and receptor desensitization as well as internalization mechanism[120]. Each AR subtype exhibits different affinities to adenosine and its agonist. For example, A_1, A_{2A}, and A_3 have a high affinity for endogenous adenosine which can be activated at nanomolar concentrations of adenosine, while A_{2B} receptor has a low affinity for adenosine and its analogues which, requires micromolar concentration of adenosine to be activated[116,127].

The cellular response to adenosine is highly dependent on the adenosine receptor subtype and the type of activated G-protein on target cells which can lead either to inhibitory or stimulatory effects[116,128]. A_1, and A_3 receptors are considered as inhibitory receptors because they bind to the Gi/o protein, and inhibit adenylyl cyclase (AC) which consequently decreases cyclic AMP (cAMP) levels[129]. This results in inhibition of the protein kinase A (PKA) and phosphorylation of the cyclic AMP response element binding protein (CREB). However, the stimulatory receptors such as A_{2A}, and A_{2B} receptors bind to Gs protein, and activate the AC, leading to an elevation of cAMP production, activation of PKA, and CREB phosphorylation[119,130]. Thus, depending on the dominant receptor in a specific tissue or cell types, cAMP can be regulated. Moreover, stimulating adenosine subtypes can activate effector mechanisms other than AC, such as PI3K, MAPKs, and extracellular receptor signal- induced kinase (ERK) in different cell types, and can affect various aspect of cellar processes, like apoptosis, metabolism and differentiation[131,132].

1.3.2 Biological characteristics of A_{2A} receptor

The human A_{2A} receptor gene is located on the chromosome 22q13 with an approximate molecular weight of 44.7 KDa[101]. Although A_{2A} receptors share a commonality in their seven transmembrane helices, both amino as well as carboxy termini are variable to the other adenosine subtypes[133]. One of the differences is the presence of four disulfide bonds in the extracellular domain that are essential for the stabilization and maintenance of the restricted conformation of the seven transmembrane helices[134]. Another key difference is that the intracellular C-terminus of A_{2A} receptor consists of 122 amino acids long, whereas other receptor subtypes consist of 30-40 amino-acids[135]. Furthermore, the C-terminus of A_{2A} receptor lacks canonical cysteine residues at the end of helix eight, which are considered as putative palmitoylation sites[136]. Thus, these differences in the C-terminus make A_{2A} receptor more flexible and able to interact with other proteins like β-arrestins, α-actinin, and calmodulin[135,137]. Similar to other ARs, A_{2A} receptor has phosphorylation sites in the intracellular carboxy-terminus such as threonine 298, which plays a key role in mediating the short term desensitization of A_{2A} receptor after ligand binding[138]. Additionally, the presence of serine 374 phosphorylation in the intracellular-terminus has an effect on A_{2A} receptor-mediated suppression of the dopamine D2 receptor agonist binding and signaling[139].

The A_{2A} subtype is broadly found both peripherally and centrally throughout the human body. However, its expression appears to be variable in tissues and organs. It is widely expressed at higher level in the striatum, the olfactory tubercle, spleen, and the immune cells, whereas low levels of A_{2A} receptors are found in neurons outside of striatum, glial cells, heart, lung ,and blood vessels[120]. It is noted that expression patterns of

adenosine subtypes are regulated by several factors such as growth factors, and inflammatory stimuli[140]. In particular, the expression of A_{2A} receptor is highly sensitive to alterations in the extracellular environment. The expression of A_{2A} receptor is modulated by different stimuli that are involved in the inflammatory milieu such as Lipopolysaccharide (LPS) and pro-inflammatory cytokines[125]. For example, the expression of A_{2A} receptor increased after exposing macrophages to LPS, which resulted in the limitation of inflammatory response[141].TNF, and IL-1 increased the expression of A_{2A} receptor mRNA and protein levels on human monocytes THP-1 and enhanced its function[142]. On the other hand, IFN-γ decreased A_{2A} receptor expression by reducing the expression of AC[143].

1.3.3 Signal transduction pathways of adenosine A_{2A} receptor

The A_{2A} receptor is increasingly recognized as an immunoregulatory effect of adenosine in the immune system by preventing exacerbation of hyperactivation of immune cells[144]. Adenosine signals through A_{2A} receptor are one of the most important mechanisms to suppress inflammation[145,146]. The stimulation of A_{2A} receptor induces a variety of intracellular signaling by preferentially interacting to a Gs protein. It leads to AC activation and elevation of intracellular cAMP levels, which has an anti-inflammatory effect[147,148]. Signaling pathways associated with A_{2A} receptor seem to be different for the peripheral system and the central nervous system. The major G-protein engaged with A_{2A} receptor is the $G_{\alpha\text{-}s}$ in the peripheral system, whereas the G_{olf} is the predominant protein in the brain that binds with A_{2A} receptor[149] in the central nervous. The binding of the A_{2A} receptor either with Gs or Golf causes the exchange of GDP for the GTP bound to the G protein α subunits as well as the dissociation of the βγ heterodimer that allows for the mediation of

downstream signaling[150,151]. Their stimulation can further stimulate AC and increase cAMP levels.

The elevation of cAMP results in protein kinase A (PKA) that phosphorylates and activates the cAMP responsive element- binding protein1 (CREB1) on serine residue-133[152]. The elevation of cAMP in PKA/CREB1 can mediate gene expression directly by interacting with gene promoters or indirectly by inhibiting the transcriptional activity of NF- kB, which consequently suppresses the expression of many pro-inflammatory cytokines such as TNF[153]. Alternatively, cAMP not only activates PKA, but also activates many other proteins, such as exchange proteins activated by cAMP (EPAC), causing alteration in the gene expression[153].

1.3.4 The action of adenosine on mast cells

Mast cells are multifunctional cells involved in allergies and many other chronic inflammatory diseases[154]. Modulating the activity of these cells plays key roles in allergic inflammation. Adenosine modulates the functions of many immune cells that are implicated in allergic asthma, such as mast cells, smooth muscles cells and eosinophils[155]. Adenosine regulates the function of MCs through binding with its receptors on the cells surface, such as A_{2A}, A_{2B} and A_3 receptors[156].

Elevated levels of adenosine are found in bronchoalveolar lavage (BAL)[157]. Adenosine levels were also raised in adenosine deaminase (ADA) deficient mouse model, causing extensive mast cells degranulation and pulmonary phenotype with asthma feature[156]. Prior research has demonstrated that inhalation or intravenous administration of adenosine in allergic asthmatics or non-allergic asthmatics subjects results in

bronchoconstrictive response[101,117]. This response is due to the capability of adenosine to enhance MCs mediators release, such as histamine and tryptase, that are found at high levels in BAL of asthmatics subjects. Adenosine potentiates mediators' release induced by immunological and non-immunological stimuli from rat peritoneal mast cells (RPMCs)[158]. It has also been noted that adenosine has dual effects on lung mast cells and that it can enhance mediators' release at low concentrations of adenosine while inhibit them at high concentrations[159]. This effect of adenosine on mast cells degranulation is believed to occur through A_3 or A_{2B} receptors[160]. It has also been shown that adenosine is directly able to activate MCs in vivo without additional stimuli[159].

1.4 MicroRNA (miRNA): Structure and function

MicroRNAs (miRNAs) are small single-stranded, non-coding RNAs of approximately 22 nucleotides that function as post-transcriptional regulators of gene expression[161]. MiRNAs exert their function upon binding to 3` untranslated region (3`UTR) of the target messenger RNA (mRNA), thereby reducing protein synthesis by either translational suppression or mRNA degradation[162]. MiRNAs play an important role in a variety of physiological and pathological processes and control many cellular processes including development, differentiation, metabolism, and apoptosis[163]. Dysregulation of miRNAs expression has been implicated in the pathogenesis of several human diseases[164].

To date, it has been estimated that miRNAs can target and regulate the expression of at least 60% of the human genes[165,166]. MiRNAs are highly conserved throughout evolution and initially identified in *Caenorhabditis elegans* (*C. elegans*) as a negative

regulator during developmental periods. They have been found in a wide range of multicellular organisms like humans, plants, animals, and viruses[167]. Lin-4 was the first miRNA discovered in the *C. elegans*, which is responsible for silencing the Lin 14 via antisense complementary to its (3`UTR) during its development[168]. Later, the discovery of another small non-coding RNA, miRNA let-7, is found in various organisms like human beings and animals. The Let -7 gene binds to a sequence in UTR of Lin-41 mRNA, causing translation repression of lin-41 mRNA[169].

MiRNAs are scattered in diverse regions of the genome that make up for 1-5 % of the human genome[167,170]. MiRNAs are expressed in various tissues and cells. However, their expression varies. Some of them are widely expressed, and others display limited expression[171]. Additionally, miRNAs can be found in body fluids, such as plasma and serum, and they are able to protect themselves from the action of blood RNAases either by existing as exosomes or by forming a complex with lipid -protein carriers, such as high density lipoprotein[172,173]. A single miRNA molecule targets numerous mRNAs, and a single mRNA molecule is targeted by multiple miRNAs, which adds complexity to the network[174]. Moreover, miRNAs are able to silence genes by either affecting epigenetic mechanisms, such as DNA methylation or histone acetylation, or targeting transcription factors[175]. Therefore, miRNAs are essential molecules that may position well to control many chronic diseases, including allergies [174].

1.4.1 Biogenesis of miRNAs

Biogenesis of miRNAs in animals consists of consecutive steps of processing that are started in the nucleus and end in the cytoplasm with several post-transcriptional

modifications[176]. MiRNAs are most commonly transcribed in the nucleus by RNA polymerase II, which generates a primary miRNA transcript with a 5`-capped and a 3` poly-A-tail[177]. The pri-miRNA is a long transcript that contains multiple miRNA sequences that are processed by a microprocessor complex formed by RNAase III endonucleases enzyme, Drosha, and a double-stranded-RNA binding protein DGCR8 (DiGeorge syndrome critical region gene8), which produces ~ 65 nucleotides long hairpin structures called precursor miRNA (pre-miRNA)[178]. The pre-miRNAs are exported from the nucleus into the cytoplasm by an exportin RanGTP complex and then cleaved by RNase III endonuclease Dicer, resulting in a small-double stranded RNA duplex[179]. Depending on the thermodynamic stability of the base pairs at the 5` of two strands, one of these strands is selected to be the mature functional strand (guide strand), whereas the other strand (passenger strand) is rapidly discarded[180]. The nomenclature of mature miRNA is determined by the direction of the miRNA strand. The mature miRNA can be derived from either 5`end or the 3`end of the precursor's duplex and are called miRNA-5p and 3p, respectively[179]. Mature miRNA (5` and 3` strands miRNA) is preferentially loaded into a RNA induced silencing complex (RISC) containing Argonaute (AGO) proteins, and the selection of one of these strands is based on the lower stability of base pairing in the second and fourth nucleotides at the 5` ends of the miRNAs duplex or at 5` U at nucleotide position 1[180,181]. After incorporation into active RISC complex, miRNAs bind with their 3`UTR mRNA molecules.

The mechanism of posttranslational silencing depends on the complementarity between miRNA and its target mRNA. When the complementarity between miRNA and its target mRNA is an exact, or nearly exact, match to each other, it leads to mRNA

degradation. On the other hand, if partial complementarity occurs between miRNA and its target, it leads to the inhibition of protein synthesis or the repression of the translation[168].

1.4.2 MiR-155

MiR-155 is encoded from a primary transcript known as B cell integration cluster (BIC) gene, which originally was identified as a common retroviral integration site in avian leukosis- virus induced lymphoma[182]. The conserved region of the BIC gene is located on chromosome 16 in mice and on chromosome 21q21 in humans (and it has been shown that there is about more than 70% identity between them)[183]. Although the level of BIC RNA is low in healthy lymphoid tissue, BIC/miR-155 is highly increased in human tissue when Hodgkin or children`s Burkitt lymphoma is present. The activation of the BIC gene causes upregulation of c-myc oncogene that accelerates the pathogenesis of lymphomas and leukemias, implying it plays a critical role in disease progression and pathology[184]. MiR-155 was identified as oncomiR and is involved in the processes of carcinogenesis for various cancer[185] . MiR-155 serves a crucial role in numerous cellular processes, such as proliferation, differentiation, apoptosis, and metabolism[186].

The dysregulation of miR-155 has been observed in many pathological disorders, such as cancer, cardiovascular disease, and autoimmune disorder[187]. MiR-155 has distinct expression in cells of hematopoietic origin. Many studies have shown that miR-155 expression is high in hematopoietic cells' progenitors than in mature hematopoietic cells including granulocytes, lymphocytes and monocytes[188]. However, the expression of miR-155 is upregulated in these cells after exposure to a variety of inflammatory stimuli, implying a potential role in mediating inflammation and immune response[189]. It has been

demonstrated that miR-155 is increased to high level in B and T cells following engagement with its receptor and that it plays an important role in the effector function of these cells[190]. In this regard, miR-155 deficient B cells displayed as defective in germinal center formation and antibody class switching, which resulted in a defective humoral immune response to T cells-dependent antigenic stimulation[191]. Mechanistically, PU.1 was found as a functional target of miR-155 in B cells that act as a negative regulator in antibody isotype switching[192]. In addition, the enzyme activation-induced cytidine deaminase (AID) is another important target of miR-155, which is required for high-affinity IgG antibody in antigen-activated B-cells[193].

Furthermore, high levels of miR-155 have also been found in the innate immune cells like macrophages and monocytes stimulated with various inflammatory stimuli[194]. MiR-155 plays important role in the macrophage polarization. The generation of proinflammatory phenotype of macrophages M1 is associated with upregulation of miR-155, whereas down regulation of miR-155 is associated with the generation of anti-inflammatory phenotype of macrophage M2, suggesting a regulator role of this molecule in these cells[185].

1.4.3 MiR-155 involvement in allergic disease

Allergic inflammation is an excessive and inappropriate immune response that is associated with marked histologic changes as well as the alteration of the expression of numerous genes and proteins. MiRNAs have been shown to serve as a posttranscriptional silencer of genes' expression that regulate multiple facets of cellular processes, and they are directly associated with many pathological conditions like allergy[195].

Dysregulation of miRNAs has been reported in asthma patients and in different models of asthma-like lung inflammation[196]. The presence of quantifiable amounts of miRNAs in biological fluids from asthma and other allergic diseases were considered as a potential biomarker for diagnosis, prognosis, and therapeutics for immune-related disease[197]. MiRNAs are expressed in different tissues and cells that contribute to the allergic tissue inflammation. MiRNAs have different expression profiles between healthy patients, human biopsy specimens and experimental models in asthma, eosinophilic esophagitis, and contact dermatitis[80].

One of these deregulated miRNAs is miR-155, which plays a role in allergen-induced model of asthma[80]. MiR-155 is greatly increased in a variety of activated cells and has a significant impact on the biology of different immune and inflammatory cells involved in allergic disease [185]. Inhibition of miR-155 was demonstrated to attenuate the clinical manifestation of allergic diseases, such as suppression of eosinophilic inflammation, mucus hypersecretion, and Th2 cells and their cytokines (IL-4, IL-5, and IL-13)[185]. Thus, the suppression of miR-155 expression could be a potential strategy for the management and treatment of allergic reactions.

1.5 The role of Resveratrol in allergic disease

Resveratrol (3,4`,5 trihydroxystillbene) is an active polyphenolic phytoalexin compound that found in a variety of plant species, such as grapes, berries, and peanuts[198]. The chemical structure of Resveratrol consists of two aromatic rings linked by a methylene bridge[199]. Resveratrol exists in two forms: cis and trans- isomers, of which the latter is more abundant, stable, and biologically active[200]. Resveratrol has garnered much attention for its

known health potential because of its remarkable pharmacological properties, such as anti-cancer, anti-oxidant, cardioprotective, immunoregulatory and anti-inflammatory effects[201,202]. Resveratrol exhibits anti-inflammatory effects by the inhibition of the secretion and release the pro-inflammatory mediators from immune cells following exposure to different stimuli by affecting on transcriptional factors such as nuclear factor-kappa B (NF-kB) and activator protein-1 (AP-1)[203]. It has been shown that Resveratrol inhibits LPS-induced inflammation of RAW264.7 macrophages[204]. In addition, it also shows an inhibitory effect on COX-1 and COX-2 as well as lipoxygenase catalytic activity, which leads to the suppression of the prostaglandins and leukotrienes production[205]. Numerous studies have described the anti-allergic effects in different animal models of asthma by suppressing airway hyperresponsiveness, eosinophilia, and mucous hypersecretion[206,207]. Resveratrol has also demonstrated the anti-allergic action through the inhibition of mast cells degranulation, which mediates via suppressing the expression levels of Syk, suggesting the important role of Resveratrol in the treatment of allergic diseases[208].

CHAPTER 2

THE EFFECT OF A_{2A} RECEPTOR IN THE REGULATION OF ALLERGIC MEDIATORS FROM HUMAN SKIN MAST CELLS

2.1 Background

Mast cells are best known as the principal effector cells in allergic diseases via a mechanism, including aggregation of FcεRI with multivalent antigen. This results in releasing various allergic mediators such as pre-stored mediators like histamine, serine protease, and de novo synthesis of lipid mediators and cytokines that play a detrimental role in the elicitation of allergic symptoms[209,210].

Adenosine is an endogenous purine nucleoside that plays a fundamental role in the modulation of numerous cellular functions involved in the immune and inflammatory responses[211,212]. Adenosine is released into the extracellular milieu under physiological conditions and at higher levels during pathological condition like hypoxia, tissue injury, and inflammation[213]. Adenosine exerts pro-inflammatory and anti-inflammatory effects through ligation to various receptors, which have been denoted A_1, A_{2A}, A_{2B}, and A_3 receptors[214]. Adenosine has been known to play an important role in allergic asthma, in part through its ability to modulate mast cells activation, in response to challenges, with a variety of stimuli[101,156]. Adenosine and its analogues can enhance MCs degranulation and evoke proinflammatory cytokines through interaction with A_{2B} and /or A_3 receptors, implying the proinflammatory effects of adenosine on mast cells[215]. However, a substantial body of evidence points toward the predominant role of A_{2A} receptor in mediating the

inhibitory effect on immune and inflammatory processes in different preclinical models of inflammation like asthma, chronic obstructive pulmonary disease (COPD) and acute lung injury[216,217]. In addition, adenosine, acting via A_{2A} receptors, suppresses the production of pro-inflammatory cytokines in different cell types[218]. The ability of A_{2A} to exert anti-inflammatory pathway may be attributed to its action as a Gs-protein coupled receptor, which ctivate adenylate cyclase (AC) and increase the intracellular cAMP concentration[104,219]. The anti-inflammatory role generated by A_{2A}R has been demonstrated *in- vitro* and *in- vivo* studies using A_{2A} selective agonist, CGS-21680, or by using mice carrying genetic deletion of A_{2A}R or selective antagonist of A_{2A} receptor which suppressed the anti-inflammatory properties[220].

Modulating adenosine receptors expression is important in regulating many inflammatory and immune systems.[221] Adenosine receptors can be regulated in response to various factors that are present in the local milieu, such as growth factors and the presence or absence of inflammatory stimuli[140]. In this regard, A_{2A} receptor expression is upregulated in response to Th1 cytokines like TNF, IL-1, and LPS in many immune cells[116,141,222] . Mast cells have been reported to express A_{2A}, A_{2B}, and A_3 but lack A_1 receptors with different level of expression depending on the mast cells origin[223]. Nevertheless, regulation of adenosine receptors expression and function in mast cells that are continuously expressed FcεRI remains obscure.

In the present study, we investigated the role of adenosine receptors subtypes, particularly A_{2A} receptors on the regulation of allergic mediators from human skin mast cells. We also investigated the effect of FcεRI signal on the expression and function of adenosine receptor subtypes in human skin mast cells to further understand the interaction

between FcεRI and adenosine receptors. We reported that A_{2A} receptor has no effect on FcεRI-induced degranulation or PGD_2 production. However, $A_{2A}R$ inhibited FcεRI–induced pro-inflammatory cytokines production from human skin mast cells. More importantly, cross-linking FcεRI modulates the expression and function of adenosine receptors. Sub-threshold stimulation of FcεRI leads to up-regulation A_{2A} and down-regulation A_3 receptors at the mRNA. The functional consequence is that mast cells with altered A_{2A} and A_3 receptors expression produce significantly more intracellular cAMP, which is known to inhibit mast cell activation. We also show that up-regulation of the A_{2A} receptors by sub-threshold of FcεRI leads to more pronounced inhibition of TNF by adenosine. Our results demonstrate that A_{2A} receptors play a vital role in the regulation of inflammatory mediators from human skin mast cells and could be a therapeutic target for treating allergic disease.

2.2 Materials and methods

The methods detailed in this study were designed to investigate the effect of A_{2A} receptors on the regulation of allergic mediators from human skin mast cells and to address the role of FcεRI signals on the modulation of adenosine receptors in order to further understand the interaction between them. Studies were performed in human skin mast cells that were isolated in accordance with the University of South Carolina Internal Review Board (IRB).

2.2.1 Isolation and Purification of Mast cells

Human skin mast cells were isolated from normal human skin tissue that was taken

from donors who underwent surgical operation. These tissues were obtained from the Cooperative Human Tissue Network of the National Cancer Institute, as approved by the Human Studies Internal Review Board at the University of South Carolina. After removing the fat from tissue, the residual tissue was cut into small strips and minced, then the minced tissue was digested with different proteolytic enzymes, such as Collagenase type 2 (Worthington Biochemical, Lakewood, NJ), and hyaluronidase and DNase I (Sigma-Aldrich, St. Louis, MO), for 3x 1 hour in HBSS wash buffer (1× HBSS, 0.04% $NaHCO_3$, 1% fetal bovine serum, 1% HEPES, 0.1% $CaCl_2$) that contained amphotericin B and Antibiotic/Antimycotic solution. After each enzymatic digestion, the dispersed cells were collected by filtration through 40 µM nylon cell strainers, washed, layered over Percoll gradient, and centrifuged. The cells were collected from buffer/Percoll interface and re-suspended at $5x10^5$ cells /ml in serum- free X-VIVO15™ media (Lonza, Walkersville, MD) containing 200 ng/ml of recombinant human stem cell factor (SCF) (PeproTech, Rocky Hill, NJ). The cells were split in 24-well plates and maintained under standard culture conditions (37°C, 5% CO_2) with weekly medium changes. The purity of primary cultured cells were assessed by using metachromatic staining with acidic toluidine blue and flow cytometry for the surface expression of FcεRI. After the purity reached 95-100%, the cells were used for experiments between 8-12 weeks.

2.2.2 Sensitization and activation of mast cells

Mature human mast cells were sensitized by overnight incubation in X-VIVO15 ™ media containing chimeric human IgE-anti-NP (human Fc + mouse Fab) (clone JW8/1; AbD Serotec, Raleigh, NC) at 1 µg/. Then the cells were activated by cross-linking FcεRI with a multivalent antigen, hapten 4-hydroxy-3-nitrophenylacetyl conjugated to bovine

serum album (NP-BSA; Biosearch Technologies, Novato, CA) (100 ng/ml) at 37°C in X-VIVO 15 ™ media containing100 ng/ml SCF or Tyrode's buffer (135 mM NaCl, 1 mM $MgCl_2$, 20 mM Hepes, 5 mM KCl, 1.8 mM $CaCl_2$, 5.6 mM glucose; pH 7.4, 0.05% bovine serum album). The cells were washed to remove unbound IgE, re-suspended at 10^6 cells/ml in X-VIVO 15™ media or in Tyrode's buffer. Mast cells were pre-treated with 5′-N-Ethylcarboxamidoadenosine (NECA), Sp-cAMP triethylammonium salt (S)- Adenosine, cyclic3`,5`-(hydrogenphosphorothioate) triethylammonium, and Rp-cAMP, triethylammonium salt (R) Adenosine, cyclic 3`, 5`-(hydrogenphosphorithioate triethylammonium, CGS-21680(4-[2-[[6-Amino-9-(NethylDribofuranuranuronamidosyl)-9H-purin-2-yl]amino]benzenepropanoic acid hydrochloride, ZM241385 (4-(2-[7-Amino-2-(2-furyl)[1, 2, 4]triazolo[2,3-a][1, 3, 5]triazin-5-ylamino] ethyl)phenol), (all from Tocris-Cookson, Ellisville, MO) for 1 h at 37°C. DMSO was used as the vehicle (Sigma-Aldrich, St. Louis, MO), and then the cells were activated with 100 ng/ml NP-BSA for the indicated amount of time.

2.2.3 β-Hexosaminidase and PGD_2 assays

Human skin mast cells were incubated with anti-NP-IgE overnight. After the pre-incubation period, the cells were washed, re-suspended, and stimulated with NP-BSA for 30 min at 37 °C in Tyrode's buffer. The degranulation reaction was stopped by placing the sample tubes on ice for 10 minutes. The cells and buffer were centrifuged to separate the supernatant from the pelleted cells. The cells were lysed with same volume of 1% Triton X-100. To assay degranulation, the activity of the secretory granule-associated enzymes β-hexosaminidase was measured in the supernatant and cell lysate by measuring the release of p-nitrophenol from the hydrolysis of p-nitrophenyl N-acetyl-β-D-glucosaminide

(pNAG; Sigma-Aldrich, St. Louis, MO) as described[224,225]. 5 µl of supernatant or cell lysate were incubated with 45µl of 1 p-NAG in a 96-well plate at 37 °C for 1 hour. The reaction was terminated by adding 150 µl of 0.2 M glycine (PH 10.7), and the absorbance was read at 405 nm with a BioTek Synergy HT microplate reader (BioTek, Winooski, VT). The results were expressed as a percentage of total β-hexosaminidase activity present in the cells, which was calculated by using the formula: % β hex release = ((supernatant)/(supernatant + lysate)) × 100. The amount of PGD2 in the supernatant was measured with a commercial enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

2.2.4 Cytokines ELISA

Human skin mast cells were pre-incubated with anti-NP-IgE as described in the previous sections. The cells were activated with 100 ng/ml NP-BSA at 37 °C in X-VIVO15^{TM} media containing SCF and 100 µg/ml soybean trypsin inhibitor (SBTI; Sigma-Aldrich, St. Louis, MO). After 24 hours of incubation, the cells and media were separated by centrifugation (2000 rpm x 5 min). TNF secretion in the supernatant was measured by enzyme linked immunosorbent assay (ELISA) in a 384 well plate as described [226]. TNF was measured using capture (purified) and detection (biotinylated) rat antibodies, and serially diluted recombinant cytokine that is common for standard curves (BD Biosciences, San Jose, CA). The plates were developed with substrate, peroxidase 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, (ABTS; Sigma-Aldrich, St. Louis, MO). The absorbance values were read at 405 nm with a BioTek Synergy HT microplate reader (BioTek, Winooski, VT) and Gen5 Data Analysis Software.

2.2.5 Real-time PCR

The RNA from activated mast cells was isolated with the RNeasy Miniprep kit (Qiagen, GmbH, Germany), and cDNA was synthesized from RNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules) according to the manufacturer's protocol. For PCR, 2 µl of cDNA was combined with 1 µl of sense and antisense primers (10 µM each) and 12.5 µl of iQ SYBR® Greens supermix (Bio-Rad, Hercules, CA), which produced a final volume of 25 µl. A hot-start protocol (95 °C for 5 min, (95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) x 35 cycles, 95 °C for 1 min, 55 °C for 1 min) was run on a CFX Connect Real Time PCR Detection System from Bio-Rad (Hercules, CA). The fold change in expression was determined by the $2^{\Delta\Delta Ct}$ method. The oligonucleotide primers used were (5'-3'; forward and reverse): A2aAR (5'-cattgcctgcttcgtcct-3'; 5'-gatgcccttagccctcgt-3'; 136 bp; NM_000675.4); A2bAR (5'-ctccatcttcagccttctgg-3'; 5'-acaaggcagcagctttcatt-3'; 236 bp; NM_000676.2); A3AR: 5'-gggcatcacaatccacttct-3'; 5'-agggccagccatattcttct-3'; 171 bp; NM_000677.3 variant 2); GAPDH (5'-caatgacccttcattgacc-3'; 5'-ttgattttggagggatctcg-3'; 159 bp; NM_002046.3).

2.2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0c for Mac OS X, GraphPad Software (La Jolla California USA), and the software is available at www.graphpad.com.

2.3 Results

2.3.1 The A_{2A} receptor signals are not required for inhibition IgE- induced human mast cell degranulation.

We had previously shown that adenosine had no influence on IgE-induced degranulation at low concentration in human skin mast cells. However, adenosine at high concentration significantly inhibited degranulation in response to cross-linking FcεRI in human skin mast cells[227]. To begin our study, we assessed degranulation by releasing β-hexosaminidase from mast cells pre-treated with increasing doses of non-selective agonist NECA, or DMSO for 1 hour instead 10 minutes in the previous studies, then the cells were stimulated for 30 minutes with (100ng/ml) NP-BSA. As seen in (Fig 2.1A), NECA had no effect on IgE-induced degranulation in human skin mast cells. However, a significant inhibition was only seen in mast cells pre-treated with NECA at 10 µM with ($p< 0.05$). These findings indicated that longer exposure to NECA (1 hour) did not increase the sensitivity of mast cells degranulation to inhibit. We worked to determine that the inhibitory effect of NECA was due to specific adenosine receptors signaling, since a lot of evidence indicated the role of $A_{2A}R$ in the down- regulation of inflammatory response in different immune cells, including lymphocytes, and macrophages[222,228]. We analyzed the effect of the A_{2A}-specific agonist CGS21680 on IgE-induced degranulation in SMC. The cells were pre-treated with (0-10µM) of CGS-21680 for 1 hour then the cells were challenged with NP-BSA (100 ng/ml) for 30 minutes. CGS-21680 had no significant difference on IgE-induced degranulation in mast cells (Figure 2.1B). Additionally, CGS-21680 inhibited the inhibitory effect of NECA on IgE–induced degranulation in SMCs, suggesting that A_{2A} receptor does not play a role in this adenosine action. Adenosine receptor subtypes were initially differentiated by their effects on adenylate cyclase

activation: A_{1A}, and A_{3A} receptors inhibited adenylate enzyme which lower the cAMP accumulation, while A_{2A} and A_{2B} receptors stimulate adenylate and increased cAMP levels[149]. Therefore, we next determined whether alteration in the concentrations of cAMP had an inhibitory effect on degranulation. We examined the effect of the activator of Sp-cAMP in mast cells. Mast cells were pre-treated with different concentrations Sp-cAMP for 1 hour, then the cells were challenged for 30 minutes with 100 ng/ml NP-BSA. Our results showed that Sp-cAMP had no effect on IgE- induced degranulation in human skin mast cells, and blocked the inhibitory effect of NECA on IgE-induced degranulation in SMCs (Figure 2.1C). Thus, alteration in the concentrations of Sp-cAMP had no effect on degranulation in SMCs. Similar data was obtained when pre-treated mast cells with dose-dependent concentrations of c-AMP antagonist, Rp-cAMP, for 1 hour and the cells were stimulated with 100 ng /ml NP-BSA for 30 minutes, showing no effect on IgE -induced mast cell degranulation and at the same time preventing the inhibitory effect (Figure 2.1D). Collectively, these findings suggest that Gαs- mediated intracellular cAMP is ineffective to inhibit mast cell release β-hexosaminidase by NECA.

2.3.2 Role of Adenosine receptor A_{2A} on PGD_2 biosynthesis

To determine the effect of adenosine on the PGD_2 production, IgE-sensitized skin mast cells were pre-treated with a dose range of adenosine for 1 hour and challenged for 30 minutes with NP-BSA (100 ng/ml). After the pre-incubation period, PGD_2 in the supernatant was measured with commercial enzyme immunoassay according to manufacturer's protocol. Adenosine produced a significant dose dependent decrease in FcεRI-induced PGD_2 biosynthesis in human skin mast cells (Figure 2.2A). Next, we determined if A_{2A} receptor is involved in mediating adenosine inhibited FcεRI-induced

PGD_2 production in SMCs. Human mast cells were pre-incubated with different concentrations of A_{2A} selective agonist CGS-21680 for 1 hour at 37C and then were challenged with NP-BSA (100ng/ml) for 30 minutes. The supernatant was used to measure PGD_2. In contrast to the finding above, no inhibitory effect was observed in response to selective agonist CGS12680 on IgE–induced PGD_2 in mast cells at all concentrations examined (0-10μM) (Figure 2.2B). Taken together, these data indicated that A_{2A} receptor has no effect on the inhibition of FcεRI-induced PGD_2 in human skin mast cells.

2.3.3 A_{2A} signaling inhibits FcεRI-induced cytokines production from human skin mast cells

In addition to triggering mast cell degranulation and PGD2 production, FcεRI-induced cytokines synthesis. To address the role of adenosine or NECA on IgE-induced cytokines production in mast cells, we did the following. First, human skin mast cells were sensitized with anti-NP-IgE overnight. Following washing, the cells were pre-treated with different concentrations of NECA (0-10 μM) for 1 hour and stimulated with NP-BSA (100 ng/ml) for 24 h. After pre-incubation period, media from human skin mast cells were centrifuged and TNF in the supernatants was measured by ELISA. As shown in (Figure 2.3A), NECA inhibited IgE- induced TNF production in a concentration-dependent manner in mast cells compared with control mast cells activated without pre-treated with NECA. Significant inhibition occurred at low concentration of NECA (1μM), and reached its maximal inhibition at the high concentration (10 μM) ($p<0.001$), indicating that adenosine receptors, particularly, A_{2A} receptors, play a key role in the regulation of TNF production in mast cells. To confirm the above findings was due to A_{2A} signals, we pre-treated human skin mast cells with different concentrations of CGS21680 (0.001-10 μM) for 10 minutes, then activated for 24 hours with NP-BSA, and secreted TNF was measured. The results

showed that CGS21680 dose-dependently inhibited IgE-dependent TNF production. Significant inhibition was seen at 1µM and 10 µM ($p< 0.001$) (Figure 2.3B). To further substantiate this observation, we repeated the same experiment by using specific antagonist ZM241388, which blocked $A_{2A}R$ signals. Sensitized human skin mast cells were pre-treated with ZM241385 (0.001-10 µM) for 10 minutes followed by adenosine (10 µM) for 10 minutes, and then challenged for 24 hours with NP-BSA (100ng/ml). As seen in (Figure 2.3C), ZM241385 prevented adenosine-inhibited TNF production. We next determined whether elevation of cAMP concentrations could be the mechanism responsible for TNF inhibition in SMCs. We first pre-treated human skin mast cells with different concentration of forskolin, which is a compound to increase cAMP production, for 1 hour, and then we challenged with NP-BSA (100 ng/ml) for 24 hours. TNF was measured in supernatant by ELISA. The results showed that forskolin inhibited TNF production in a dose dependent manner ($p< 0.05$), suggesting cAMP mechanism is responsible for the inhibition of IgE-induced TNF production (Figure 2.3D). Altogether, these data demonstrated the involvement of A_{2A} signals in the inhibition of TNF production by cAMP mechanism in human skin mast cells.

2.3.4 FcεRI stimulation modulates adenosine receptors mRNA expression

Many studies have been reported that mast cells expressed A_{2A}, A_{2B}, and A_3 receptors in different species[155,229-231]. To determine the effect of FcεRI signals in the regulation adenosine receptors in mast cells, human skin mast cells were stimulated with anti-FceRI monoclonal antibody, 22E7, in dose-dependent manner for 3 hours. After incubation period, mast cells and medium were separated by centrifugation. RNA was isolated from mast cells and the qRT-PCR was used to quantify changes in adenosine

receptors mRNA. Degranulation was determined by measuring the activity of β-hexosaminidase. Investigation of adenosine receptors in human skin mast cells from this study revealed that mRNA level of A_{2A} receptor was significantly increased, whereas the mRNA level of A_{3A} receptor was decreased in dose-dependent manner. A_{2B} was not affected. Our data showed that simulating these cells with 1 ng/ml of 22E7 was sufficient to induce a maximal increase in the expression of A_{2A}R mRNA, and decrease A_{3A}R (Figure 2.4A). Overlying the normalized data revealed that stimulation FcεRI with 22E7 at 1 ng/ml induces significant changes in A_{2A}R and A_{3A}R with minimal induction of degranulation (Figure 2.4B). We further confirmed these finding above in Time-course for FcεRI-induced changes in adenosine receptors. SMCs were stimulated with optimal (100ng/ml) or sub-optimal (1 ng/ml) concentrations of the anti-FcεRIα monoclonal antibody 22E7 for 1, 3, 6, and 24 hours. At each time point, we collected total RNA from mast cells and used RT-PCR to quantify the changes in adenosine receptors expression. A shown in (Figure 2.5A and B), stimulating FcεRI either at optimal or sub-optimal concentrations resulted in an increased A_{2A}R and decreased A_{3A}R in a time dependent manner. The maximal increase in A_{2A} receptor was observed at 3 hours, whereas the maximal decrease in A_{3A}R occurred at 6 hours. The changes in A_{2A} and A_{3A} receptors expression appeared to return to baseline by 24 hours. A_{2B} receptor did not change at each time point. We also found that mast cells degranulation occurred when FcεRI was stimulated with 22E7 at 100 ng/ml, whereas stimulation FcεRI with 22E7 1 ng/ml did not induce degranulation (Figure 2.5C).

Taken together, these data demonstrated that cross-linking FcεRI regulates the expression of adenosine receptors at mRNA levels. To determine if similar changes occur when FcεRI is crossed-linking with NP-BSA, which is more physiologically-relevant

stimuli than 22E7, SMCs were primed with anti-NP IgE for 3 hours incubation, then challenged with NP-BSA for 3 hours. RT-PCR was used to quantify the changes in the adenosine receptors' expression. The data showed that IgE/Ag cross-linking induced an increase in $A_{2A}R$, decreased $A_{3A}R$, and $A_{2B}R$ was not affected (Figure 2.6). Thus, cross-linking FcεRI with sub-optimal concentration of monoclonal anti-FcεRI or with multivalent Ag, result in altered adenosine receptors by increasing $A_{2A}R$ and decreasing $A_{3A}R$ expression.

2.3.5 Human skin mast cells with increased $A_{2A}R$ and decreased $A_{3A}R$ due to sub-optimal stimulation of FcεRI express increased cAMP

As observed above, FcεRI cross-linking stimulation led to increased A_{2A} receptor and decreased A_3R receptor at mRNA level. These receptor subtypes exhibit differential effect signaling by interaction with G- proteins. A_{2A} receptor signals via Gαs-adenylyl cyclase-cAMP, whereas A_3 receptor signals via Gi-adenylyl cyclase- cAMP. To determine if the changes in $A_{2A}R$ and A_3 mRNA following low-level stimulation of FcεRI translated to changes at the protein levels, human skin mast cells were stimulated with sub-optimal concentration of 22E7 (1ng/ml) for 6 hours, washed and re-suspended in the Tyrode's buffer in the presence or absence of adenosine (10 µM) for 10 minutes. Intracellular cAMP was measured by a commercially available kit. As expected, intracellular accumulation of cAMP in mast cells with altered $A_{2A}R$ and $A_{3A}R$ were significantly elevated following treatment with adenosine (10µM) compared with the control cells, indicating the predominant role of A_{2A}Receptor in accumulation cAMP following FcεRI stimulation (Figure 2.7).

2.3.6 Efficiency of the up-regulation of $A_{2A}R$ on FcεRI-induced TNF production in human skin mast cells

To pinpoint the functional significance of A_{2A} receptor upregulation, we next determined the sensitivity of human skin mast cells to $A_{2A}R$-mediated inhibition of cytokines production in response to cross-linking FcεRI. For IgE-induced activation, mast cells were sensitized with anti-NP-IgE overnight. Following washing, these cells were stimulated with sub-optimal concentration of NP-BSA (1ng/ml) for 6 hours. After washing, the cells were pre-treated with adenosine (10μM) for 1 hour. After the pre-incubation period, the cells were stimulated with or without IL-33 (10ng/ml) for 24 hours. The cells and medium were collected and then centrifuged. TNF was measured in the supernatant by using ELISA. As seen in (Figure 2.8), adenosine has a more inhibitory effect on TNF production in the cells pre-treated with IL-33 following cross-linking FcεRI, compared to the cells non stimulated with cross linking FcεRI or with IL-33 alone. Thus, these data indicated that up-regulation of $A_{2A}R$ can shift mast cells to anti-inflammatory phenotype.

2.4 Discussion

Adenosine has been suggested to play an important role in the pathogenesis of allergic asthma[232]. Mast cells represent pivotal players that contribute in orchestrating allergic events through the production of pre-stored mediators from granules, and production of lipid mediators and cytokines that are sustained in allergic inflammation[36,41]. Adenosine modulates mast cells release mediators through interaction with its receptors that are then expressed on these cells[93,160].

In this study, we investigated the receptor subtype mediating the suppressive effect of adenosine on allergic mediators release from human skin mast cells. We further tested

the hypothesis that FcεRI stimulation modifies adenosine receptors expression. Our data show that NECA at physiological concentrations had no effect on IgE- induced degranulation in human skin mast cells, but it significantly inhibited IgE- induced degranulation at higher concentration. Our finding of the effect of NECA on IgE-induced degranulation in SMCs in vitro is consistent with our previous studies[159]. Although we increased incubation period exposure to NECA (1hour) before challenging it with antigen, no difference was observed between the cells incubated for 10 minutes in the previous studies, or the cells incubated for 1 hour in our study. In contrast, a previous study showed that mast cells had biphasic response to adenosine or its analogue in which low concentration of adenosine (1μM) can enhance FcεRI-mediated degranulation in human lung mast cells by 25%, whereas using the high concentration of adenosine (1mM) limited or decreased mast cell degranulation by 75%[159,233].

Most of the anti-inflammatory effects of adenosine are produced by the activation of the A_{2A} receptor. Therefore, we used a specific agonist of A_{2A} receptor CGS-21680 to investigate the role of A_{2A} receptor on IgE-induced degranulation in human skin mast cells. Our observation demonstrated that CGS-21680 was unable to inhibit IgE-induced degranulation in mast cells. Additionally, activation of the A_{2A} receptor inhibited the inhibitory effect of NECA on the IgE-mediated degranulation of human skin mast cells, which suggested that A_{2A} receptor is not involved in the inhibition of degranulation in mast cells. Our results are similar to the previous studies which demonstrated that A_{2A} receptor has no role on Ag/IgE-induced degranulation in BMMCs both *in vitro* and *in vivo*[234]. In contrast, other studies demonstrated that adenosine can inhibit IgE-induced degranulation in human umbilical cord cells (HUCBMCs) through binding to A_{2A} receptor, suggesting

the involvement of the A_{2A} receptor in adenosine-mediated inhibition of degranulation[230]. It was also demonstrated that stimulation A_{2A} receptor plays an important role in the suppression of mast cells degranulation in the murine heart and decreasing reperfusion injury[235]. Adenosine can perform a differential function on target cell depending on the adenosine receptor expression and G-protein coupled they engaged. The stimulatory receptors (A_{2A} , A_{2B}) receptors activate AC through coupling to Gs leading to an increase in cAMP accumulation, whereas the inhibitory receptors (A_1 and A_3) inhibit AC by coupling to Gi/0 which leads to a decrease in cAMP concentrations[149,236].

We next tested whether cAMP- dependent signaling mediated inhibition of degranulation in SMCs. Our data showed that Sp-cAMP specific activator of cAMP had no effect on degranulation and prevented the inhibitory effect of NECA on this process. Similar data was obtained when pre-treated mast cells with Rp-cAMP, the inhibitor of cAMP, showed no effect on IgE-induced degranulation and blocked the inhibitory effect of NECA on degranulation. Thus, these data raise the possibility that adenosine receptors have no effect to the observed NECA inhibition in mast cells degranulation, indicating that intracellular mechanism rather than cAMP is responsible for the inhibition of degranulation in mast cells.

It has been known that adenosine receptors have modulatory pattern following interaction with adenosine or analogues on mast cells[230], and many explanations have clarified the differential effects of adenosine and its analogues on IgE-mediated degranulation in mast cells. One of these explanations is that mast cells encompass heterogenous cell type that are derived from the bone marrow, and are migrated into the peripheral tissues where they acquire various phenotypic properties and functional

plasticity in response to dynamic microenvironmental factors which affect the expression and functions of cell receptors, including adenosine receptors[5]. Moreover, the longer exposure to adenosine or its analogues and the initiation to degranulation stimulus might have an influence on the properties of adenosine receptors and the outcome of adenosine[223]. In addition to degranulation, activation mast cells lead to the release of arachidonic acid metabolites like PGD_2, which plays an important role in allergic asthma. We determined the effect of adenosine in IgE-induced PGD_2 production. Our present finding showed that adenosine significantly inhibited IgE-induced PGD_2 production in human skin mast cells. We next examined the effect of $A_{2A}R$ in IgE-induced PGD_2 production. We showed that CGs-21680 has no effect on FcεRI induced PGD_2 production in a dose-dependent manner, suggesting that A_{2A} receptor is not involved in adenosine-inhibited PGD_2. It has previously been demonstrated that NECA has on effect on IgE-induced lipid mediators in mouse mast cells[234]. Collectively, $A_{2A}R$ signaling has no effect on the early phase response (degranulation and PGD_2), which occurred within minutes following cross-linking FcεRI. We next investigated the effect of NECA on IgE- induced cytokines production. we showed that NECA inhibited Ag/IgE induced-TNF production in human skin mast cells. The inhibition of TNF by NECA was seen in the low concentration of NECA, suggesting that this effect was due to the high affinity A_{2A} receptor effect rather than the low affinity A_{2B} receptor, which requires high concentrations of adenosine to be activated. This observation was confirmed when pre-treated mast cells with different concentrations of A_{2A} receptor agonist CGS-21680 on IgE-induced cytokines production showed that CGs-21680 inhibited IgE-induced TNF production. Further supporting this notion is that this inhibition was blocked by using A_{2A} antagonist ZM241385.

We also explored the possible signaling mediated by NECA. We found that pre-treated human skin mast cells with different concentrations of forskolin inhibited IgE-induced TNF production, suggesting that increased cAMP levels are associated with suppression of Ag/IgE- induced cytokines production in mast cells. Our finding of the inhibitory effect of A_{2A} receptor on IgE-induced cytokines production are consistent with many humans and murine studies that have ascribed an anti-inflammatory effect of the A_{2A} receptor in the regulation of inflammatory mediators in many pathological condition[237]. Hue *et al.* showed that A_{2A}-Gs signaling plays an important role in the inhibitory effects of NECA on IgE-induced pro inflammatory cytokines production in murine mast cells[234]. Activation A_{2A} receptor decreased the elevated levels of pro-inflammatory cytokines like IL-6,TNF,and IL-8 and increased anti-inflammatory cytokines like IL-10, leading to the attenuation of inflammatory status[238].

Collectively, our data indicated that the inhibitory effect of NECA on IgE-induced TNF production is mediated by A_{2A} receptor. We also found that cross-linking FcεRI stimulation in mast cells increased the expression of A_{2A} receptor while concomitantly decreasing A_3 receptor, but A_{2B} receptor was not affected. It is also noted that maximal increase of A_{2A}, and decrease A_3 receptors occurs at sub-optimal concentration of 22E7 with minimal induction of degranulation. We also found that A_{2A}R upregulation reaches maximal increase at 3 hours, whereas down-regulation of A_3 receptor reaches maximal decrease at 6 hours. A_{2B} receptor was not affected at all time points or dose-dependent changes. We also observed that the amount of transcripts of these receptors returned to baseline at 24 hours. Our finding also demonstrated that there was an increase in cAMP response in cells with upregulation of A_{2A}R and a decrease in A_3R in the presence of

adenosine, compared with minimal accumulation of cAMP in non-activated cells, suggesting that cross-linking FcεRI causes upregulation of A_{2A} receptor at protein levels. It has been recognized that increased intracellular cAMP are associated with inhibition mast cell function[231].

Altogether, these data suggest that cross-linking FcεRI stimulation altered the expression of A_{2A} and A_3 receptors, not only at mRNA level but also at protein levels. Furthermore, up-regulation of A_{2A} receptors led to a more significant decrease of TNF production through an increase in cAMP compared with the control cells ($p<0.05$). It has been noted that the potency and magnitude of adenosine can be affected by many factors, like receptor density and functionality of adenosine signaling pathways coupled to adenosine receptor[116]. These results parallel to the previously reported changes in A_{2A} receptor in different cells types treated with various inflammatory stimuli such as cytokines and LPS[142,218,239]. In contrast, a previous study showed that decreased A_{2A} receptor and increased A_{2B} receptor in response to IL-4 treatment enhanced the pro-inflammatory effect of adenosine on mast cell mediators[223].

The expression of adenosine receptors are markedly sensitive to alteration in the local milieu, which reflects the capacity of adenosine to function as both pro-inflammatory and anti-inflammatory mediator[149]. Thus, modulation of adenosine receptors could be one strategy to treat various inflammatory diseases[217].

In conclusion, this study demonstrates that adenosine inhibits FcεRI-induced TNF production from human skin mast cells. A_{2A} signals are involved in the inhibitory effect of adenosine in FcεRI-induced TNF production via cAMP mechanism. Our results showed

that stimulation of FcεRI could significantly increase the expression level of A_{2A} and decrease A_{3A} receptors at mRNA and protein levels, and that these changes accompany the anti-inflammatory effect of adenosine on human mast cells. We demonstrate that selective up-regulation of A_{2A} receptor following FcεRI cross-linking promotes mast cells to shift from pro-inflammatory phenotype into anti- inflammatory phenotype, suggesting that the differential up-regulation of A_{2A} receptor could be considered as a potential negative feedback through a mechanism involving accumulation cAMP.

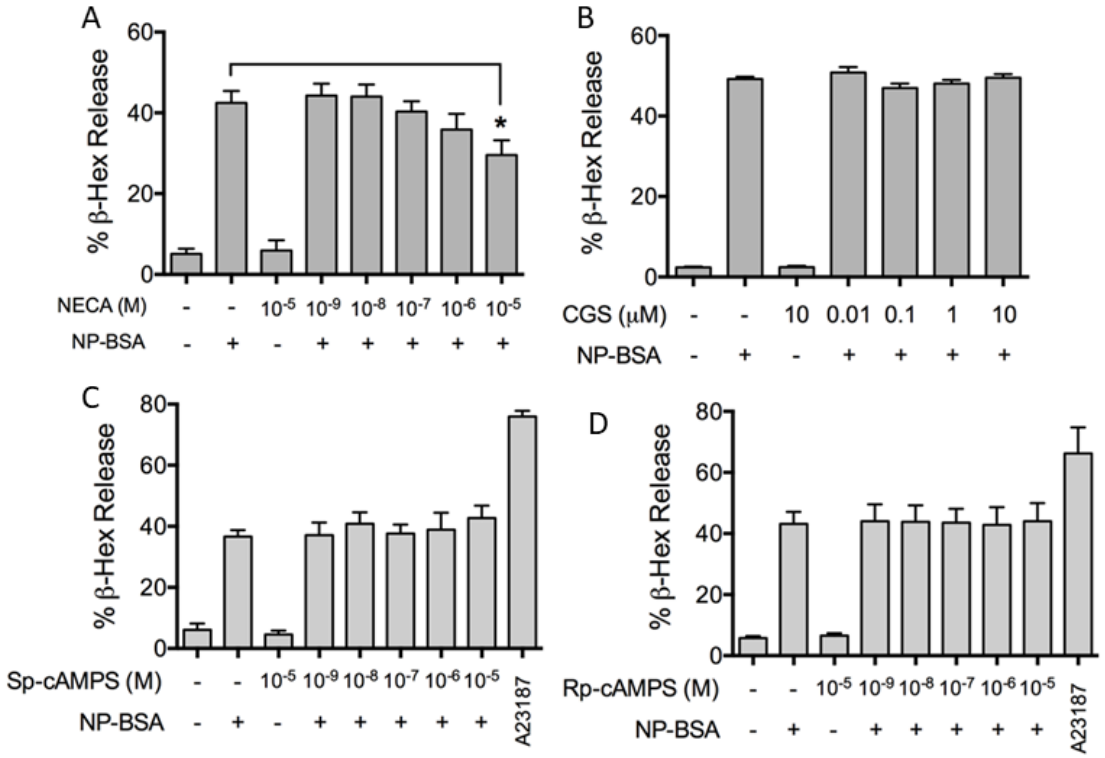

Figure 2.1 $A_{2A}R$ signals are not responsible for inhibiting IgE-mediated degranulation in human skin mast cells. Human skin mast cells that were sensitized with anti-NP IgE were pre-treated for 1 hour with (A) stable adenosine analog NECA ,(B) $A_{2A}R$ agonist (CGS-21680), (C) cAMP agonist (Sp-cAMP), and (D) cAMP antagonist (Rp-cAMP), and then challenged with antigen NP-BSA (100ng/ml) for 30 minutes at 37°C). Degranulation was determined by β-hexosaminidase release assay . Data are expressed as the mean ± SEM of values obtained from independent experiments with human skin mast cells n = 3 different donors. *, $p<0.05$ Student` s t-test.

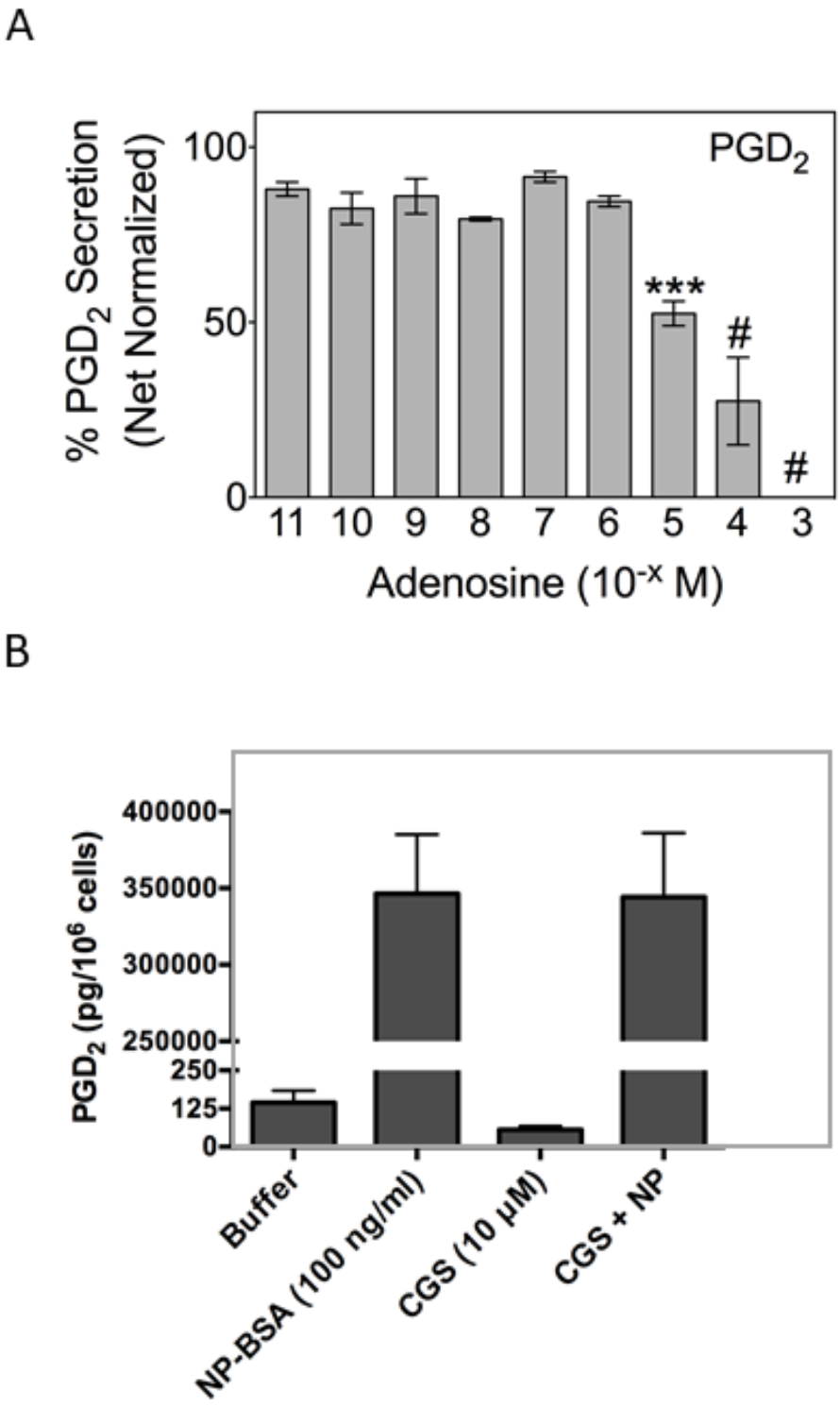

Figure 2.2 The effect of $A_{2A}R$ on FcεRI-induced PGD_2 biosynthesis of human skin mast cells. Mast cells were sensitized with anti-NPIgE, then pre-treated for 10 minutes with (A) adenosine, (B) A2aAR agonist CGS21680, and then activated with NP-BSA for 30 minutes at 37°C. Secreted PGD2 in supernatant was measured by enzyme immunoassay (EIA). *** ($p < 0.01$),# ($p < 0.001$) by one-way ANOVA.

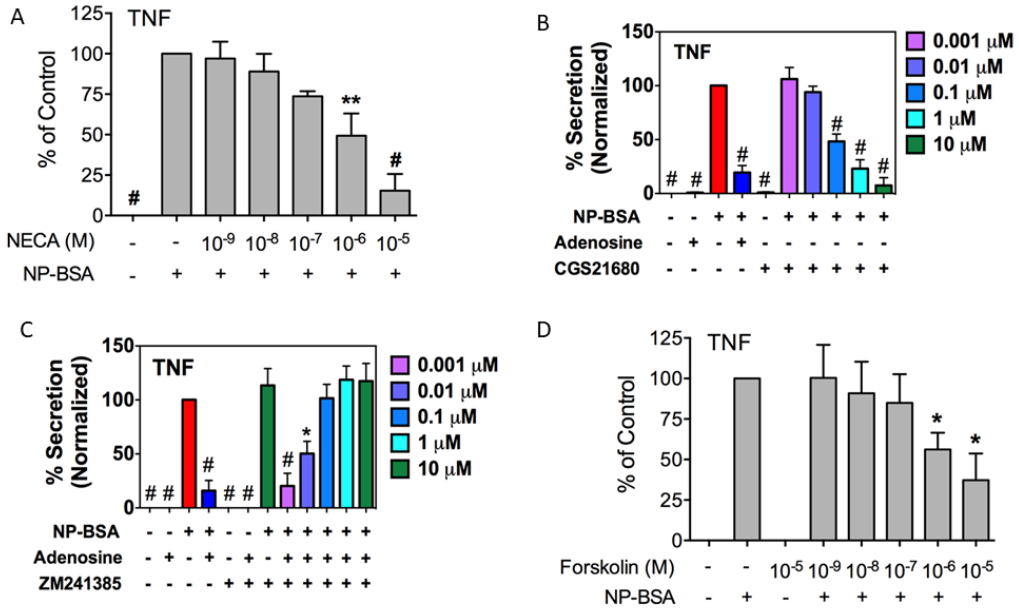

Figure 2.3 The effect of $A_{2A}R$ signal on FcεRI-induced TNF production from human skin mast cells. IgE sensitized human skin mast cells were pre-treated for 1 hour with (A) NECA, (B) $A_{2A}R$ specific agonist CGS21680, (C) $A_{2A}R$ specific antagonist ZM241385, and (D) Forskolin, and then activated with NP-BSA (100ng/ml) for 24 hours. Secreted TNF in supernatant was measured by ELISA. Data shown is expressed as mean ± SEM of values from skin mast cells from different donor tissue in independent experiments. * $P<0.05$, **($P< 0.01$) and # ($p< 0.001$) by one-way ANOVA with Bonferroni post-test.

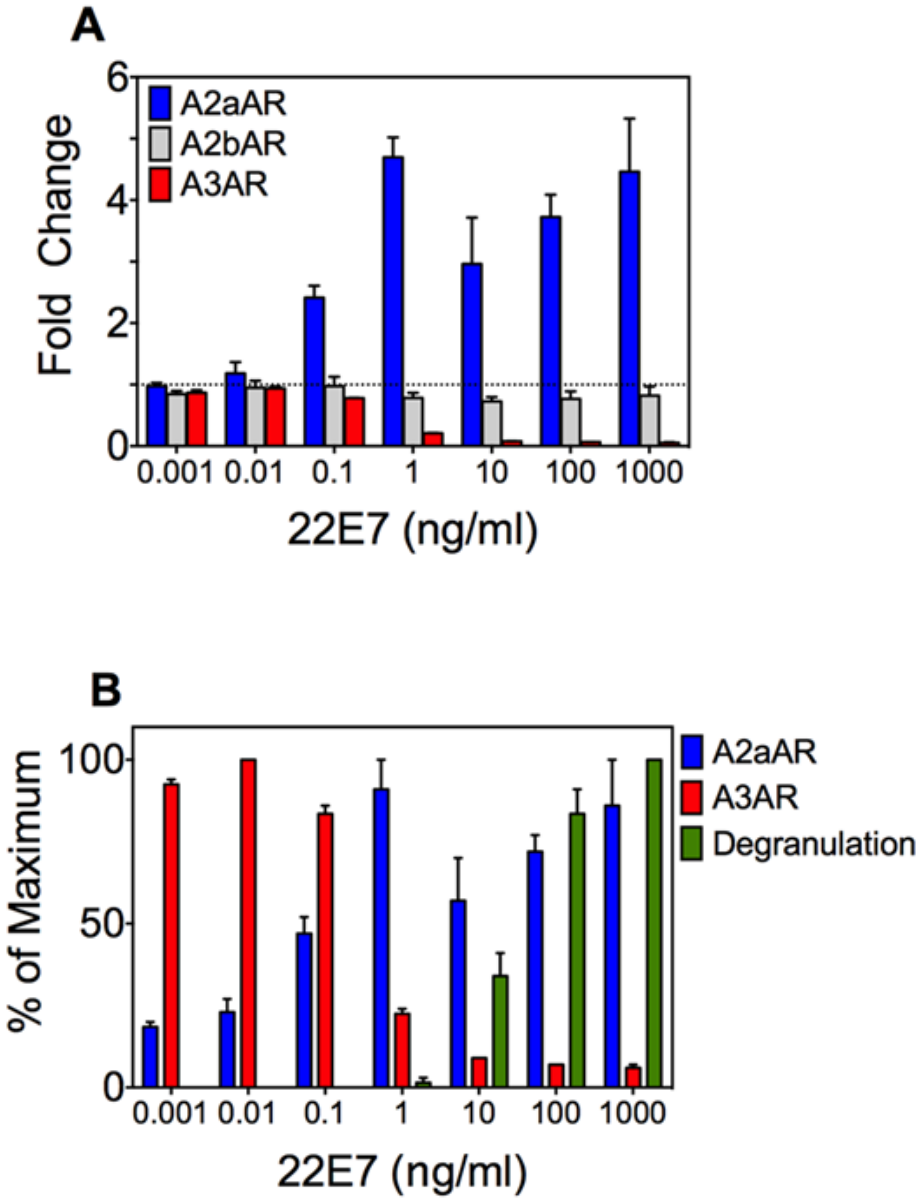

Figure 2.4 The effect of FcεRI stimulation on the modulation adenosine receptors in human skin mast cells in dose dependent manner. (A) qRT-PCR analysis of $A_{2A}R$, $A_{2B}R$ and $A_{3A}R$ in human skin mast cells stimulated for 3 hours with 22E7 at1 pg/ml-1µg/ml. Fold change, expressed as mean ± SEM, was determined by the $2^{\Delta\Delta Ct}$ method comparing simulated mast cells to non-stimulated mast cells. (B), Overlay of normalized FcεRI- induced changes in $A_{2A}R$ and $A_{3A}R$ with degranulation demonstrating that stimulation with 22E7 at 1 ng/ml induces maximal increase of $A_{2A}R$ and decrease of $A_{3A}R$. Fold changes ≥ 2- fold are considered significant.

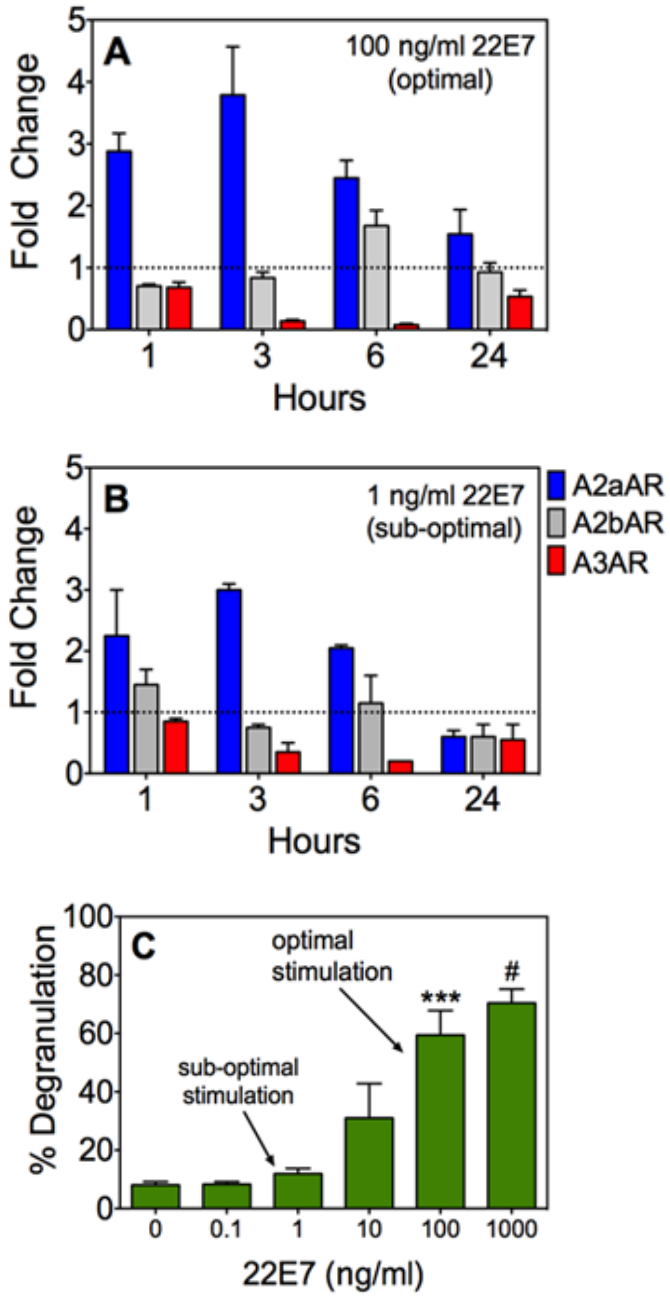

Figure 2.5. Time course analysis of adenosine receptor expression following FcεRI stimulation. qRT-PCR analysis of $A_{2A}R$,$A_{2B}R$ and $A_{3A}R$ in human skin mast cells stimulated for 1, 3, 6, and 24 hours with mAb 22E7 at 100 ng/ml (A) or 1 ng/ml (B). Fold change was determined by the $2^{\Delta\Delta Ct}$ method comparing stimulated mast cells to non-stimulated mast cells. Fold change ≥ 2-fold are considered significant. (C) Degranulation dose response to 22E7 showed that optimal stimulation with 22E7 at 100ng/ml induces degranulation, whereas sub-optimal stimulation with 1 ng/ml does not. Degranulation was determined by β- hexosaminidase release assay.

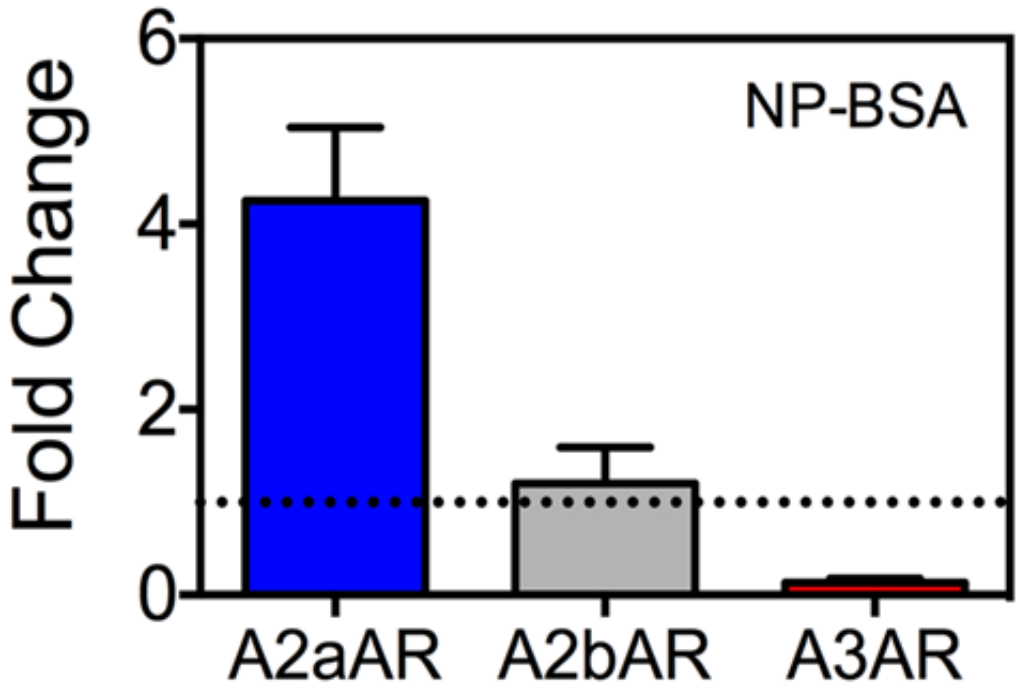

Figure 2.6 IgE/Ag cross-linking induces changes in adenosine receptors expression. qRT-PCR was used to measure the changes in adenosine receptors. Human skin mast cells were sensitized with anti-NP IgE, and then activated with multivalent antigen (NP-BSA) (100ng/ml) for 3 hours. NP-BSA increases $A_{2A}R$ and decreases $A_{3A}R$ in human skin mast cells. Fold change was determined by the $2^{\Delta\Delta Ct}$ method comparing stimulated mast cells to non-stimulated mast cells. Fold changes $\geq$ 2-fold are considered significant.

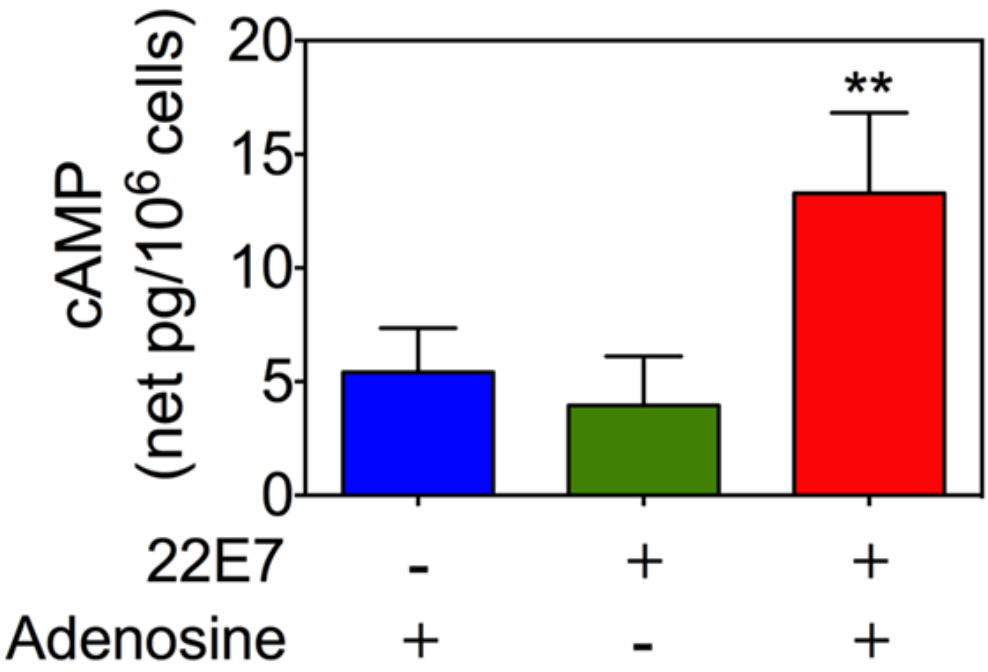

Figure 2.7 Functional expressions of adenosine receptors following crosslinking FcεRI signal. Human skin mast cells were stimulated with 22E7 (1 ng/ml) for 6 hours, washed, and re-suspended in buffer with or without adenosine for 10 minutes. Intracellular cAMP was measured by commercially available kit. Accumulation cAMP is increased in human skin mast cells with upregulation of $A_{2A}R$ and down regulation of $A_{3A}R$ following suboptimal stimulation FcεRI upon exposure to adenosine ** ($p< 0.001$).

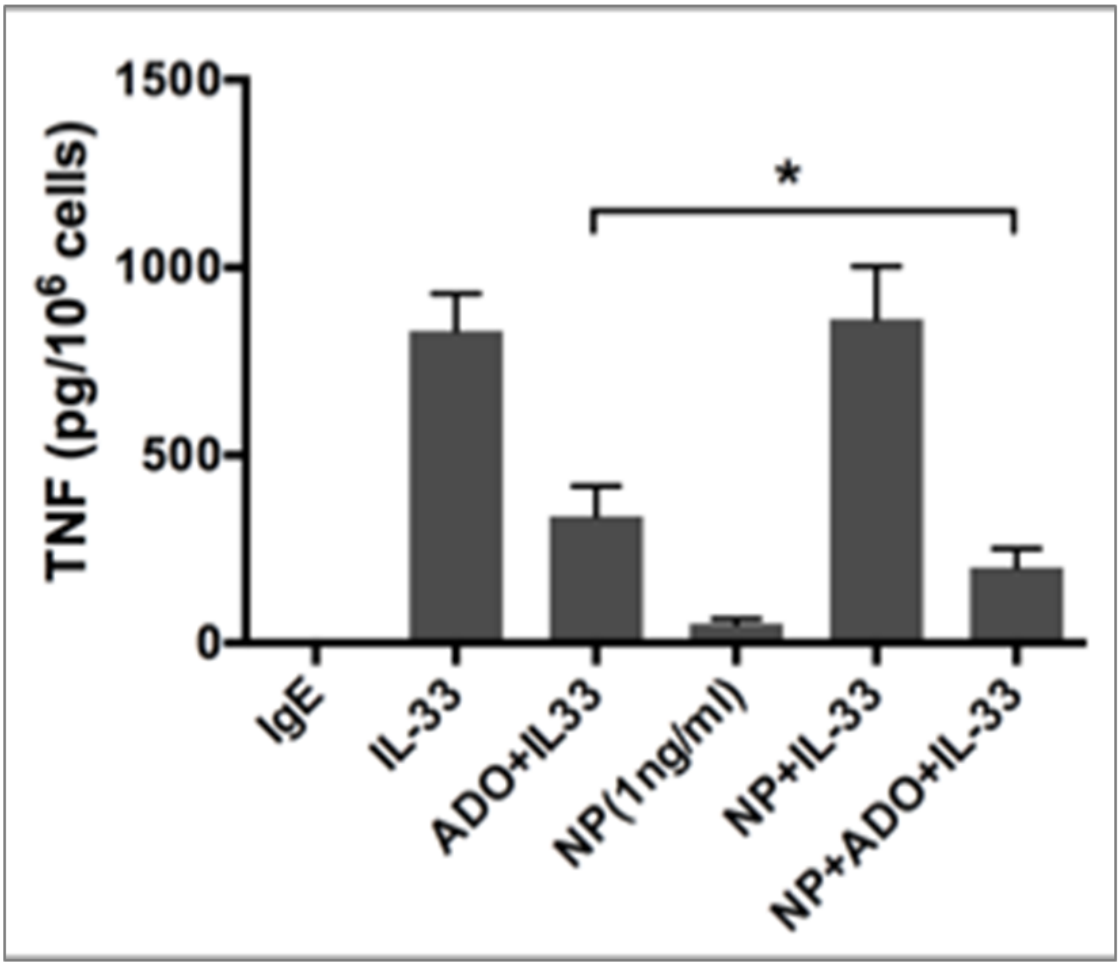

Figure 2.8 Efficiency of the up-regulation of $A_{2A}R$ on FcεRI-induced TNF production in human skin mast cells. IgE-sensitized human skin mast cells with anti-NP IgE, stimulated with sub-optimal concentrations of NP-BSA (1ng/ml) to crosslinking FcεRI for 6 hours, were washed, pretreated with adenosine for 1 hour, then stimulated with IL-33 (10ng/ml) for 24 hours. Secreted TNF in supernatant was measured by ELISA measured by ELISA.* P <0.05.

CHAPTER 3

MIR-155 IS A POSITIVE AND NEGATIVE REGULATOR OF MAST CELL RELEASE INFLAMMATORY MEDIATORS

3.1 Background

Mast cells are a major driver of allergy and other chronic inflammation through releasing various inflammatory mediators that contribute to allergic diseases[240]. These cells can be highly regulated by miRNAs. MiRNAs are non-coding RNA molecules that regulate gene expression of various mediators[81]. It has been demonstrated that miRNAs play an important role in allergy and asthma pathogenesis[174]. miRNAs exhibit different expression profiles in asthmatics, allergic rhinitis subjects compared with health subjects[81]. Several miRNAs have been reported to play important roles in regulation of mast cell activation, like miR221, miR223 and others[241]. In addition, miR-155 was reported to control mast cells activation and anaphylaxis[242]. MiR-155 is upregulated in different activated immune cells, implying the effector function of miR-155 in the cells[185]. It has been reported that miR-155 is highly increased in an allergen- induced model of asthma[195]. In this study, we used human skin mast cells and wild type and miR-155 knockout (KO) to address the role of miR-155 in regulating the release of inflammatory mediators from mast cells. Our results showed that miR-155 expression was induced following FcεRI cross-linking with multivalent antigen in human skin mast cells and mouse BMMCs. We found that miR-155 did not influence FcεRI-induced mast degranulation. Moreover, the amount of β-hexosaminidase activity was the same in both types of mast cells.

We also showed that miR-155 has no effect on leukotrienes C4 secretion in these cells. In support of this, we observe that ALOX5 expression was similar in WT and miR-155 KO BMMCs at mRNA and protein levels. In contrast, FcεRI-induced expression of COX-2, which is directly involved in prostaglandin biosynthesis, was severely diminished in the absence of miR-155. In addition, miR-155 KO significantly reduced the levels of TNF, IL-6, and IL-13 following FcεRI cross-linking, but the level of these cytokines was increased compared to WT following LPS treatment. The phosphorylation of Akt was significantly decreased in miR-155 KO compared to WT, whereas p38, p42/p44 phosphorylation were not affected. These data suggest the regulatory role of miR-155 in mast cell release mediators. Therefore, modulation of miR-155 expression could support targets to therapeutically target and modulate mast cell response.

3.2 Materials and methods

The methods detailed in this section were designed to address the role of miR-155 in the regulation of mast cell function by investigating its effects on degranulation, eicosanoid biosynthesis, gene expression and cytokines production. This study was performed on human skin-derived mast cells that were isolated in accordance with the Human Studies Internal Review Board (IRB) of the University of South Carolina and BMMCs that were generated in vitro from wild and miR-155KO mice in accordance with animal use protocol approved by the Institutional Animal Care and USC Committee (IACUC) at the University of South Carolina.

3.2.1 Bone Marrow-Derived Mast Cells (BMMCs), IgE Sensitization, and Activation

Bone marrow was isolated from femurs and tibias of sex and age-matched (8-12 weeks old) miR-155$^{-/-}$ and C57BL6 mice that were housed at the University of South

Carolina and that were used in accordance with animal use protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina. After flushing the bone marrow from the bones with media and filtering through a 40μm filter, bone marrow cells were cultured with complete RPMI 1640 media supplemented with 10% FCS and 10ng/ml each of murine recombinant stem cell factor (SCF) and interleukin-3 (IL-3). The cultures were maintained under standard conditions (37ºC, 5% CO_2) with weekly media changes, and used for experiments when >95% of the cells were FcεRI$^+$. Prior to each experiment, BMMCs were sensitized with IgE by culturing overnight in media containing anti-DNP IgE (0.1μg/10^6 cells) (generously provided by Dr. Daniel Conrad, Virginia Commonwealth University). For activation, BMMCs were washed to remove unbound IgE and were treated with DNP-BSA at concentrations and time points indicated.

3.2.2 Human Skin Mast Cells, IgE Sensitization, and Activation

Human skin mast cells (SMCs) were isolated and purified from fresh surgical specimens of human skin tissue. Skin tissues were obtained from the Cooperative Human Tissue Network of the National Cancer Institute, as approved by the Human Studies Internal Review Board (IRB) of University of South Carolina. SMCs were cultured in X-VIVO 15™ media containing SCF (100 ng/ml) with weekly media changes. SMCs (10^6 cells/ml) were sensitized with IgE by incubating overnight in media containing 1 μg/ml chimeric human anti-NP IgE (clone JW8/1; AbD Serotec) at 37ºC. After washing and re-suspending the cells in Tyrode's-BSA buffer, SMCs (10^6 cells/ml) were activated with NP-BSA (Biosearch Technologies) using the indicated concentration and time-point at 37ºC.

3.2.3 Degranulation, Cytokine, and LTC_4 release assays

FcεRI-induced degranulation and Leukotriene C4 (LTC4) release were determined by standard β-hexosaminidase release assay and enzyme immunoassay, respectively. IgE-sensitized BMMCs (10^6 cells/ml) were activated with DNP-BSA at the indicated concentrations for 30 minutes in Tyrode's-BSA buffer (135 mM NaCl, 1 mM $MgCl_2$, 20 mM Hepes, 5 mM KCl, 1.8 mM $CaCl_2$, 5.6 mM glucose; pH 7.4, 0.05% bovine serum albumin). After the activation period, BMMCs and supernatant were separated by centrifugation, and BMMCs were lysed with 1% Trixon X-100. For degranulation, 10µl of supernatant and lysate was mixed with 10µl of 1mM p-nitrophenyl N-acetyl-β-D-glucosaminide (PNAG; Sigma-Aldrich) in a 96 well plate, and incubated for 1hour at 37ºC. The reaction was terminated and the color change was induced with 200µl/well of 0.1M Na_2CO_3/$NaHCO_3$ buffer, and absorbance was read at 450 nm. Percent β-hexosaminidase release was calculated from the absorbance values according to the formula: % β-hexosaminidase release = ((supernatant)/(supernatant + lysate)) × 100. LTC_4 in the supernatant was measured with a commercial enzyme immunoassay (Cayman Chemical) according to the manufacturer's instructions. For cytokine determination, BMMCs (10^6 cells/ml) were activated with anti-DNP IgE or stimulated with lipopolysaccharide (LPS) at the indicated concentrations for 24 hours in complete RPMI 1640 media supplemented with SCF and IL-3. TNF, IL-6, and IL-13 in the cell-free media were measured with commercial enzyme linked immunosorbent assay (ELISA) (R&D Systems). Absorbance measurements were taken on a BioTek Synergy HT microplate reader, and cytokine concentrations were determined using Gen5 Data Analysis Software.

3.2.4 Gene Expression Analysis

Gene expression was determined by quantitative real-time PCR. IgE-sensitized BMMCs were activated with DNP-BSA at the indicated concentrations and time-points, and RNA was extracted with miRNeasy kits. Inactivated cells were used as a control for the experiment. For miR-155 analysis, cDNA was synthesized with miScript II RT with HiFlex buffer, and PCR was carried out with miScript SYBR Green and miScript Primer Assays for human and mouse miR-155-5p, and SNORD96A was used as the control gene. PCR was carried out with 2ng of cDNA per reaction in a hot start protocol: (95°C × 15 min, (94°C × 15 sec, 55°C × 30 sec, 70°C × 30 sec) x 35 cycles). All miRNA kits were purchased from Qiagen and used according to the manufacturer's instructions. For COX-2 and ALOX5 analysis, cDNA was synthesized with the iScript cDNA Synthesis kit, and PCR was performed using iQ SYBR® Green Supermix (Bio-Rad). PCR was carried out with 200ng of cDNA per reaction in a hot-start protocol: (95°C × 5 min, (95°C × 30 sec, 55°C × 30 sec, 72°C × 30 sec) x 35 cycles, 95°C × 1 min, 55°C × 1 min). Validated oligonucleotide primers (Sigma-Aldrich) that were used included COX-2 (F:5'-ACTGCTCAACACCGGAATTT-3', R: 5'-CAAGGGAGTCGGGCAATCAT-3'), ALOX5 (F: 5'-CAGGAAGGGAACATTTTCATC-3', R: 5'-AGGAAGATTGGGTTACTCTC-3'), and β2 microglobulin (B2M) (F: 5'-TGGGTTTCATCCATCCGACA-3', R: 5'-CTGCTTACATGTCTCGATCCC-3'). Analysis was performed on a CFX Connect Real Time PCR Detection System (Bio-Rad). The fold change in expression was determined by the $2^{\Delta\Delta Ct}$ method.

3.2.5 miR-155 genotyping

MiR-155 genotyping was performed using a modified version of a protocol provided by Jackson Laboratories. Genomic DNA was extracted from WT and miR-155 KO BMMCs by incubating it with Direct PCR Lysis Reagent (cell) + Proteinase K solution (Viagen Biotech) overnight in a 55ºC water bath followed by a 1 hour incubation at 85ºC. The DNA was precipitated with ethanol + NaOAc, re-suspended in water, and 50ng per reaction was amplified with iTaq Universal SYBR Green Supermix (Bio-Rad) in a reaction mix containing wild type (5'-AATCATTCCTGAGGG CTACC-3') or mutant (5'-GCCTGAAGAACGAGATCAGC-3') forward primer and a common primer (5'-GGAAACGTGGGTCTCCTTAC-3') with the protocol 94ºC × 5min, (94ºC × 5min, 61.8ºC × 1min, 72ºC × 30sec) x 36 cycles, 72ºC × 3min. For visualization, the PCR products were loaded onto a 1.5% TBE gel containing ethidium bromide and electrophoresed. The expected band sizes were 165bp for *miR-155*$^{+/+}$ and 226bp for *miR-155*$^{-/-}$.

3.2.6 Flow Cytometry

The BMMCs (10^6/ml) were washed and re-suspended in FACS buffer (1% BSA, 0.04% NaN_3 in PBS) on ice. FcγRs were blocked with rat anti-mouse CD16/32 (Clone S17011E) (1µg/10^6 cells) for 20 minutes on ice. The cells were stained with FITC-labeled anti-mouse FcεRIα mAb (clone MAR-1) or IgG isotype control (clone HTK888) (BioLegend) (1µg/10^6 cells) for 20 minutes on ice. The cells were washed twice in FACS buffer and fixed with 2% paraformaldehyde. Data was collected using a FACSAria II cell sorter and was analyzed with FlowJo v10 software (FlowJo, LLC).

3.2.7 Immunoblotting

Whole cell lysates were prepared from BMMCs that were activated as indicated. BMMC activation was terminated immediately by the addition of ice-cold PBS. The BMMCs were pelleted by centrifugation and lysed (10^7 cells/ml) with Tris-Glycine SDS Sample Buffer (Life Technologies) containing 1% β-mercaptoethanol and 1mM Na_3VO_4. Equivalent volumes were loaded onto 10-12% Tris-Glycine polyacrylamide gels and separated by SDS-PAGE. The separated proteins were then transferred onto nitrocellulose membranes with Towbin's Transfer Buffer (25μM Tris, 192mM Glycine, 20% Methanol) using a semi-dry transfer apparatus (Bio-Rad). After transfer, the membranes were blocked for 1 hour at room temperature with Odyssey Blocking Buffer (LI-COR Biosciences). Two-color staining was performed by incubating the blots overnight at 4°C with the following combination of primary antibodies (Cell Signaling Technology): rabbit polyclonal anti-p38 MAPK + mouse monoclonal anti-phospho-p38 MAPK (Thr180/Tyr182)(28B10), rabbit polyclonal anti-p44/42 (Erk 1/2) + mouse monoclonal anti-phospho-p42/44 (Erk1/2) (Thr202/Tyr204) (E10), rabbit polyclonal anti-Akt + mouse monoclonal anti-phospho-Akt (Thr308)(L32A4), or rabbit monoclonal anti-ALOX5 (C49G1) + mouse monoclonal anti-β-actin (8H10D10). After the incubation period, the blots were washed and incubated for 1 hour at room temperature with the secondary antibodies goat anti-rabbit IRDye 680RD + goat anti-mouse 800CW (LI-COR Biosciences). The blots were then washed and scanned on an Odyssey® CLx Infrared Imaging System and were analyzed with Image Studio Software version 3.1.4 (LI-COR Biosciences).

3.2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 6.0c for Mac OS X, GraphPad Software (La Jolla California USA), and the software is available for download at www.graphpad.com

3.3 Results

3.3.1 FcεRI crosslinking upregulates miR-155 expression in human and mouse mast cells

To determine the miRNAs that are potentially involved in IgE-stimulated mast cells, we performed a miRNA array of human skin mast cells in stimulated and non-stimulated mast cells. MiRNAs array analysis revealed that miRNAs were altered following crosslinking FcεRI. We identified 10 miRNA that were significantly ($p < 0.01$) upregulated, and 11 downregulated (Figure 3.1). Notably, miR-155-5P was the most significantly upregulated in activated mast cells. We confirmed the miRNA array data with quantitative RT-PCR analysis of SMCs (Figure 3.2A), and further demonstrated that miR-155-5p expression was also induced in C57BL6 BMMCs following FcεRI crosslinking (Figure 3.2B). Thus, FcεRI signaling induces miR-155-5p expression in human and mouse mast cells.

3.3.2 MiR-155 positively regulates the FcεRI-induced prostaglandins pathway, but has no effect on LTC4 synthesis or degranulation

To investigate the effect of miR155 expression in regulating mast cells function, we compared BMMCs from WT and miR-155KO. The genotype was confirmed by PCR (Figure 3.3A) and quantitative RT-PCR (Figure 3.3B). We also determined the effect of miR155 in the surface expression of FcεRI by FACS (Figure 3.3C) and the content of the

β-hexosaminidase (Figure 3.3D). No significant difference in the IgE receptor expression or the contents of β-hexosaminidase was observed between WT and KO BMMCs, indicating that miR155 has no effect on mast cells development. Next, we assessed the role of miR-155 deficiency on IgE-mediated mast cell activation.

For functional studies, BMMCs were sensitized with anti-DNP IgE ($0.1\mu g/10^6$ cells), and then challenged with DNP-HSA (0.1-100 ng/ml) to FcεRI crosslinking. We found that miR-155 had no effect in IgE-mediated degranulation, which was indicated by the release of β-hexosaminidase (Figure 3.4A). We also observed that the miR-155 had no effect on mast cells degranulation in response to stimulation with calcium ionophore, suggesting that miR-155 has no effect on IgE and non-IgE mediated degranulation in BMMCs (Figure 3.4B). Next, we determined the effect of miR-155 deficiency on the arachidonic acid pathways leading to LTC_4 and PGD_2. We analyzed the effect of miR-155 on COX-2 expression, which is a key enzyme in eicosanoid pathways leading to prostaglandins. IgE-sensitized BMMCs were activated with DNP-HSA for 5, 10, 20, and 40 minutes. Changes in expression of COX-2 expression were determined with qRT-PCR. As shown in (Figure 3.5A), FcεRI induced COX-2 expression was highly diminished in miR-155 KO BMMCs, indicating that FcεRI- induced PGD_2 biosynthesis is defective in the absence of miR155. Because we are working on mouse samples that contain antibodies that interfere with PGD_2 ELISA kit, we could not measure PGD_2. Interestingly, FcεRI -induced LTC4 was not affected in the absence of miR-155, although a slight but insignificant increase was observed (Figure 3.5B) Accordingly, ALOX5 expression, a key enzyme in the leukotriene pathway, was expressed at similar mRNA and protein levels in WT and miR-155 KO BMMCs (Figure 3.5C and D). Together, these data demonstrate that

miR-155 positively regulates the FcεRI-induced prostaglandin pathway, but does not regulate the leukotriene or degranulation pathways in mast cells.

3.3.3 MiR-155 has divergent effect on IgE-dependent and LPS-stimulated cytokines production in mast cells

To determine the role of miR-155 on IgE-mediated cytokine production, BMMCs from WT and miR-155 KO mice were sensitized with anti-DNP IgE (0.1µg/10^6), and then challenged with DNP-HSA (10ng/ml) for 24 hours. Secreted TNF, IL-6, and IL-13 in the cell-free supernatants were measured with ELISA. As shown in (Figure 3.6), miR-155 KO BMMCs secreted significantly lower amounts of TNF, IL-6, and IL-13 compared to WT BMMCs. In order to determine the inhibitory role of miR-155 deficiency on cytokines, production was limited to FcεRI signals or to similar signaling pathways. WT and miR-155 KO BMMCs stimulated with (LPS) (0.1-10µg/ml) activated Toll like Receptor (TLR4). In contrast, with FcεRI crosslinking, TNF, IL-6, and IL-13 were secreted at significantly greater amounts from LPS-stimulated miR-155 KO BMMCs compared to WT (Figure 3.7). Together, these data demonstrate that miR155 has divergent effects on cytokine production induced by FcεRI and TLR4, indicating that the targets of miR-155 are distinct in the FcεRI and TLR4 pathways.

3.3.4 FcεRI-induced Akt phosphorylation is inhibited in miR-155 deficient mast cells

Akt is known to play a major role in FcεRI-induced cytokine production from mast cells[243]. Therefore, we compared Akt activation in WT and miR-155 KO BMMCs following FcεRI crosslinking. As predicted, based on the observed impairment in cytokine production in the absence of miR-155 (Figure 3.8**)**, IgE-dependent Akt phosphorylation was severely attenuated in miR-155 KO BMMCs. On the other hand, p38 and p42/44 (ERKs) phosphorylation was unaffected, indicating that miR-155 specifically targets the

Akt pathway and not early FcεRI-proximal events that broadly control FcεRI signaling. Indeed, it was reported that miR-155 deficiency had no effect on activation of FcεRI-proximal src kinases Fyn and Lyn, which negatively and positively regulate FcεRI signaling pathways, respectively[242]. Akt is known to be downstream of the phosphoinositide 3-kinase (PI3K) pathway and a direct substrate of phosphoinositide-dependent protein kinase 1 (PDK1)[244]. However, we did not detect any difference in expression of the PI3K subunits p85 or p101, or PKD1 in miR-155 KO BMMCs compared to WT cells, indicating that miR-155 is a target of Akt- independent PI3K pathways.

3.4 Discussion

MiRNAs have been recognized as posttranscriptional regulators with enormous importance in many cellular processes[241]. MiR-155 plays a key role in the pathogenesis of allergic diseases[245]. Although much evidence indicates its role in allergic disease, few studies address the role of miR-155 in mast cells function. Therefore, elucidating the function of miR-155 in mast cells is greatly beneficial for controlling and treating allergic diseases like asthma. In this study we identified 21 dysregulated miRNAs following crosslinking FcεRI in SMCs. Among them, 11 were downregulated, and 10 were upregulated. MiR-155-5p was significantly upregulated miRNAs in IgE-stimulated mast cells, suggesting that miR-155 play a regulatory role in mast cells function. To further explore the role of miR-155 in mast cells, we compared the effects of miR-155 from WT and miR-155 from KO BMMCs on IgE-mediated mast cells activation. Our study demonstrated that miR-155 has no effect in IgE-dependent degranulation. In contrast, a previous study showed that absence of miR-155 enhanced mast cells degranulation and Lamp 1 expression following FcεRI crosslinking[242]. However, this study also reported that

FcεRI-induced phosphorylation of syk and src kinases, critical signaling proteins that regulated FcεRI- induced degranulation was unaffected in miR-155 KO BMMCs. Moreover, the reported % β-hexosaminidase release was only on the order of 15% following FcεRI crosslinking of WT BMMCs with antigen at a concentration (20 ng/ml) that should have induced a > 50 % effect. For comparison, 10 ng/ml antigens induced 51.3 ± 1.8 % β- hexosaminidase.

We also found that inhibition miR-155 has no effect in mast cells stimulated with calcium ionophore A23187, indicating that miR-155 has no role in regulating mast cells degranulation that are activated by IgE or non-IgE mechanism. Eicosanoids like leukotrienes and prostaglandins are important proinflammatory mediators released within minutes following mast cells activation, and play an important role in various pathological conditions like allergic asthma[26]. Biosynthesis of these mediators starts with conversion of arachidonic acid by cyclooxygenase and lipoxygenase pathways into prostaglandins and leukotrienes[246].

Many miRNAs have been reported to play a key role in the regulation of eicosanoid pathways[246]. In our study, we demonstrate that miR-155 has no effect on leukotrienes secretion. However, FcεRI-induced COX-2 was significantly diminished ($p < 0.01$) in the absence of miR-155, suggesting that miR-155 plays a critical role in PGD_2. MiR-155 has been found to increase COX-2 expression in the smooth muscles of human asthmatics airways, and non-asthmatics patients indicated the positive correlation between miR-155 and COX-2 expression[247]. A similar finding was observed in cockroach allergen-induced mouse model of asthma[195].

For further insight into the role of miR-155 during mast cells activation, we examined its effect on cytokines production by stimulated BMMCs from WT and miR-155 KO with two different signaling. Our finding demonstrated that miR-155 regulated cytokines production in mast cells. Inhibition of miR-155 in FcεRI-induced cytokines production result in suppressing production of proinflammatory cytokines TNF, IL-6, and IL-13, all of which play an important role in inflammatory and allergic responses. In contrast, a certain previous study had demonstrated that miR-155 deficiency enhanced IL-6, TNF, and IL-13 compared to WT[242]. It has been revealed that inhibition of miR-155 positively regulated the expression of Th2 IL-5,and IL-13[248]. Several miRNAs have demonstrated their role in regulating cytokines production in mast cells[249,250]. Interestingly, inhibiting miR-155 promoted TNF, IL-6, and IL-13 in LPS- treated BMMCs. Thus, our data suggest that miR-155 is a positive regulator in FcεRI induced cytokine production, and a negative regulator in LPS- stimulated cytokines production. Many studies have indicated the critical role of miR-155 in the inflammation and modulation immune response[251].

MiR-155 was reported to inhibit or promote inflammation depending on the inflammatory stimulant involved[251]. The AKT/PI3K signaling pathway is involved in mediating many aspects of mast cells functions, like maturation, activation, survival and apoptosis[252]. As the PI3K conformation changes, phosphorylation of amino acids residues Ser 473 and Thr 308 by PDK1 is required for Akt activation[253]. Many previous studies have reported that the PI3K/Akt signaling pathway playa an important role in the regulation of mast cells activation and allergic diseases[252,253]. In this study, the phosphorylation of Akt to be involved in cytokine production was severely inhibited in miR-155 KO BMMCs.

However, we did not detect any difference in expression of the PI3K subunits p85 or p101, or PKD1 in miR-155 KO BMMCs compared to WT cells. These findings are inconsistent with a previous study which demonstrated that miR-155 KO BMMCs increased Akt and the downstream of the PI3K[242].

These data demonstrate that miR-155 is a positive regulator of FcεRI-induced Akt activation independent of PI3K. These findings may point towards the effect of miR-155 in regulating FcεRI- dependent mechanism like cytokines and prostaglandin production, indicating the essential role of miR-155 in regulating mast cells response. In conclusion, the current study identified that miR-155 was significantly up-regulated in human and mouse mast cells, and that inhibition attenuated IgE-induced COX-2 expression and pro-inflammatory cytokines production, but augmented the production of these cytokines in LPS-stimulated mast cells. These results highlight the regulatory role of miR-155 on mast cell response and indicate how miR-155 may serve as a novel approach for the treatment of allergic diseases.

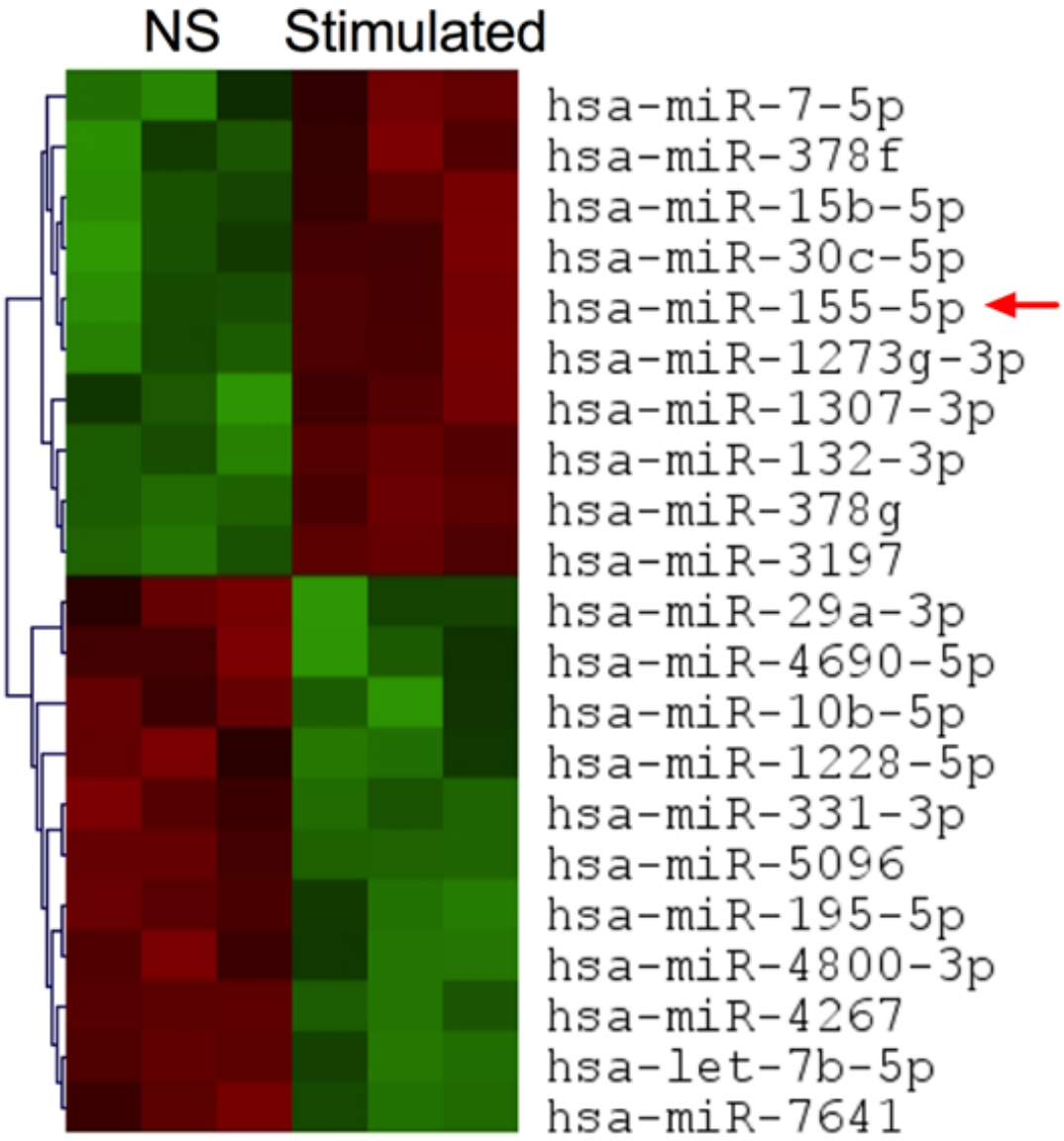

Figure 3.1 FcεRI crosslinking alters miRNAs profile in human skin mast cells. Heat map shows significant ($p<0.01$) alterations in specific miRNA in sensitized human skin mast cells that were stimulated, or not (NS), with multivalent antigen (NP-BSA). RNA was isolated with miRNeasy kit, and miRNAs profile was performed

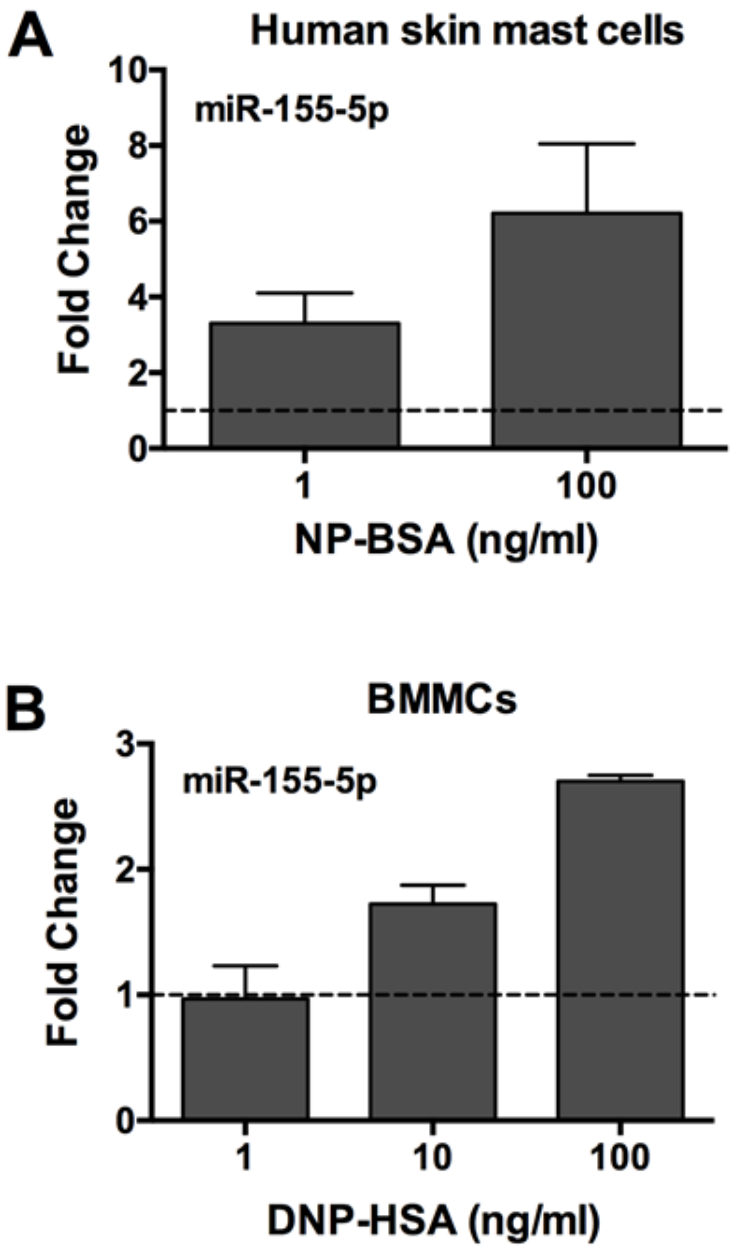

Figure 3.2 qRT-PCR were performed to determine the expression of miR-155 in human and mouse mast cells. (A) miR-155 expression in human skin mast cells. IgE-sensitized SMCs were activated with different concentrations of NP-BSA for 6 hours. (B) miR-155 expression in WT BMMCs. BMMCs from WT were sensitized, activated with different concentrations of DNP-HSA for 6 hours. The expression of miR-155 was determined by qRT-PCR.

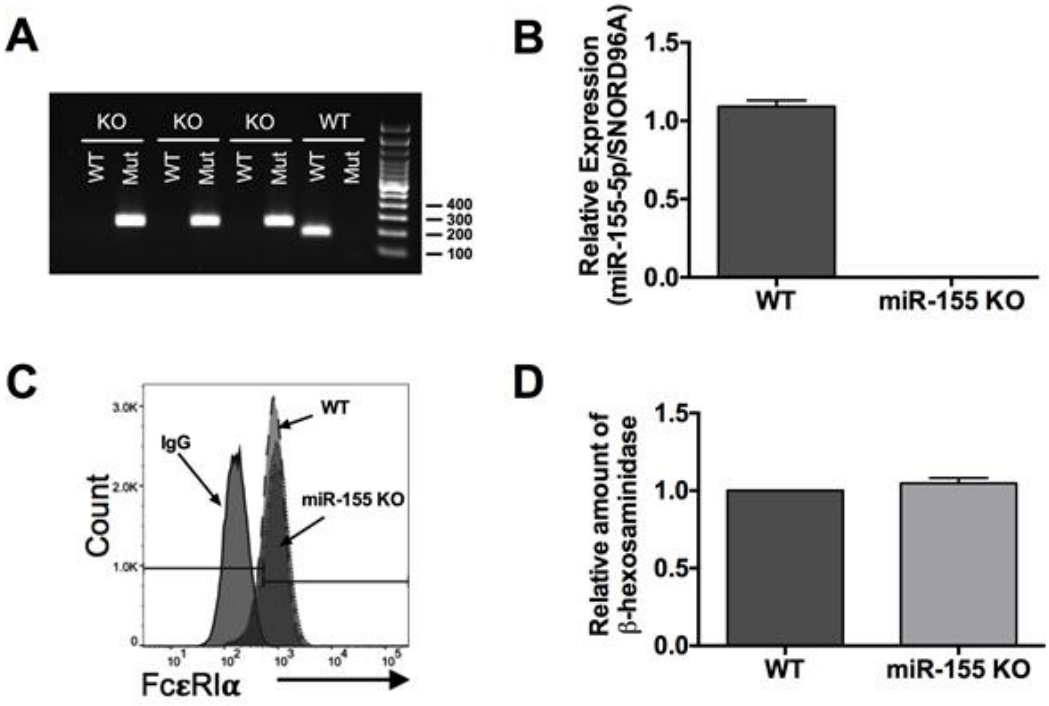

Figure 3.3. The effect of miR-155 on development of mast cells (A) miR-155 genotyping of BMMCs from WT and KO BMMCs was determined by PCR. The expected band sizes were 165bp for miR 155+/+ and 226bp for miR-155-/-. (B) qRT-PCR was compared to miR-155 expression in WT and miR-155 KO BMMCs (C) Expression of FcεRI surface expression in WT, and miR-155 KO BMMCs was measured by FACS. (D) The total contents of β- hexosaminidase in the WT and miR-155 KO BMMCs.

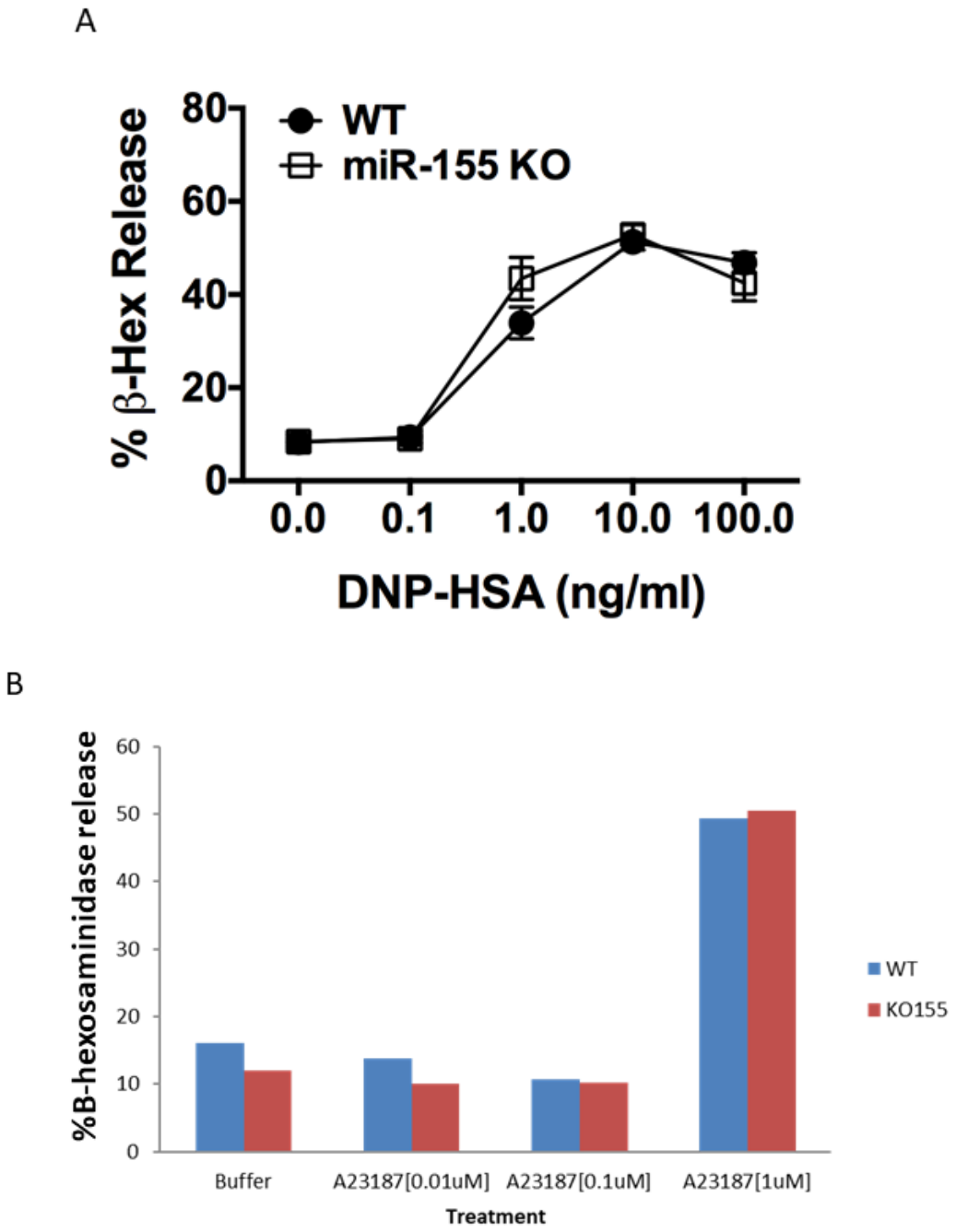

Figure 3.4 The effect of miR-155 on IgE and non IgE-induced degranulation in BMMCs. (A) BMMCs from WT, miR-155 KO were sensitized with anti-DNP IgE and then challenged with DNP-HSA (1-100 ng/ml) for 30 minutes. Degranulation was determined by β-Hexosaminidase assay. (B) BMMC from WT, and miR-155 KO BMMCs were pre-treated with different concentrations of calcium ionophore A23187 for 30 minutes. Degranulation was determined by β-hexosaminidase assay .

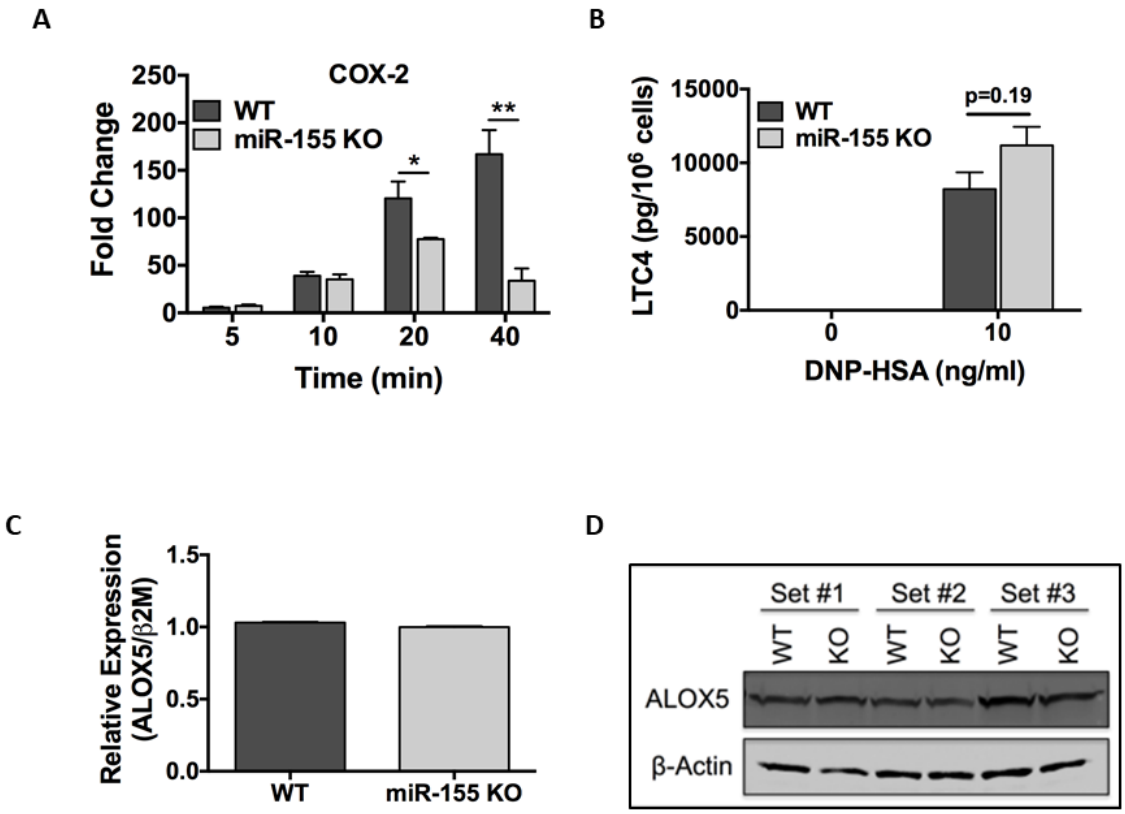

Figure 3.5.The effect of miR-155 on eicosanoids production in BMMCs. (A) Time-course of COX-2 expression sensitized BMMCs were activated with DNP-HSA (10 ng/ml) for 5, 10, 2, and 40 min, and COX-2 expression was determined by qRT-PCR. * ($p< 0.05$), ** ($p< 0.01$) (B). LTC4 concentrations in WT and miR-155 KO were sensitized with anti-DNP IgE and then challenged with DNP-HSA (10ng/ml) for 20 minutes. LTC4 was measured by enzyme immunoassay. (C) Relative expression of ALOX5 expression by qRT–PCR. (D) Protein level of ALOX5 by Western Blot.

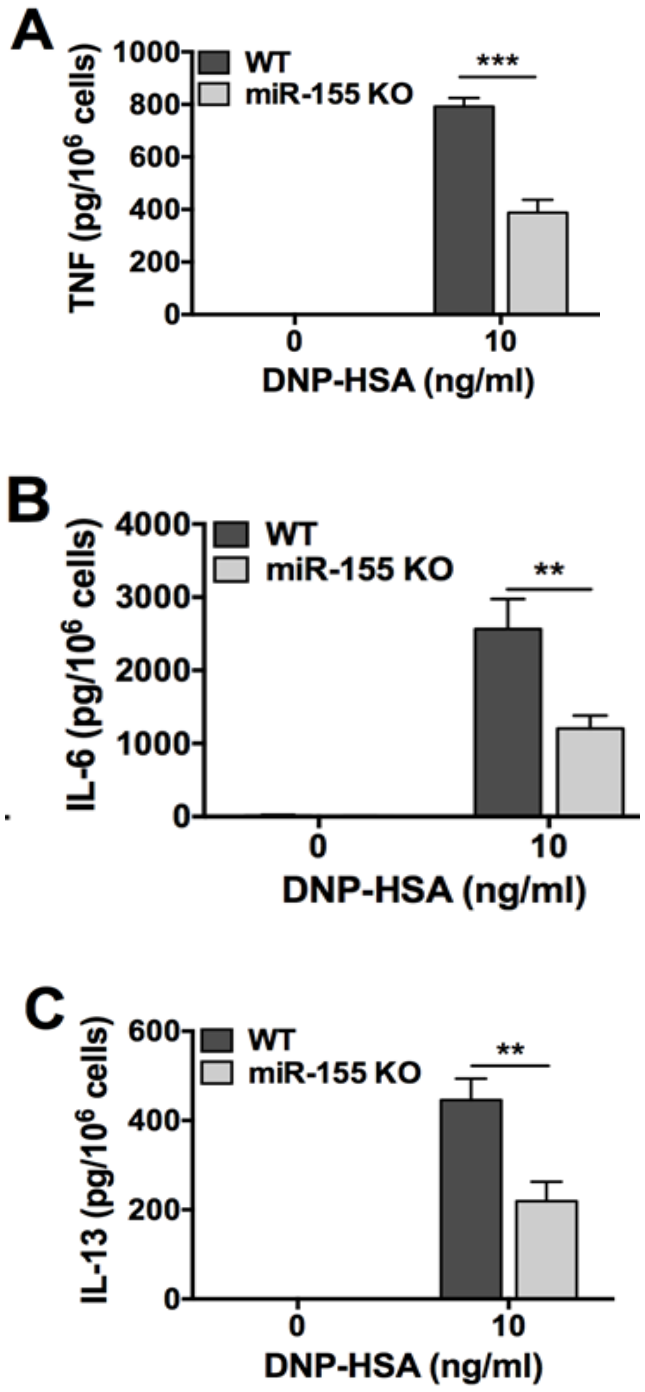

Figure 3.6 The effect of miR-155 on FcεRI-induced cytokines production from WT and miR-155 KO BMMCs. WT and miR-155 KO BMMCs were sensitized with anti-DNP IgE and then challenged with DNP-HSA (10 ng/ml) for 24 hours. Cytokines in the cell-free supernatants were measured with ELISA **, $p<0.01$; ***, $p<0.001$ by Student's t-test (n=3).

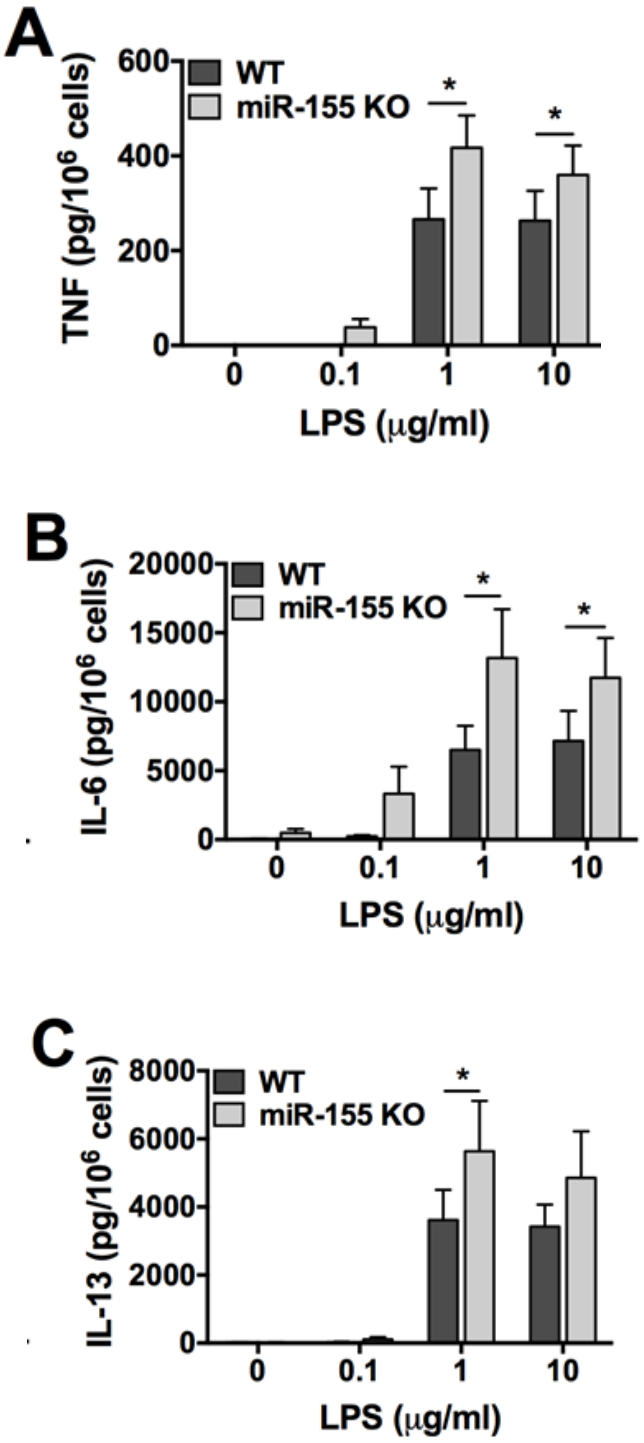

Figure 3.7 The effect of miR-155 on LPS-induced cytokine production in BMMCs . WT and miR-155 KO BMMCs were stimulated with LPS (0.10μg/ml) for 24 hours. Cytokines in cell-free supernatant were measured with ELISA. . *, $p<0.05$ by Student's t-test (n=3).

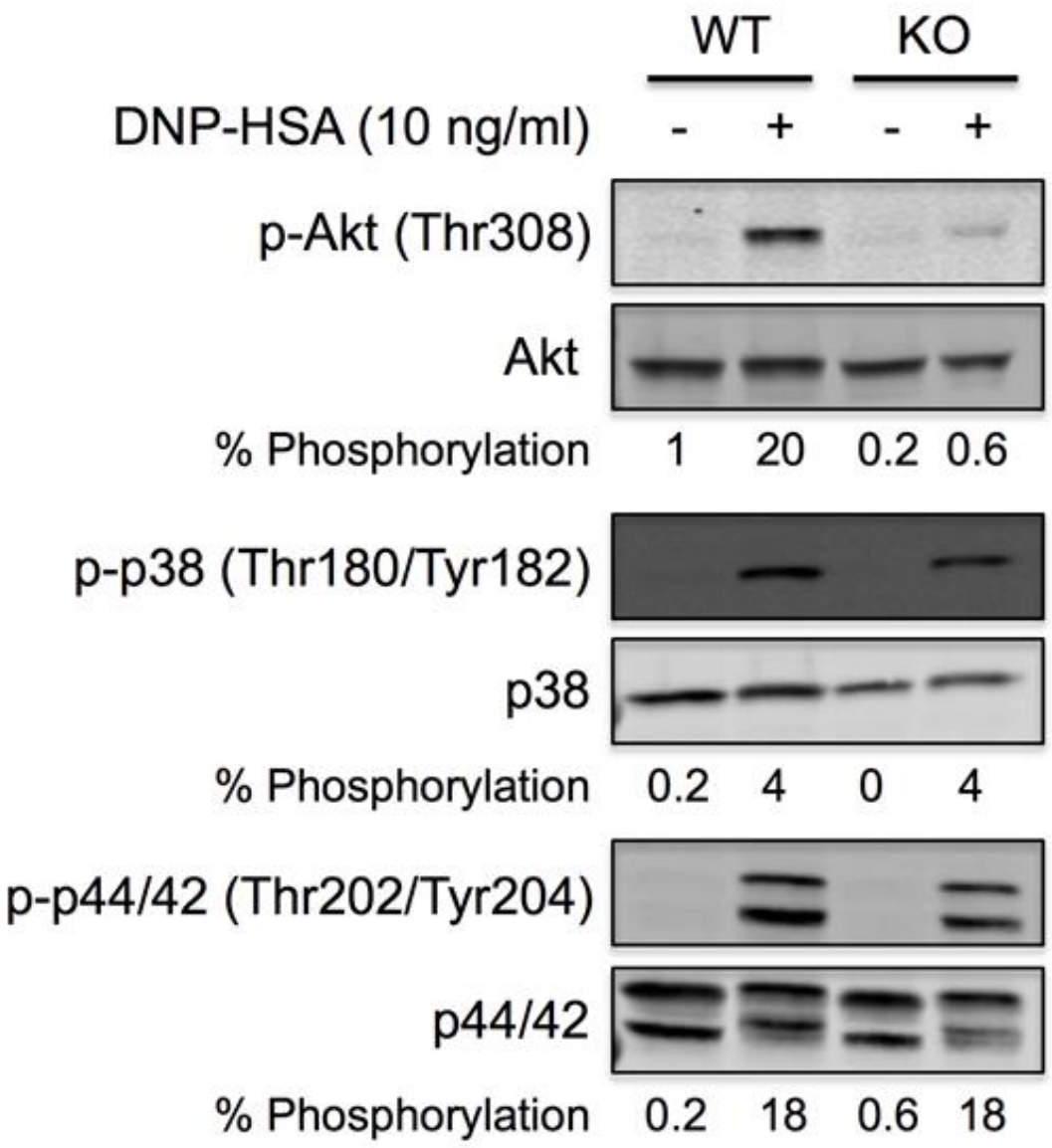

Figure 3.8 The effect of miR-155 on FcεRI-induced phosphorylation of Akt, p42/44 and p38 in BMMCs. Phosphorylation of Akt, p38, and p 42/ p44 l was determined by quantitative infrared Western blotting of whole cell lysates of sensitized mast cells activated for 5 minutes with 10 ng/ml DNP-HSA. Fold induction of phosphorylation was determined from fluorescent signal values obtained with an infrared imager. The blots shown are representative of 3 independent experiments with mast cells from WT, and miR-155 KO BMMCs.

CHAPTER 4

RESVERATROL INHIBITS FCƐRI-INDUCED COX-2 EXPRESSION OF MAST CELLS VIA SUPRESSION OF MIR-155

4.1 Background

Resveratrol (3,5,4`trihydroxy stilbene) is a natural polyphenolic compound found in various plants species such as grapes, berries, peanuts, and some medicinal herbs[200]. Resveratrol, which has a stilbene structure, exists into two isoforms: trans isomers which is the active and abundant form, and the cis isomers[199]. Resveratrol has been shown to have many biological and pharmacological activities, including anti-oxidant, anti-cancer, anti-allergic, and anti-inflammatory effects[206]. The anti-allergic effect of Resveratrol has been demonstrated in acute mouse models of allergic airway inflammation and in murine mouse models of asthma[207]. Resveratrol attenuates allergic asthma by reducing Th2 inflammatory cytokines such as IL-4, and IL-5. Furthermore, oral administration of Resveratrol inhibits bronchial hyper-reactivity, lung eosinophilia, and mucus secretion. Resveratrol has also been shown to inhibit the release of allergic mediators from murine BMMCs[254]. Additionally, the anti-inflammatory effects of resveratrol have also been attributed to the inhibition of COX-2 expression and NFkB[255]. The inhibitory effect of Resveratrol on COX-2 expression has also been demonstrated in SMCs following activation[256]. However the mechanism by how Resveratrol inhibits FcεRI-induced COX-2 expression has not been identified. Resveratrol has the ability to modulate the activity and expression of miRNAs that play important roles in the regulation of target gene expression. They are involved in

the regulation of different cellular processes such as proliferation, apoptosis, metabolism, and are linked with different pathologies[161]. The mechanism of Resveratrol-mediated inhibition of FcεRI-induced COX-2 expression at the miRNA levels is not fully understood. In this study, microarray analysis identified that Resveratrol inhibits the FcεRI-induced miR-155 expression in human skin mast cells. We demonstrated that FcεRI-induced COX-2 expression was inhibited in WT BMMCs and further failed to inhibit COX-2 in miR-155 KO BMMCs following Resveratrol treatment. Our results showed that Resveratrol inhibits FcεRI-induced COX-2 and miR-155 expression in mast cells, leading to the increase of ATF3 expression as a possible mechanism to therapeutically target and modulate COX-2-PGD_2 biosynthesis.

4.2 Materials and methods

The experiments described in this part were designed to identify the mechanism of regulation for Resveratrol to inhibit COX-2 expression through modulation miR155 expression in mast cells, and to assess the target or the repressor. This study was performed on in-situ-matured human skin mast cells and bone marrow- derived mast cells (BMMCs) from WT, KO 155 BMMCs.

4.2.1 Isolation, purification, and culture of human skin mast cells and BMMCs

Human skin mast cells and BMMCs were isolated, purified and cultured in their corresponding media as described in chapter 2.2 and 3.2

4.2.2 IgE sensitization, and activation in human skin mast cells and BMMCs

Mast cells (10^6cells/ml) were sensitized with anti-NP IgE (human) or anti-DNP IgE

(mouse) (1 µg/ml) overnight. Mast cells were pre-treated with Resveratrol (Sigma-Aldrich, St. Louis, MO) or DMSO (vehicle) for 1 hour at 37°C, and then activated with 100 ng/ml NP-BSA or 10 ng/ml DNP-HSA for the indicated amount of time.

4.2.3 Microarray Analysis

MiRNA array analysis on human skin mast cells was performed. IgE- sensitized human skin mast cells with anti-NP IgE were pre-treated with Resveratrol (10µM) for 1 hour, and then activated for 3 hours with NP-BSA. RNA was isolated with miRNeasy kit, and miRNAs profile was performed.

4.2.4 Gene Expression Analysis

Gene expression was determined by quantitative real-time PCR. IgE-sensitized hSMCs or BMMCs were pre-treated with Resvertrol (10 µM) for 1hour, and then activated or not with NP-BSA or DNP-HSA for 3 hours, and RNA was extracted with miRNeasy kits. For miR-155 analysis, cDNA was synthesized with miScript II RT with HiFlex buffer, and PCR was carried out with miScript SYBR Green and miScript Primer Assays for human and mouse miR-155-5p and SNORD96A as the control gene. PCR was carried out with 2ng of cDNA per reaction in a hot start protocol: (95°C × 15 min, (94°C × 15 sec, 55°C × 30 sec, 70°C × 30 sec) x 35 cycles). All miRNA kits were purchased from Qiagen and used according to the manufacturer's instructions. For COX-2, cDNA was synthesized with the iScript cDNA Synthesis kit, and PCR was performed using iQ SYBR® Green Supermix (Bio-Rad). PCR was carried out with 200ng of cDNA per reaction in a hot-start protocol: (95°C × 5 min, (95°C × 30 sec, 55°C × 30 sec, 72°C × 30 sec) x 35 cycles, 95°C × 1 min, 55°C × 1 min). Validated oligonucleotide primers (Sigma-Aldrich) were used:

COX-2 (F:5'-ACTGCTCAACACCGGAATTT-3', R: 5'-CAAGGGAGTCGGGCAATCAT-3'), ALOX5 (F: 5'-CAGGAAGGGAACATTTTCATC-3', R: 5'-AGGAAGATTGGGTTACTCTC-3'), and β2 microglobulin (B2M) (F: 5'-TGGGTTTCATCCATCCGACA-3', R: 5'-CTGCTTACATGTCTCGATCCC-3'). Analysis was performed on a CFX Connect Real Time PCR Detection System (Bio-Rad). Fold change in expression was determined by the $2^{\Delta\Delta Ct}$ method.

4.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0c for Mac OS X, GraphPad Software (La Jolla California USA), and the software is available at www.graphpad.com.

4.3 Results

4.3.1 Resveratrol down-Regulation FcεRI-induced miR-155 expression in human primary skin mast cells

MiR-155 plays an essential role in the regulation of immune response[161]. We had previously shown that Resveratrol inhibited FcεRI-induced COX-2 expression in human skin mast cells, which is responsible for prostaglandin biosynthesis[256]. Thus, we determined the effect of Resveratrol on FcεRI-induced miRNAs expression in human primary skin mast cells by performing a miRNAs array analysis. To do so, human skin mast cells were sensitized with anti-NP IgE, washed to remove unbound IgE, pre-treated with Resveratrol (10μM) for 1hour, and then activated, or not, with NP-BSA (100 ng/ml) for 3 h. Total RNA was extracted with miRNeasy kit, and miRNA expression profiling was

performed. As seen in (Figure 4.1A), Resveratrol altered miRNA expression profile following cross-linking FcεRI. We found that Resveratrol downregulated FcεRI-induced miR-155 expression. The Ingenuity Pathway Analysis (IPA) indicated that Activating Transcription Factor 3 (ATF3) was increased with Resveratrol treatment, suggesting that it could be the link between miR-155 and COX-2 expression (Figure 4.1B).

4.3.2 A positive correlation between miR-155 and COX-2 expression

Real-time qRT-PCR analysis in human skin mast cells validated the miRNA array analysis and IPA and confirmed that activation mast cells through cross-linking FcεRI induces the production of COX-2 and miR-155 expression, whereas treatment with low concentration of Resveratrol inhibits FcεRI-induced COX-2 and miR-155 expression in human skin mast cells with increasing ATF3, indicating that ATF3 could be the intermediate COX-2 repressor that connects miR-155 to COX-2 expression (Figure 4.2 A and B).

4.3.3 Resveratrol inhibits FcεRI- induced COX-2 expression and fails to further inhibit COX-2 in BBMCs

To further corroborate that Resveratrol has the same effect on FcεRI-induced COX-2 expression in BMMCs. WT and miR-155KO BMMCs were sensitized with anti-DNP IgE, pretreated with Resveratrol (10 µM) for 1 hour, and then activated for 3 hours with DNP-HSA (10 ng/ml). RNA was isolated with RNeasy kit, and qRT-PCR was performed. As shown in (Figure 4.3), Resveratrol significantly inhibited the FcεRI-induced COX-2 expression in WT BMMCs ($p<0.01$). Moreover, Resveratrol fails further to inhibit FcεRI-induced COX-2 expression in miR-155 KO BMMCs.

4.4 Discussion

This study demonstrates the anti-inflammatory effects of Resveratrol on mast cells function by suppressing the expression of pro-inflammatory markers like COX-2 expression, which plays an important role in the production of PGD_2. Moreover, we confirmed the effect of Resveratrol on mast cells function by down-regulation of miR-155 and up-regulation ATF3. Resveratrol is a non-flavonoid polyphenol that is reported to possess different biological and pharmacological properties such as anti-inflammatory, anti-cancer, anti-infective, protective activity concerning cardiovascular system, and anti-allergic effects via multiple molecular mechanisms[206]. Among these pharmacological properties of Resveratrol is its inhibitory effect in asthma and other allergic diseases. The anti-allergic activity of Resveratrol has been associated with the suppression of the hallmark manifestation of an allergic reaction, which is the attenuation of the allergenic re-exposure by suppression of the adhesion and migration of peripheral B-cells, the inhibition of IgE, and the IgG1 and Th2 cytokine production (IL,4, IL-5 and IL-13) in sensitized mice[257]. Resveratrol was reported to modify the miRNA profiles expression that play an important role in the regulation of the gene expression attributed to allergic disease[258]. Their role has been recognized in various allergic diseases such as asthma, eosinophilic esophagitis, allergic rhinitis, and others in different murine models, making them a potential therapeutic and diagnostic candidate through modulating the expression[174]. MiR-155 has been reported to regulate mast cells function and anaphylaxis in mice[242]. In the present study, we observed that Resveratrol inhibited FcεRI-induced COX-2 expression in WT BBMCs. This finding is consistent with a previous study conducted in human skin mast cells[256]. Moreover, we found that Resveratrol fails further

to inhibit FcεRI-induced COX-2 expression in miR-155 KO BMMCs, indicating that miR-155 has similar anti-inflammatory effect of Resveratrol on FcεRI-induced COX-2 expression. Then, we investigated the mechanism by how Resveratrol inhibited FcεRI-induced COX-2 expression in mast cells and identified the potential role of miRNA in mediating this inhibition. The miRNA array and IPA analysis was performed in human skin mast cells to measure the miRNA associated with COX-2 inhibition. The results showed that Resveratrol significantly downregulated FcεRI-induced miR-155-5p in human skin mast cells, and upregulated ATF3 expression. Furthermore, we found the positive correlation between miR-155-5p and Cox-2 expression in human mast cells, following FcεRI crosslinking, was downregulated with Resveratrol treatment. Because miRNAs can function as inhibitors of target genes, these results indicate that miR-155 targets a negative regulator of COX-2, rather than COX-2 directly. Previous studies have indicated that miR-155 was shown to directly repress the inositol phosphatase SHIP-1 and Suppressor of Cytokine Signaling-1 (SOCS-1), both of which play an important role in the regulation of COX-2 expression[259]. ATF3 is a member of the ATF/CREB protein family of leucine Zip transcription factors. ATF3 was known to repress COX-2 expression in macrophages during the development of the inflammatory response[260]. In conclusion, this study shows that Resveratrol inhibits FcεRI-induced COX-2 expression in mast cells and miR-155 mediated this response to Resveratrol in mast cells. Collectively, Resveratrol inhibited FcεRI- induced miR-155 and COX-2 expression with upregulation of ATF3, providing a novel mechanism to ameliorate allergic inflammation by affecting the mast cell release mediators.

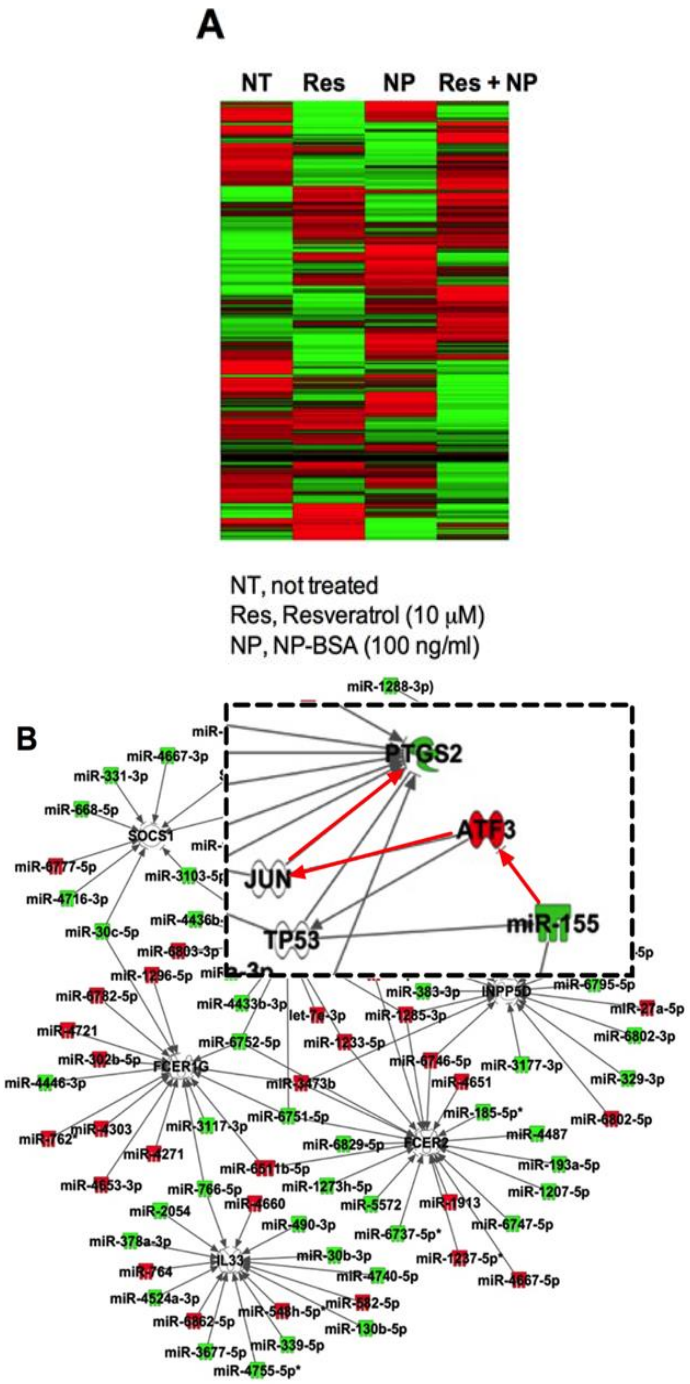

Figure 4.1 The effect of Resveratrol on FcεRI-induced miRNA expression in human skin mast cells. Anti-NP IgE sensitized human skin mast cells were pre-treated or not with Resveratrol (10 μM) for 1 hour and then activated by crosslinking FcεRI with NP-BSA (100 ng/ml) for 3 hours. (A) Heat map. (B) Ingenuity Pathway Analysis (IPA®) comparing FcεRI-activated (NP) and Resveratrol + NP samples (NP + Resveratrol).

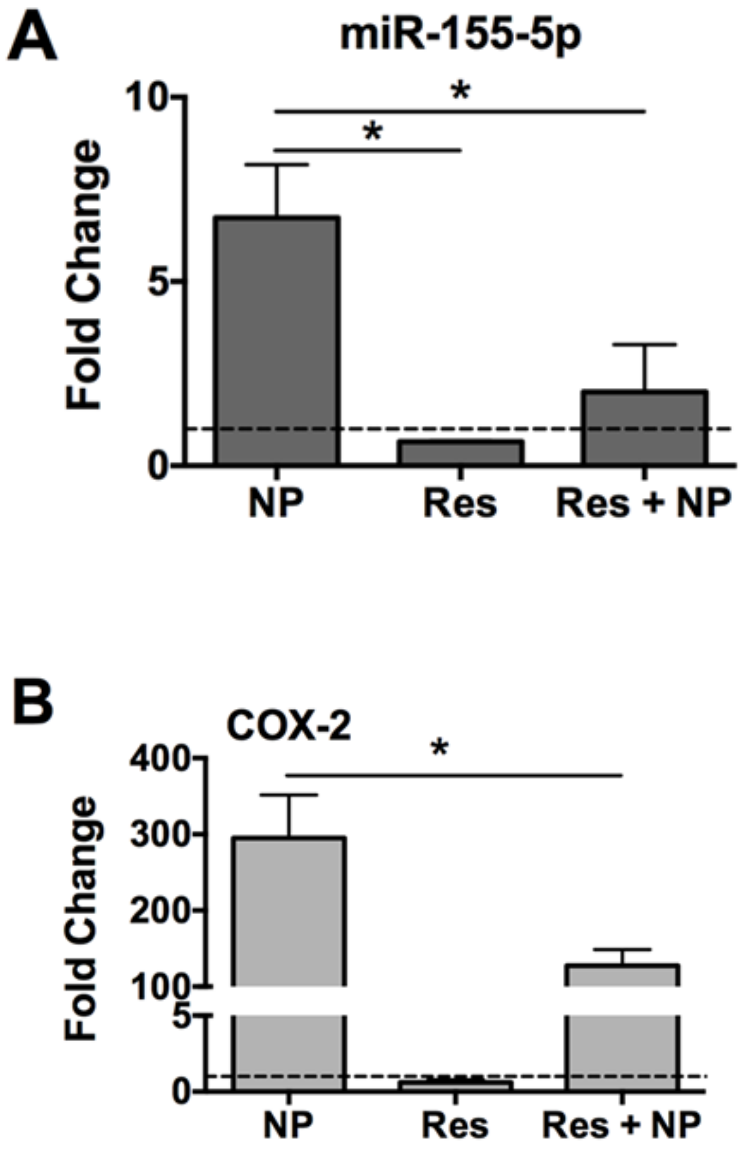

Figure 4.2 Positive correlation between miR-155 and COX-2 expression in human skin mast cells. qRT-PCR to validate the miRNA array analysis and Ingenuity Pathway Analysis (IPA). Resveratrol at low concentrations inhibits FcεRI-induced expression of miR-155 (A) COX-2 (B). The data demonstrate a positive correlation between miR-155 and COX-2 expression. Thus, indicating that miR-155 targets a repressor of COX-2. *, $p<0.05$ by Student's t-test. n=3 different donors.

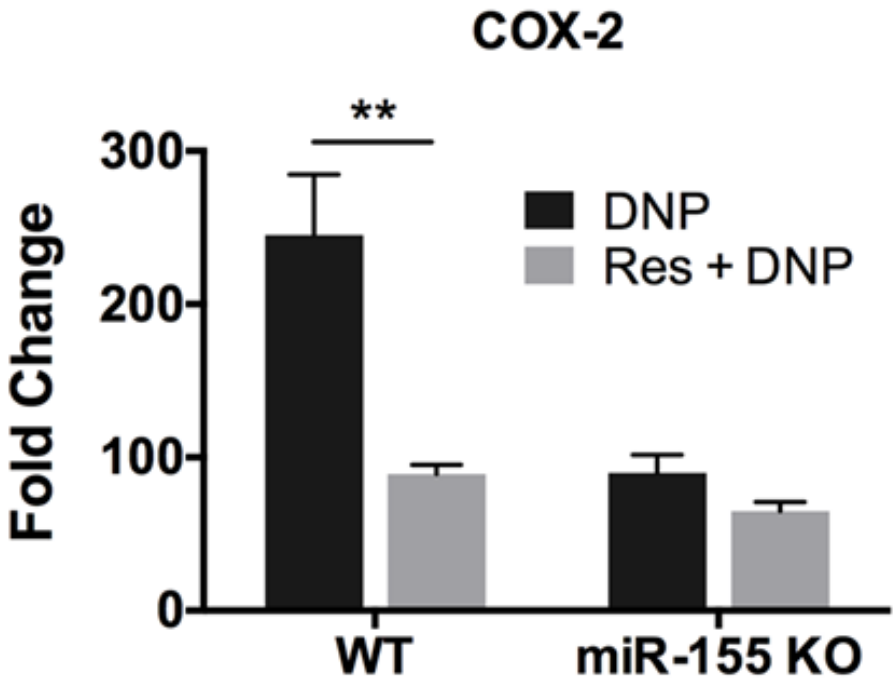

Figure 4.3 The effect of Resveratrol on FcεRI-induced COX-2 expression in BMMCs. qRT-PCR of WT and miR-155 KO BMMCs that were pre-treated or not with Resveratrol (10 μM) for 1 hour and activated with DNP-HSA (100 ng/ml) for 3 hours. **, $p<0.01$ by Student's t-test.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTION

Mast cells play a critical role in allergic disease through the release of various bioactive mediators. Today, the inhibition of mediators release from inflammatory cells like mast cells is considered an important therapeutic strategy to treat various inflammatory disorders, including allergic diseases. The data described here provides new knowledge to help us understand the factors and molecules that are responsible for the regulation of the mast cells response. Additionally, this study identifies their role in the allergic inflammation and finds potential therapeutic targets.

Adenosine receptors have been reported to play an important role in allergic asthma by inducing bronchoconstriction and modulating mast cells response. In this study, we tested the efficiency of A_{2A} receptors agonist in regulating mast cell mediators released from human skin mast cells. Several novel findings are revealed from our study. We report that adenosine inhibits FcεRI-induced TNF production from SMCs. Activation of A_{2A} receptor has been sufficient to mediate the inhibition of TNF production in SMCs and this inhibition is linked to the elevation of cAMP production. In contrast, A_{2A} receptors have no effect on degranulation or PGD2. These data demonstrate that A_{2A} signals have no effect on the early phase reaction, but they impact the late phase reaction. We also show differential expression of adenosine receptors subtypes in human SMCs. We report that A_{2A} receptors are the predominant subtype following crosslinking FcεRI, whereas

expression of the A_3 receptors is low in the activated mast cells. We also noted that A_{2B} receptors were not affected by crosslinking FcεRI.

These data suggest that FcεRI signal exhibits immunomodulatory effect in part by modifying adenosine receptors on mast cells, which could be one way to regulate allergic reaction. The upregulation of the A_{2A} receptors lead to the production of more cAMP levels in these cells following exposure to adenosine, identifying SMCs as a good model to act as effector cells in the limitation of inflammatory response. Moreover, we found that upregulation A_{2A} receptor suppresses further TNF production, indicating the important role of A_{2A} receptors in regulating mast cells in tissue under homeostasis and pathological conditions. MiRNAs, negative regulator molecules, can control the coordinate expression of multiple genes and proteins that drive cellular function[251].

Although many studies have indicated a role of miR155 in allergic diseases, there are only limited functional data of the role of miR-155 on mast cells function. In the current study, we characterized the role of miR-155 on the regulation of mast cells mediators. We demonstrated that miR-155 expression is increased in human and mouse mast cells, indicating a regulatory role in these cells. We compared IgE-dependent degranulation and secretion of eicosanoids and cytokines from wild type and miR-155 KO BMMCs. We found that IgE-dependent degranulation was not affected by the absence of miR-155. Moreover, the amount of β-hexosaminidase was the same in both types of mast cells. Leukotriene C4 secretion also was not affected. Accordingly, ALOX5 expression was similar in WT and miR-155 KO BMMCs. However, FcεRI-induced expression of COX-2, which is directly involved in prostaglandin biosynthesis, was severely impaired in miR-155 KO BMMCs, indicating a positive role for miR-155 in PGD_2 biosynthesis. In the term

of cytokines, IgE-induced TNF, IL-6, and IL-13 were significantly decreased in miR-155 KO BMMCs compared to WT. In support of this notion, we found that FcεRI-induced phosphorylation of Akt was severely impaired in miR-155 KO BMMCs. Interestingly, miR-155 KO BMMCs produced significantly more TNF, IL-6, and IL-13 in response to LPS treatment compared to WT BMMCs. These findings elucidated the role of miR-155 in regulating mast cells response. Overall, these data demonstrated that miR-155 is involved in the regulation of allergic diseases in the context of mast cells.

Future experiments will be needed to determine if overexpression of miR-155 could enhance the proinflammatory effect of miR-155 on COX-2 and cytokines production in response to crosslinking FcεRI. To do so, human LAD2 mast cells or mouse mast cells will be transfected with synthetic has-miR-155-5p mimic or mmu-miR-5p mimic. After transfection, the cells will be sensitized with anti-NP (human), or anti-DNP-BSA (mouse) overnight at 37C, and then challenged with NP-BSA or DNP-BSA (100ng/ml). Changes of COX-2 expression will be monitored by Western Blot and qRT-PCR, and the levels of cytokines will be determined with ELISA. Future work will also be needed to validate other miRNAs that were detectable in mast cells following crosslinking FcεRI to study their function in mast cells by using qRT-PCR. This validation will help us to assess whether other miRNAs may provide a potential mechanism in regulating mast cells.

Resveratrol is a natural polyphenol that exerts many biological and pharmacological activities such as anti-inflammation, anti-cancer, and anti-allergic properties. Resveratrol modulates the expression and activation miRNAs[261]. In this present study, we assessed the mechanism of how Resveratrol inhibits FcεRI-induced COX-2 expression in MCs. This inhibitory effect of Resveratrol is orchestrated by downregulation

miR-155 and the increased ATF3. We also found that Resveratrol fails to inhibit FcεRI-induced COX-2 expression in miR-155 KO BMMCs, indicating that miR-155 and Resveratrol has similar effect on FcεRI-induced COX-2 expression. This study provides a novel mechanism on how Resveratrol inhibits COX-2 expression by attenuating miR-155, therefore showing how Resveratrol can be used as a therapeutic approach in the treatment of allergic diseases.

Future experiments will be needed to validate the expression of ATF3 by qRT-PCR. In these future experiments, we will sensitize BMMCs with anti-DNP-IgE overnight, pre-treated with Resveratrol for 1 hour and then activated with DNP-BSA. ATF3 expression will be measured by qRT-PCR and Western Blot. We will also determine the effect of Resveratrol on FcεRI-induced COX-2 expression in mast cell's overexpressed miR-155.

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