EXPLORING HETEROGENEITY IN MAST CELL'S MEDIATOR RESPONSE TO DIFFERENT ANTIGENS AND PATHOGENIC CHALLENGES

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ABBREVATIONS

µg	Microgram
µl	Microliter
µM	Micromolar
APC	Antigen Presenting Cells
BCG	Bacillus Calmette Guerin
BMMC	Bone Marrow Derived Mast Cells
BSA	Bovine Serum Albumin
CFU	Colony Forming Unit
DMSO	Dimethyl Sulphoxide
DNP-BSA	Dinitrophenyl-Bovine Serum Albumin
EDTA	Ethylenediamine Tetra Acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FcεRI	Fcε Receptor I
FACS	Fluorescence Activated Cell Sorter
FBS	Fetal Bovine Serum
FITC	Fluorescein-isothiocynate
FSC	Forward Scatter
hr	hour
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic-acid
HRP	Horse Radish Peroxidase
HSC	Hematopoietic Stem Cells
Ig	Immunoglobulin
IgE	Immunoglobulin E
IP	Immuno Precipitation
IFN	Interferon
IL	Interleukin
kDa	Kilodalton
MCs	Mast Cells
MCETS	Mast Cell Extracellular Traps
MCP	Mast-cell Progenitors
mg	Milligram
mV	Millivolt
MHC	Major Histocompatibility Complex

mins	Minutes
MMC	Mucosal Mast Cell
PE	Phycoerythrin
PBS	Phosphate Buffered Saline
RBL-2H3	Rat Basophil Leukemia cell line
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
SEM	Standard Errors of the Means
SSC	Side Scatter
VEGF	Vascular Endothelial Growth Factor
XL	Cross-linked

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ABSTRACT

Mast cells (MCs) are densely granulated tissue dwelling cells that are widely distributed throughout the body, especially in the periphery where there is direct contact between host and external environment including incoming antigens and pathogens. Activation of MCs leads to secretion of pro-inflammatory and immune- regulatory mediators by the process of exocytosis. Furthermore, MCs are able to regranulate secretory granules after one set of exocytosis. In this study, secretory response of MCs to multiple allergen challenges, and cytokines and chemokine expression by MCs to different treatments were analyzed. RBL-2H3 mast cell line was stimulated by allergen for multiple times and its response on mediator released was analyzed by β-hexosaminidase assay. The difference in β-hexosaminidase release was significant during both challenges; secondary and tertiary when compared to primary challenge. Mast cells regranulate and degranulate again in different challenges at least up to two times and can be challenged with antigen again and again. Further, mast cells were treated with different triggers and the secretion of various pro-inflammatory cytokines (IL-4, IL-5, IL-6, IL-13, TNF-α) and chemokine (MIP-α) were studied at mRNA level by using semi- quantitative RT-PCR. At first, PCR was standardized in terms of annealing temperature, number of thermocycle and amount of template for these molecules. These standardized conditions further exploited to study their expression levels in RBL-2H3 and to determine their modulation under various antigenic or pathogenic conditions. Finding suggests that MCs can be differentially activated by various triggers to release cytokines and chemokine. By understanding the nature of stimuli and specific MCs response to it, MCs can selectively be modulated during inflammatory or infectious disease conditions. Further elucidation of the molecular mechanisms involved in mast cell activation and exocytosis by different stimuli may provide new therapeutic avenues for treating allergic disorders.

Key words: Mast cells (MCs), cytokines, exocytosis, allergen, β -hexosaminidase assay, RT-PCR

Chapter 1
Introduction

1.1 Background

Mast cells (MCs) are specialized tissue based secretory cells of hematopoietic origin that have a pivotal function in innate as well as adaptive defense to pathogens and in various inflammatory and immunoregulatory responses (Galli et al., 2005). MCs are located throughout the body, especially in the periphery where there is direct contact with external environment, incoming antigens and pathogens i.e. epithelial layer of blood vessel, gastrointestinal tract ,skin and many more (Stone et al., 2010). Mast cells are the first cell to interact with invading pathogens or infection along with other immune cells because of its location in the host. Earlier mast cells were only viewed for their detrimental effect on type I hypersensitivity reaction. Beside of its involvement in hypersensitivity, they are also engaged in damaging effects including atherosclerosis, allergy, rhinitis, contact dermatitis, cancer and many more. Despite its damaging responses it also have numerous beneficial functions against bacterial infection and allograft tolerance to the host (Wernersson et al., 2014). Mast cells are elongated irregularly or ovoid shape cells with the ovoid nucleus. They are up to 20µm in diameter. MCs contain metachromatic electron dense secretory granules which looks like lysosomes and can be stained by cationic dyes. This staining property of mast cells is the main basis for discovery of MCs in the late 1800s by Paul Ehrlich (Crivellato et al., 2003).

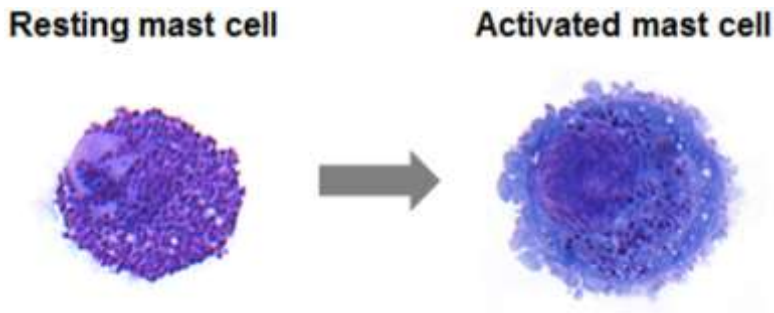


Figure 1.1: Giemsa stain of a resting and activated human intestinal mast cell (Reference; Lorentz et al., 2012)***.***

Based on the staining property of mast cell; mast cells in rodents are divided into two major subtypes; connective tissue mast cell (CTMC) and mucosal mast cell (MMCs). MMCs cells are T-cell dependent cells for their proliferation but CTMCs cells are not depended on T-cell for their proliferation. CTMCs cells are dependent on fibroblast growth factors including stem cell growth factor. MMCs and CTMCs are also different in context of mediator's storage. MMCs have large amount of chondroitin sulfate and less amount of histamine whereas CTMCs contains large amount of heparin and histamine. In human, Mast cells are divided on the basis of presence of enzyme tryptase and chymase. Mast cells are of two types in human. They are MC_{TC} having both enzyme tryptase and chymase and MC_T cells having only tryptase enzyme (Metcalfe et al., 1997; Welle, 1997). Recently new types of mast cell containing tryptase and carboxypeptidases is found (Welle, 1997). The mast cells in human being also differ from

each other on the basis of their location in the body. As growth and development of mast is influenced by different growth factors and cytokines present in surrounding environment, the mast cells present in urinary tract is different to the mast cell present in cardiac, epithelium airway tract and skin. Some are different in context of their morphology while other is different with respect to mediators release and activation by allergen.

Mast cells are originated in similar manner as other immune cell types. Hematopoietic stem cells (HSC) derived from bone marrow have self-renewing capacity. Whenever HSC further developed into multipotent progenitors (MPPs), it loses its self-renewing capacity. Going through hematopoietic lineage, MPPs further differentiate into either common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs). During their development, CMPs further divide into megakaryocyte/erythrocyte progenitors (MEPs) and granulocyte/monocyte progenitors (GMPs). Then further GMPs develop into committed mast cell progenitor (MCp). The committed MCp is then regulated by different transcriptional factors like CCAAT/enhancer binding protein a (C/EBP α), Micropthalmia-associated transcriptional factor (MITF), GATA–binding protein (1, 2, 3), Hairy and enhancer of split-1 (hes-1) transcriptional factors (Dahlin et al., 2015). The committed mast cell progenitor is distributed through blood to different tissue and organ. The McP mature only when they are localized in their own designated tissue. Mature mast cells are different than immature mast cells.

Mast cells number increases in IgE mediated hypersensitivity reaction including rhinitis, arthritis, asthma, connective tissue related disorders, osteoporosis, chronic renal disease, chronic liver disease, neoplastic diseases like lymphoma and leukemia and many more. In parasitic as well as in mastocytosis, mast number increases rapidly. Mast cell expresses a large array of receptors on its surface. Mast cells are KIT (CD117) and FcεRI positive. MCs may express FcγRIIa (CD32a), FcγRIa, β_2-androgenic receptor, adenosine receptor A2B and prostaglandin (PG) E_2. It also express complement protein receptors like $C3_a$ and $C5_a$ receptors, IL (3,4,5,9,10) receptors, Interferon receptors, CCR5, CXCR4, CXCR2, toll-like receptors (TLRs), nerve growth factor receptors and many more depending upon its location (Stone et al., 2010).

Allergen specific IgE increases when the host detects any stimuli. Aggregation of FcεRI receptors by allergen recognized by IgE activates mast cell and this interaction is the main basis for anaphylaxis and other inflammatory diseases. The density of FcεRI receptor on the surface of mast cell increases in the presence of high level of free IgE in serum. Mast cells are also activated by other receptors. After mast cell activation as shown in figure 1.2, it starts releasing mediators. Mediators in mast cell are majorly subdivided into three categories including preformed, newly synthesized and denovo synthesized cytokines, chemokines, and lipid mediators. Mediators are stored in secretory granules. Granules fuse with the plasma membrane and their content are released into the environment by the process of degranulation or exocytosis.

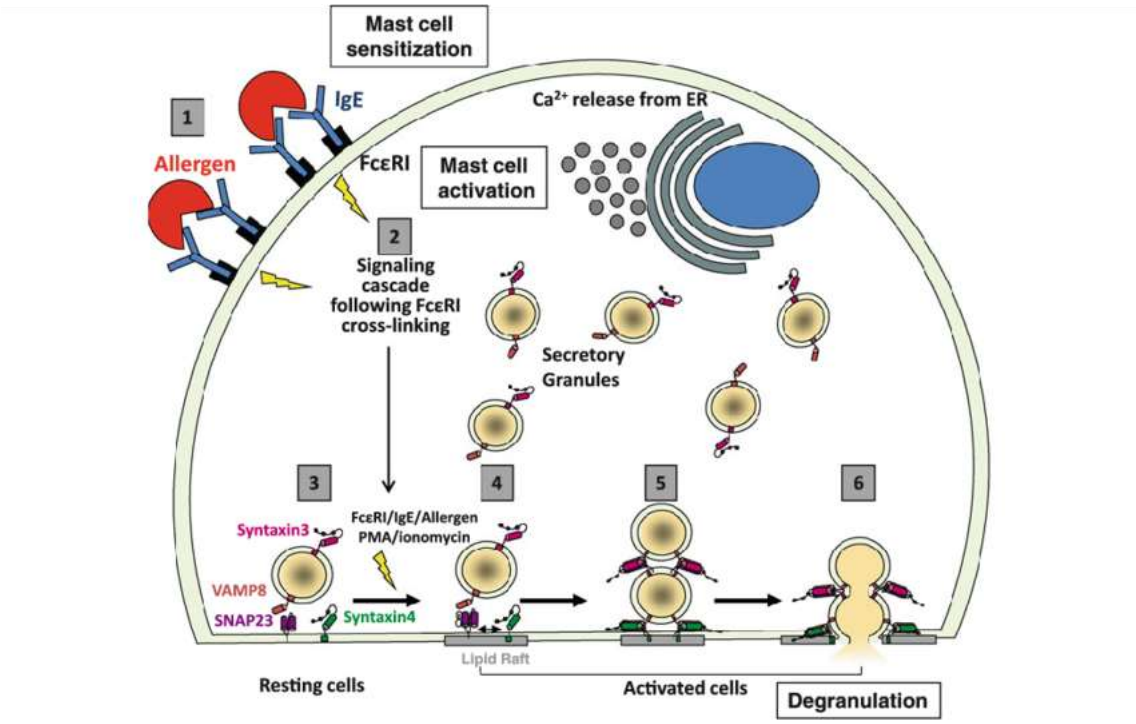


Figure 1.2 Sensitization, Activation and Degranulation of mast cells. *(1) Whenever MCs are exposed to any allergen for the first time, allergen specific IgE is produced. IgE binds to FcεRI on the surface of the mast cells. Repeated exposure to same allergen binds to IgE bounded on the surface of the mast cell. This leads to the cross-linking of FcεRI and activation of mast cells. (2) Crosslinking of FcεRI activates signaling cascade. (3) In resting cells, terminal-SNARE (SNAP 23 and Syntaxin 4) is located in plasma membrane whereas vesicle-SNARE (VAMP-8) and Syntaxin 3 (t-SNARE) is located in the membrane of secretory granules. (4)SNAP-23 is phosphorylated upon activation, followed by recruitment of Syntaxin 4 into lipid rafts and forms t-SNARE complex. (5) VAMP8 on the surface of secretory granule interacts with the t-SNARE complex Syntaxin4/SNAP23 on the plasma membrane to form a stable four-helix bundle. (6) This interaction brings the opposing membranes into close proximity, leading to the fusion of the vesicle and membranes leading towards release of inflammatory mediators* (Reference; Wesolowski et al., 2011).

Preformed mediators including serine proteases such as chymase and tryptase, biogenic amines such as histamine, carboxypeptidases, proteoglycans like heparin and chondroitin sulfates are abundant in granule and are released within minutes of activation (Stone et al., 2010). After activation there will be phosphorylation of mitogen activated protein kinase (MAPK) and increases intracellular calcium level which leads mast cells to produce and release neoformed mediators such as phospholipid metabolites prostaglandin D2, Leukotrienes B4, E2, C4, and Platelet Activating Factor (Boyce, 2007; da Silva et al., 2014) later on after about an hour of mast cell activation, there will be transcriptional activation of many cytokines and chemokines along with lipid mediators including TGF-β, IL-10, IL-4, IL-5, IL-6, IL-1, IL-2, IL-3, IL-12, IL-18, TNF-α, CCL5, CXCL8, MIP- 1α, MIP-1β, and MCP-1 are neosynthesized. Different growth factors like SCF, GM-CSF, β-FGF, NGF, PDGF, TGF-β, VEGF, reactive nitrogen species (Nitric

Oxide) and Complement Factor C3 and C5 (Burd et al., 1989; da Silva et al., 2014; Marshall, 2004).

Mast cells are regarded as critical effector cells in regulation of a variety of physiological functions, including vascular and bronchial homeostasis, wound healing, innate and adaptive immunity, hair follicle cycling, vasodilation, regulation of bone growth, remodeling, mineral homeostasis, angiogenesis, bacterial, and parasite elimination. Since mast cells generate and release biologically active mediators; it regulates the functions of many organs and tissues (Krystel-Whittemore et al., 2016). Traditionally, mast cells are known for its role in allergic inflammatory diseases. However, it has also been implicated other large variety of additional diseases including pathological processes (allergic reactions) atherosclerosis, contact dermatitis, cancer and arthritis (Wernersson et al., 2014). The mechanisms of action of mast cells mediators on their targets may be similar but the outcome of the mediator interaction can differ with respect to location of the cells. For example the wound healing process versus airway remodeling process or bronchoconstriction process in airway versus motility disturbances in intestine.

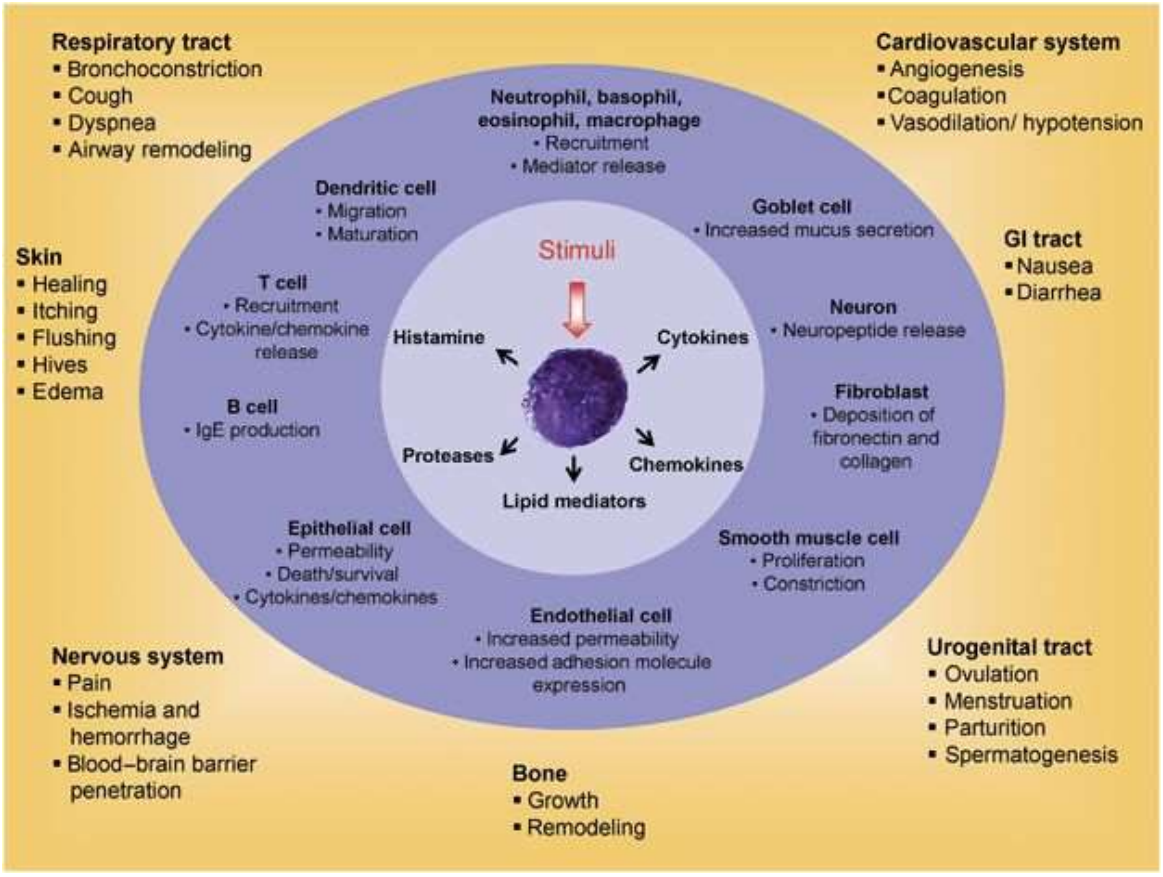


Figure 1.3: Role of MCs in homeostasis and diseases. *Activated MCs release large array of mediators that have role in regulating homeostasis and pathological processes* (Reference; Chen et al., 2010).

Mast cell activation through high affinity for IgE receptor (FcεRI) by polyvalent allergen recognized by IgE leads to the initiation of immediate type hypersensitivity as well as

delayed type hypersensitivity. Immediate reaction (minutes to hour) is mediated by preformed mediators and delayed reaction by cytokines and chemokines within 6-24 hour. Immediate reaction includes erythema, edema, itching, sneezing, rashes, cough, vomiting, diarrhea, cramping in gastrointestinal tract and hypertension whereas late phase reaction is characterized by the leukocytic influx and may persistent asthma (Stone et al., 2010).

1.2 Current studies

Recently, immunological laboratories are focused on the study related to mast cells as mast cells are capable to influences other immune cells directly or indirectly. Though researches are ongoing, detailed about mast cells biology is not still known. So researches are focused on studying mast cell biology meticulously. Expression and functional significance of activation of mast cells by different stimulus and the receptors involved in it are being studying (Zane et al., 2005). Further more detail mechanism involved during exocytosis such as SNARE mechanism, degranulating mechanism are being studied (Balseiro-Gomez et al., 2016.; Lorentz et al., 2012; Naskar et al., 2017). Similarly research related to cytokines strategy, their profiling and their response on treatment with different products is being focused so that novel therapeutics can be invented for treatment of mast cells related disorders (Bagnasco et al., 2016; Woolley et al., 2000). In order to develop new type of therapeutics for mast cell related disorders, different modulation in mast cell biology is being undergoing and being studying (Kubo et al., 2003).

1.3 Research Hypothesis

a) Null hypothesis:

$H_0$1: The amount and type of mediator release from mast cells differ in response to different triggers

H_o2: The amount of mediator released decreases upon subsequent multiple antigenic challenges

b) Alternative hypothesis:

$H_1$1: The amount and type of mediator release from mast cells do not differ in response to different triggers

$H_1$2: The amount of mediator released remains constant or increases upon subsequent multiple antigenic challenges

1.4 Objectives

General Objectives

To explore heterogeneity in mast cell mediator response to different antigens or pathogenic challenges.

Specific Objectives

1. Comparision of the growth kinetics of RBL-2H3 cell in two different media.

2. Study of mast cells mediator release on primary, secondary and tertiary challenges.

3. Analysis of effect of multiple sensitizations on surface expression of mast cell receptor.

4. Designing primers for the mediators that are released by mast cells during degranulation.

5. Heterogeneity study in mast cells response after treating mast cells with different antigens and pathogens/pathogenic products.

1.5 Rational of study

Mediators released by mast cells are highly studied but the differential release of mast cells mediators and mechanisms involved in these secretory pathways are not exactly defined yet. Furthermore, the release of mediators in response to different stimuli is also poorly understood. Immunological study on mast cell is challenging because of several factors like difficulty in isolation and culture as well as in their maintenance. In this study, we use rat basophilic leukemia (RBL-2H3) cell line for studying heterogeneous response of mast cell to different stimuli. RBL-2H3 cell line is an efficient and reliable experimental research tool towards understanding mast cell biology. It is already known that mast cells can recover from antigenic challenge by regranulation of granules over repeated rounds of degranulation (Xiang et al., 2001) but the response of mast cells after regranulation may or may not be the same as the original primary response of mast cells to antigen/allergen or pathogenic challenge is not known. Different findings on antigen and pathogenic challenges led to the hypothesis that the amount and type of mediator release from mast cells may differ in response to different triggers. Study on extent and type of stimuli involved and mediators released will help to improve our understanding of the signaling pathway leading to degranulation and to identify pathways leading to degranulation eventually understanding the regulatory and/or allergic role of mast cells. Mast cells releases mediators and undergoes recovery event simultaneously. There may be heterogeneity in mast cells response to different challenges and also responses may vary in subsequent challenge with different agents. Hence, we set up experiments to explore this heterogeneity in mast cells response to various antigen and allergen or pathogenic challenges. The results will help us to explore how mast cells may regulate host responses through differential mediator release under different challenges.

Chapter 2
Review of literature

Mast cells, developed from hematopoietic lineage are major effector cells of the immune system. They are present throughout the body, including mucosal surface and connective tissue such as the lung, skin and have functions in diverse processes in both maintenances of body physiology and pathophysiological disorders. Mast cells are originated from pluripotent progenitor cells and mature under the influence of transcriptional factors along with growth factors. Because of their location, mast cells act during the first line of defense against invading pathogens and external stimuli (Krystel-Whittemore et al., 2016).

2.1 Functions of Mast cells

Mast cells are multifunctional cells with major effectors in allergic reactions. They have a role in immune modulation as well as physiological functions like Inflammation, homeostasis, tissue repair, angiogenesis, innate immunity, adaptive immunity, immune tolerance, vasodilation, fibrosis, bacterial and parasite elimination. Additionally, they also regulate functions of many cell types such as macrophages, dendritic cells, T cells, B cells, eosinophils, endothelial cells, epithelial cells, and fibroblasts. Mast cells are capable of secreting both pro-inflammatory as well as anti-inflammatory cytokines (da Silva et al., 2014a; Krystel-Whittemore et al., 2016). Pro-inflammatory cytokines including interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) stimulate a number of other immune cells to produce inflammatory mediators whereas anti-inflammatory cytokines control the response of pro-inflammatory cytokines. The balance between these two types of cytokines regulates physiological functions whereas their misbalance leads to inflammation (Frenzel et al., 2013).

2.1.1 Mast cells in allergy

Mast cells are the main players involved in the pathophysiology of allergic disease, mainly in IgE mediated hypersensitivity reaction in the skin, airway, and gastrointestinal tract. Allergic inflammation includes vascular permeabilization, smooth muscle contraction, and induction of mucous secretion (T C Moon et al., 2009). Allergic reactions are multi-specific that occurs immediately or acute phase or even in the late phase. The allergic reaction is characterized by leukocytic infiltration at the site of inflammation, initiation of an acquired immune response, persistent inflammation, tissue remodeling, and fibrosis. When an allergen is encountered for the first time in the host, immune responses of the host stimulate B cells to secrete IgE antibody. Secreted IgE circulates in the blood and binds to an IgE-specific receptor (a kind of Fc receptor called FcεRI) on the surface of mast cells. IgE coated mast cells are sensitized to the allergen (Janeway, 2001). Re-exposure to same antigen/allergen results in cross-linking of adjacent FcεRI-bound IgE and then consequent aggregation of surface FcεRI to causes activation of mast cells. Activated mast cells secrete diverse group of metabolically active mediators, which are either stored or *de novo* synthesized in cytoplasmic granules. The examples include histamine, serotonin, proteases, lipid-derived mediators (PGD2, LTB_4, LTC_4, LTD_4, and LTE4) and cytokines. These molecules are released by mast cells either by the process of degranulation or exocytosis (Rao et al., 2008).

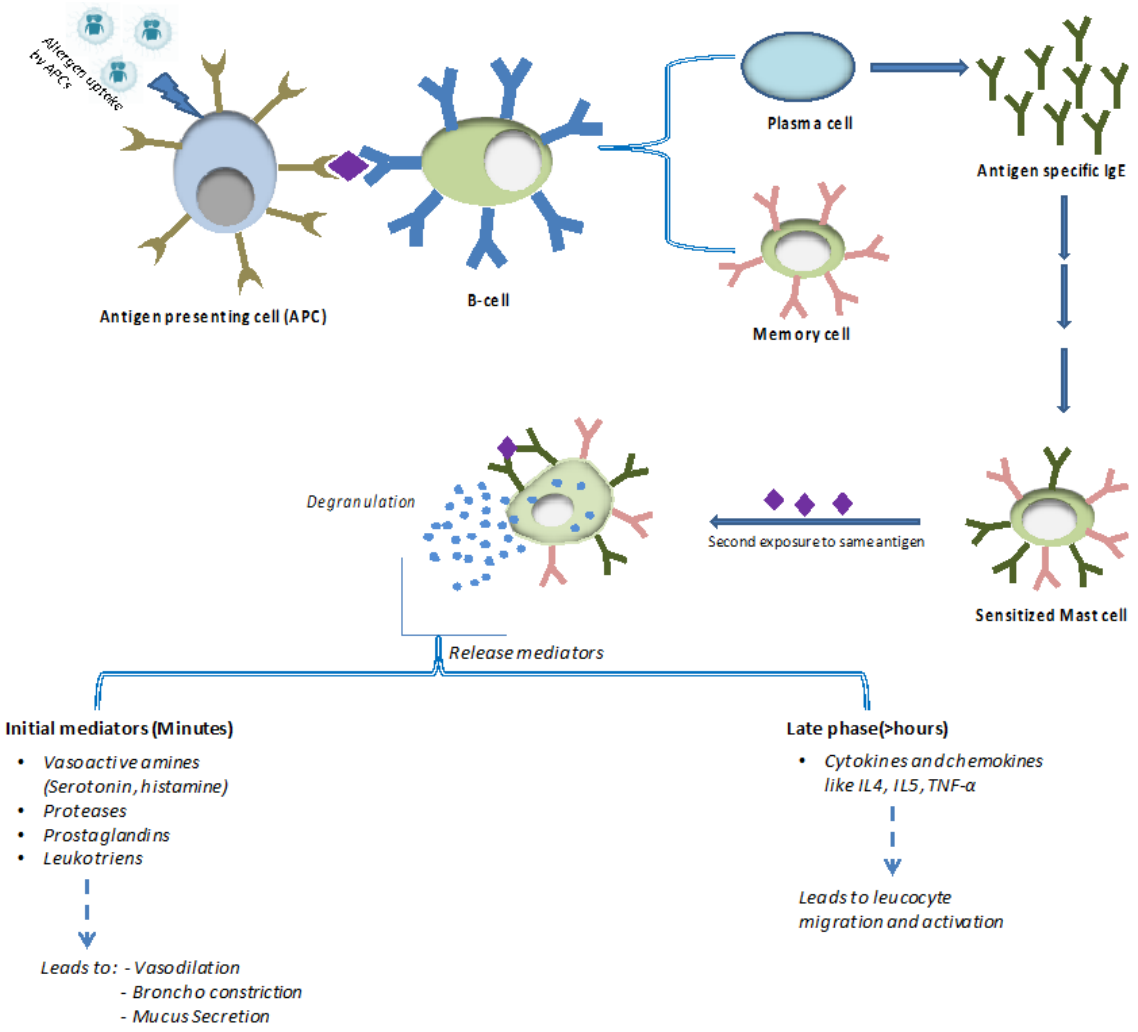


Figure 2.1: Mechanism and pathogenesis involved in Type I Hypersensitivity*. Antigen presenting cell present antigen to B-cells leading to activation of B-cells to form antigen specific IgE- secreting plasma cells and memory B-cells. The secreted IgE molecules bind to IgE- specific Fc receptors on mast cells and blood basophils. Second exposure to same allergen leads to crosslinking of bound IgE, triggering the release of biologically active mediator from mast cells (as well as by basophils). The mediators cause Broncho constriction, vasodilation and leucocyte migration and activation (Adapted from; Decker, 2006)*

Mediators like cytokines and chemokines are produced from new transcripts and are therefore secreted after an hour of initial mast cell activation (da Silva et al., 2014b). The effect of mast cell mediators can be systemic or localized. If this exocytosis response is localized to the airways, then a person might suffer from asthma or any other respiratory problem. The systemic response can result in anaphylaxis, the catastrophic immune response that can rapidly result in death if not treated in time. The inflammation and functional changes associated with early phase responses to antigens typically resolve within a few hours. However, in some individuals late phase reaction develops at the site of antigen challenge, after few hours of antigen exposure (Berkowitz et al., 2014; Grimbaldeston et al., 2006; Obata et al., 2007; Oka et al., 2013).

2.1.2 Mast cells in innate immunity

Mast cells are localized at the junctions where the external environment meets the internal host environment placing them amongst the first cells to interact with invading pathogens or infections. Mast cells recognize harmful antigens by binding to the pathogen directly or with the help of pattern associated molecular pattern (PAMPs) on the surface of the pathogen (Marshall, 2004). PAMPs are recognized by Toll-like receptors (TLRs) and receptors for complement, which are mainly expressed by mast cells. As antigen binds to the receptors, mast cells are activated to release an inflammatory mediator which in turn helps to eliminate pathogens.

TLRs are activated by both Gram-positive bacteria and Gram-negative bacteria as well as to some extent by mycobacteria. Recognition of lipopolysaccharides of Gram-negative bacteria by TLRs triggers mast cell to release proinflammatory cytokines (TNFα, IL-1, IL-6) without degranulation (Marshall, 2004a; Metz et al., 2008). On the other hand, Gram positive bacterial products like peptidoglycan stimulate mast cell degranulation as well as histamine release via Toll-like receptors. Mast cells also have a role in the elimination of bacteria and helminthes by IgE-independent defense mechanism that involves direct (Opsonin Independent) and indirect (Opsonin dependent) mode of clearance. Elimination is aided by inflammatory mediators like IL-25, IL-33 and Thymic stromal lymphopoietin (TSLP) that increase vascular permeability, fluid accumulation and recruit other immune cells such as eosinophils, dendritic cells, NK cells, and neutrophils. Additionally, mast cells produce antibacterial products, such as cathelicidins, defensins, and piscidins. Mast cells also contribute to antiviral responses by recruiting $CD8^{+}$T cells, which produce IFN-α and IFN-β (Kulka et al.,2004.; Zane et al., 2005). Mast cells are very important as it have direct role in early innate immune response to *Leishmania tropica* and *Leishmania donovani.* During Leishmania infection, their interaction is targeted as successful candidate to develop therapeutics for Leishmaniasis (Naqvi et al., 2017). One of the major functions for which mast cells were studied was its role against parasitic infections. When mast cells are activated by crosslinking IgE and allergens; release of mediators from the mast cell increases vascular permeability and smooth muscle contraction, which helps to expel the parasites from the gastrointestinal tract by inducing vomiting or diarrhea or from the respiratory tract by coughing or sneezing (Strauss-Albee et al., 2014).

2.1.3 Mast cells in adaptive immunity

Mast cells are involved in an adaptive immune function via secretion of mediators, which helps in dendritic cells (DCs) maturation, activation, function and recruitment to the tissue or their migration to local draining lymph nodes (McLachlan et al., 2008). Along with dendritic cells, mast cells also process and present antigens via MHCI and MHCII (Stelekati et al., 2009). Mast cells also involve in Th2-dominated immune response by releasing cytokines including IL-5, IL-13, and IL-10 which are especially important in the pathogenesis of inflammation and immune defense against bacteria and parasites. Additionally, mast cells release tumor necrosis factor (TNFα), which can activate cytotoxic T cells directly (Nakae et al., 2006). Mast cell also lead to induction of class-switch recombination in B cells to generate different isotype of antibody (Palm et

al., 2016). Biologically potent mediators that are secreted in response to stimuli during innate immune response influences the function of dendritic cells, T cells and B cells. Thus mast cells are involved in acquired immune responses (Galli et al., 2005).

2.1.4 Mast cells in wound healing

Recent studies have found out that MCs are involved in the pathogenesis of connective tissue disorders including wound healing. They are involved in different stages of wound healing, which is conceptually divided into three phases i.e. inflammation, proliferation, and remodeling (contraction). These processes are tied up together in a sequence of cellular events which is triggered by an initial injury (Oskeritzian, 2012). Tumor growth factor-β1 is the main stimulator of fibroblast and wound contraction. Mast cells upregulate α-smooth muscle actin of fibroblast and are thus involved in wound healing. Vascular endothelial growth factor and transforming growth factor beta both produced by mast cells account for fibroblast proliferation as well as angiogenesis. Tryptase and histamine help in collagen synthesis and its deposition on extracellular matrix (ECM). Tryptase also activates fibroblast matrix metalloproteinase-2 (MMP-2). After collagen deposition in the wound, fibroblasts differentiate into myoblast which resembles contractile smooth muscles cells. For wound contraction both mast cells and fibroblasts are required and are dependent on presence of tryptase and histamine. After wound contraction, oxygen and vital nutrients are supplied to the repairing cells and proliferating tumor cells which have a high metabolic activity by the process of angiogenesis. The number of mast cells is directly related to the degree of angiogenesis. The appearance of neo-vasculature indicates its angiogenesis in injured area (Ng, 2010).

2.2 Mast cells receptors

Large repertoires of receptors are expressed on the surface of mast cells. Among all the receptors some are mast cells activating receptors while others are mast cells inhibiting receptors.

2.2.1 Mast cells activating receptors

There are large numbers of mast cells activating receptors. Chemokine receptors like CXCR3, CX3CL1, CCL1-5, CCL11, CCL13, CCL17-18, CCL22, CCL26-27, and CCL120 are expressed on mast cells which are involved in mast cells activation. Chemokines are small cytokine like proteins that regulate trafficking of leukocytes under normal as well as in inflammatory condition. Receptors especially CXCR3 and CCL5 (RANTES) are important for the migration of mast cell precursors to the target tissue where the mature occurs. Interleukins (ILs) like IL-15, IL-13, IL-17, IL-18 receptors are also expressed on mast cell surface. ILs are special types of glycoproteins that mainly communicate with leukocytes. Thymic stromal lymphopoietin receptors are also expressed by mast cells and initiate allergic inflammation and lead to TH2 responses. Furthermore, Toll-like receptors (TLRs) and various others including sphingosine-1-phosphate (S1P) are expressed on mast cells and provide immunity against bacteria, viruses and other parasites. S1P receptors are crucial for mast cell degranulation and release of chemokine, cytokine and lipid mediators from activated mast cells in both human and rodent. It also provides antiviral immunity. Various amines receptors including histamine and serotonin receptor is also expressed on the surface of mast

cells. Likewise, purinergic receptors are also expressed which mediates the relaxation of gut smooth muscle in response to Adenosine 5'-triphosphate (ATP). Similarly corticotricotrophin releasing hormone receptors (CRHR), Endocannabinoids receptors are also expressed. All these receptors directly or indirectly activate mast cells and lead towards degranulation (Migalovich-sheikhet et al., 2012).

2.2.2 Mast cell inhibitory receptors

Activation of mast cells through ITAMs containing receptors can be regulated negatively by receptors having immunoreceptor tyrosine-based inhibition motifs (ITIMs). Inhibitory receptors are equipped to modulate mast cell functions and examples of such receptors include FcγRIIB, CD300, CD72, sialic acid binding Ig-like lectins (Siglec), mast cell function-associated antigen (MAFA), signal regulatory protein α (SIRPα), leukocyte immunoglobulin-like receptor (LIR) complex and many others (Li & Yao, 2004). These receptors on the surface of mast cells modulate the function of mast cells by inhibiting the downstream signaling from receptors with tyrosine kinases i.e. FcεRI and c-kit. ITIMs motif containing receptors deliver opposite signals, which phosphorylate by Src-family protein kinases and then recruit either SHIP or SHP-1/SHP-2. The recruited phosphatases inhibit the signaling pathways of activating receptor and eventually raise activation thresholds (Migalovich-sheikhet et al., 2012).

2.3 Mast Cells Activation

Mast cells are well known for sensing wide range of unusual signals rapidly and selectively in the host and signal may be immunological or non-immunological. Varieties of receptors are present on cell surface of mast cells enabling them to interact with pathogen or allergen directly or indirectly. Upon activation, mast cells release a plethora of biologically active mediators which alert immune system to increase existing response. Traditionally mast cells activation was categorized as "IgE dependent" and "IgE independent" mechanisms. But recent studies have discovered various criteria to study various ways of mast cell activation as mentioned below (Rao et al., 2008).

2.3.1 Direct Activation of MCs by Pathogens

MCs first recognize pathogen invasion and alert other immune cells near the area of pathogen exposure. They recognize pathogens through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), which are activated in response to conserved pathogen-associated molecular patterns (PAMPs)(Abraham et al., 2015). TLRs form a link between innate and adaptive immunity. Mast cells have different types of TLRs. They are TLR 1, 2, 3, 4, 6, and 9. TLR 2, 4 and 3 directly bind to peptidoglycan of the bacterial cell wall, lipopolysaccharide (LPS) and viral double strand RNA respectively. Mast cell activation through TLR and FcεRI pathway has a synergistic effect on cytokine production, increasing cytokine transcription by enhancing mitogen-activated protein kinases(MAPKs) (Rao et al., 2008). Mast cells also contribute to acute inflammatory events through engagement of TLR2 and TLR4. TLR-3 mediated activation of fetal-skin-derived mouse MCs induces pro-inflammatory cytokine production whereas similar activation of cultured human MCs induces the expression of type 1 interferon. TLR1 and TLR6 are expressed both in human and rodents and are important because they are functional heterodimers with TLR2 enabling responses to many mast cell activators.

TLR7 and TLR8 are important for viral infection as they recognize single-stranded RNA sequence(Marshall, 2004a). CD8 protein mediate mast cell activation by direct interaction with the pathogen, which binds to fimbrial protein (FimH) on gram negative bacteria such as *E. coli, M. tuberculosis,* and *S. aureus* (Abraham et al., 2015). Mast cell recognizes *S. cerevisae* through Dectin-1 receptors (Marshall, 2004a).

2.3.2 Indirect Activation of MCs by Pathogens (FcR mediated activation)

Mast cells possess receptors for Fc portion of IgE and IgG. FcεRI and FcγRI are high-affinity receptors for IgE and IgG respectively, whereas FcγRII and FcγRIII are low-affinity receptors for IgG. Although all of these receptors are expressed in mast cells, their expression level changes based on the type of stimuli. FcεRI-mediated activation is mostly studied in case of allergy. IgE and FcεRI-mediated mast cell activation provides immunity against parasites. Fcγ receptors bind only with antigen that is coupled with IgG. Fcγ regulates mast cell activation in both ways i.e. positively and negatively (Abraham et al., 2015).

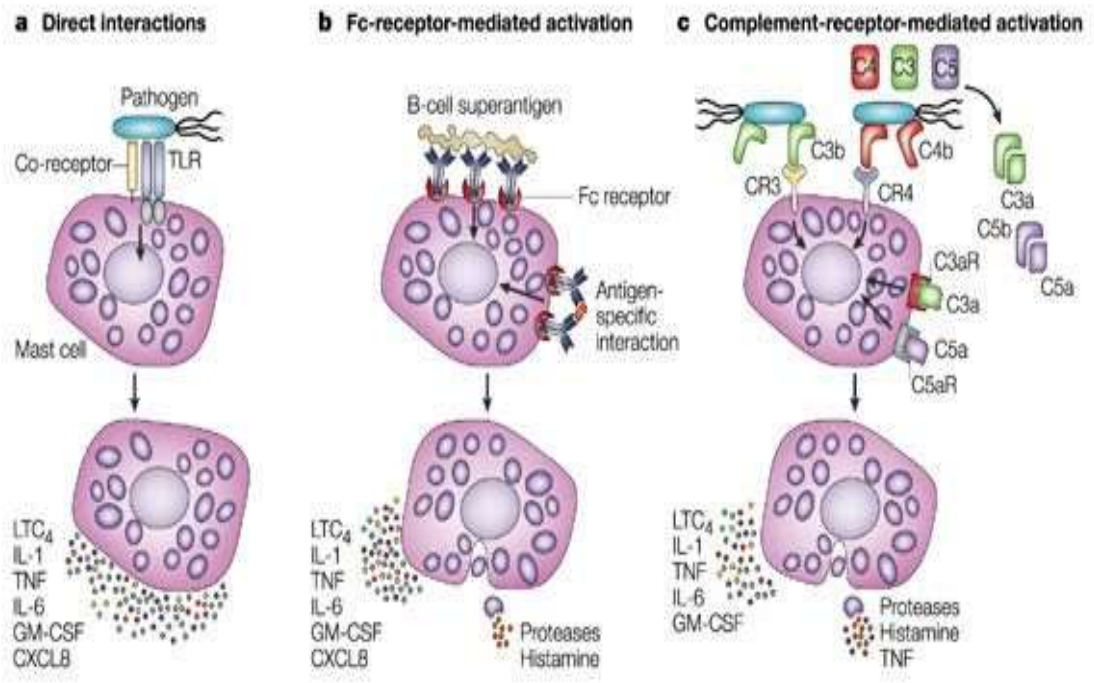


Figure 2.2: Activation of mast cells by pathogens. *Direct and indirect mechanism of activation of mast cells. (a) Direct interactions include Toll-like receptor (TLR)-mediated events (b) Fc-receptor-mediated activation leads to the degranulation of mast cells and the subsequent release of large array of newly synthesized mediators, resulting from either antigen- specific interactions with antibody or the actions of B-cell super-antigens. (c) Complement-receptor-mediated activation of mast cells can occur through various receptors for complement components* (*Reference;* Marshall, 2004b).

2.3.3 Complement Receptor-Mediated Activations of MCs:

Complement pathway is an effector component of innate immunity as it is involved in defense and pathogen clearance. Complement system consists of various serum proteins and cell-surface receptors that functions by opsonizing, chemotaxis, activating leukocyte and lysing the cells. Mast cells have complement receptor (CR) 3, CR4 (CD11c-CD18), and CR5. Expression of these receptors depends on the level of cytokines of mast cell environment. There is heterogeneous expression of these receptors at heterogeneous anatomical locations. Mast cells are activated by complement protein like C3a and C5a (CD88) and are important for host defense against bacterial infection. Integrin α2β1 on mast cells surface also acts as receptor for complement protein C1q and provides defense against peritonitis (Rao et al., 2008). C3 and C4 receptor deficient mice are more susceptible to bacterial infection (Marshall, 2004a).

2.3.4 Activation of MCs by pathogen-associated substances:

Mast cells can also be activated by various pathogen associated substances like wasp venom, snake venom or mosquito saliva after breaching the skin barrier. Mastoparan (14-amino acid peptide) of wasp venom induces mast cell degranulation efficiently (Abraham et al., 2015)

2.4 Mast cells mediators

Mast cells are highly granulated cells, which secrete a diverse array of biologically active mediators. Upon activation a large array of inflammatory mediators are released which in turn have a diverse role in hemostasis, tissue repair, angiogenesis, and innate as well as adaptive immunity (Metcalf et al., 1997). Broadly there are three groups of mediators i.e. preformed mediators (prestored in cytoplasmic granules in normal condition), neoformed mediators (derived from plasma & vesicle membrane lipids) and neosynthesized cytokines and chemokines. The regulation of these mediators depends on the type of triggers and receptor involved in activation by stimuli (Galli et al., 1999). Contents in the granules are released through degranulation or exocytosis and granules are capable to re-granulate. MCs are unique cells as they remain functional after exocytosis and degranulation (da Silva et al., 2014a). Stimuli and receptor involved in activation define the type of mediator release.

2.4.1 Preformed MCs mediators

Mast cells are rich in preformed mediators like amines, hydrolases, proteases, cytokines and chemokines, which are stored in secretory granules. Upon activation of mast cells, the secretory granules of mast cell fused with the plasma membrane followed by release of all the intercellular contents to extracellular environment within minutes. Biogenic amines such as Histamine, Serotonin, Dopamine and Polyamines are prestored. Histamine is the main player in inflammatory reaction and causes vasodilation, smooth muscle contraction, increased capillary permeability, and bronchoconstriction. Proteases like tryptase-βI, βII, βIII, -γ, δ, chymase-1, cathepsin G, granzyme B, carboxypeptidases are stored. These enzymes are in active state and constitute nearly 25% of total protein content in mast cell. These proteases are involved in several pathophysiological states

such as arthritis, allergic airway inflammation, abdominal aortic aneurism formation, tumor angiogenesis and many more. Granules exhibit presence of several lysosomal enzymes including β-hexosaminidase, β-glucuronidase, β-D-galactosidase, Arylsulphatase A, Cathepsins (C, B, L, D and E). Others active enzymes like kinogenases, Heparanase, Angiogenin and active Caspase-3 are also stored. Proteoglycans (Heparin and Chondroitin sulphate), cytokines (TNF-α, IL-4, IL-8,IL-15), Chemokines (CCL5, eotaxin, MCP-1, MCP-3, MCP-4), Growth Factors (TGF-β, bFGF-2, VEGF, NGF, SCF), hormones (Peptides Corticotrophin-Releasing Hormone, Endorphin), Peptide (Endothelin-1, Cathelicidin, Substance P, Vasoactive Intestinal Peptide) and other eosinophil major basic protein are prestored in mast cells (da Silva et al., 2014b; Valent et al., 1990).

2.4.2 Neoformed MCs mediators

Neoformed mediators (eicosanoids) are released from MCs when intracellular calcium level is up regulated and mitogen-activated protein kinase (MAPK) is phosphorylated. Prostaglandin and Leukotrienes are major eicosanoids. Prostaglandins contribute to leukocyte recruitment, increase in vascular permeability, mucus production and activation of nerve cells. Leukotrienes (LT) contribute to defense against bacterial infections. LT-C4 is the most abundant and released via energy dependent export mechanism. LT-B4 is released in less quantity and recruits other immune cells to provide immunity (da Silva et al., 2014a).

2.4.3 Neosynthesized (late phase) MCs mediators

Late phase mediators are synthesized and released after hours of activation. They are synthesized only after transcriptional activation of mast cells. Neosynthesized mediators mostly include cytokines and chemokines. Mast cells are capable to synthesize both proinflammatory as well as anti-inflammatory cytokines. Cytokines and chemokines like TGF-β, interleukins family (IL-1,2,3,4,5,6,12,18), TNF-α, CCL5, CXCL8, Macrophage inflammatory protein(MIP-1α,1β) and Monocyte Chemoattractant Protein-1 are neosynthesized and are released after an hours. Chemokines recruit other immune cells to the site of infection. Different growth factors like SCF, GM-CSF, β-FGF, NGF, PDGF, TGF-β, VEGF, reactive nitrogen species (Nitric Oxide), reactive oxygen species and Complement Factor C3 and C5 are also released after an hour (Burd et al., 1989; da Silva et al., 2014b; Marshall, 2004b).

Table 2.1 : Details of cytokines released by mast cells

S.N.	Name of cytokines	Pre-stored/ Newly synthesized	Other Cells types beyond mast cell	Functions	References
1	IL-4	Prestored in mouse and human mast cell line and newly synthesized in mouse BMMCs and mouse PMCs	Constitutive in Basophils, eosinophil and T-cells.	• Development of Th2 cells & allergic reaction. • Influences mast cell function & differentiation and growth. • Development, proliferation and survival of monocyte, macrophage, B-cells and T-cells.	(Church, 1995; Moller, Henz, Grutzkau, Lippert, & Schwarzt, 1998)
2	IL-5	Prestored in human mast cell and newly synthesized in mouse PMCs.	Macrophage, dendritic cells, NK cells and leukocyte including eosinophils, neutrophils.	• Influence immune responses. • Growth factor & chemo attractant for eosinophils	(Akatsu, 2011; Church, 1995; Mukai, Tsai, & Saito, 2018)
3	IL-6	Prestored in human mast cells, mouse BMMCs, nasal mast cell, mouse in vivo &newly synthesized in mouse cell lines, Rat PBMCs, CBMCs	Macrophages and neutrophils.	• Inflammation, pathogenesis of asthma or allergic response. • Mast cell growth and survival. • Development and proliferation of Neutrophils, eosinophils and basophils.	(Church, 1995; Gordon, Burd, & I, 1990; Moller et al., 1998; Stanley & Lacy, 2018)
4	IL-13	Newly synthesized in human CBMCs, PBMCs &	T-cells, Basophils, eosinophils and epithelial cells.	• Type 2 immune responses & function overlap with IL-4. • Host defense against parasitic	(Mukai et al., 2018)

		mouse BMCMCs		infection.	
5	TNF-α	Pre-stored in human skin mast cell, rodent mast cells & newly synthesized in BMCMCs, PMCs, human lung mast cell, Rat PMCs.	Macrophages, NK cells, Neutrophils.	• Leukocyte and neutrophil recruitment at the site of inflammation. • Migration of dendritic cells to draining lymph nodes. • Sentinels during such host responses. • Gastric inflammation and bacterial immunity.	(Gordon et al., 1990; Stanley & Lacy, 2018)
6	MIP-1α (CCL3)	Newly synthesized in Mouse cell line, BMCMCs, CBMCs.	Macrophages	• Mast cell migration towards macrophages, monocytes, eosinophils and basophils.	(Moller et al., 1998)

2.5 Exocytosis

An exocytosis is a form of active transport. Molecules like protein or transmitters are expelled out of the cell through an energy dependent active transport process. Mast cells are specialized secretory cells, whose secretions have various functions ranging from hemostasis to pathology. Mast cell exocytosis occurs in response to various stimuli, such as IgE receptor crosslinking, complement components, and various other peptides. These stimuli can be from either endogenous or exogenous environmental sources like pollen, venom or any other peptide. The receptor for IgE (FcεRI) is heterotetrameric and is composed of one α-subunit, one β-subunit, and two γ-subunits. The α-subunit of FcεRI has a high affinity for IgE whereas β-and γ-subunits contain immune receptor tyrosine-based activation motifs (ITAMs). IgE receptor aggregates when FcεRI-bound IgE molecules are cross-linked by different stimuli. Aggregation of IgE receptors phosphorylates ITAMs consequently activating tyrosine protein kinases (FYN, LYN, and SYK) which further phosphorylate adaptor proteins including GRB2-associated binding protein 2, phospholipase Cγ (PLCγ) and phosphoinositide 3-kinase (PI3K). This signal then activates different proteins to produce diacylglycerol, inositol-1,4,5-trisphosphate ($InsP_3$) and phosphatidylinositol-3,4,5-trisphosphate which leads to calcium mobilization and the activation of protein kinase C, thereby leading to mast cell degranulation. Exocytosis

is mediated by multiple membrane fusion (granule-granule fusion) or the fusion of cytoplasmic secretory granules with the plasma membrane.

Fusion of plasma membrane is regulated by SNARE (SNAP receptor) proteins. SNAREs are present on vesicle membranes as well as in target membranes. SNAREs on vesicle membrane are called as vesicle SNAREs or v-SNAREs whereas SNAREs on target membrane are called as target SNAREs or t-SNARE. V-SNAREs are small; type 2 integral membrane proteins that make up the vesicle-associated membrane protein (VAMP)/synaptobrevin family. Out of the many v-SNAREs, VAMP 8 and VAMP 7 are predominantly expressed on the granules of mast cells. t-SNAREs can exist as heterodimers consisting of one protein of the syntaxin family (syntaxin 3, syntaxin 4) and one from synaptosomal-associated protein 23 (SNAP23) family (Puri et al., 2003). The phosphorylation of SNAP-23 protein regulates the process of dynamic membrane association in exocytosis of mast cells (Naskar et al., 2017).Membrane fusion is aided by SNARE proteins along with active involvement of numerous accessory proteins. Calcium sensor complexin II and GTPases of the RAB family are accessory proteins. RAS-related protein RAB27 is crucial for mast cell degranulation. Synaptotagmins (especially synaptotagmin II) are calcium sensors and promote membrane fusion events. In humans, tetraspanin CD63 is closely associated with degranulation and exocytosis. It is required for IgE-mediated degranulation of mast cell and anaphylaxis (Kraft et al., 2013)

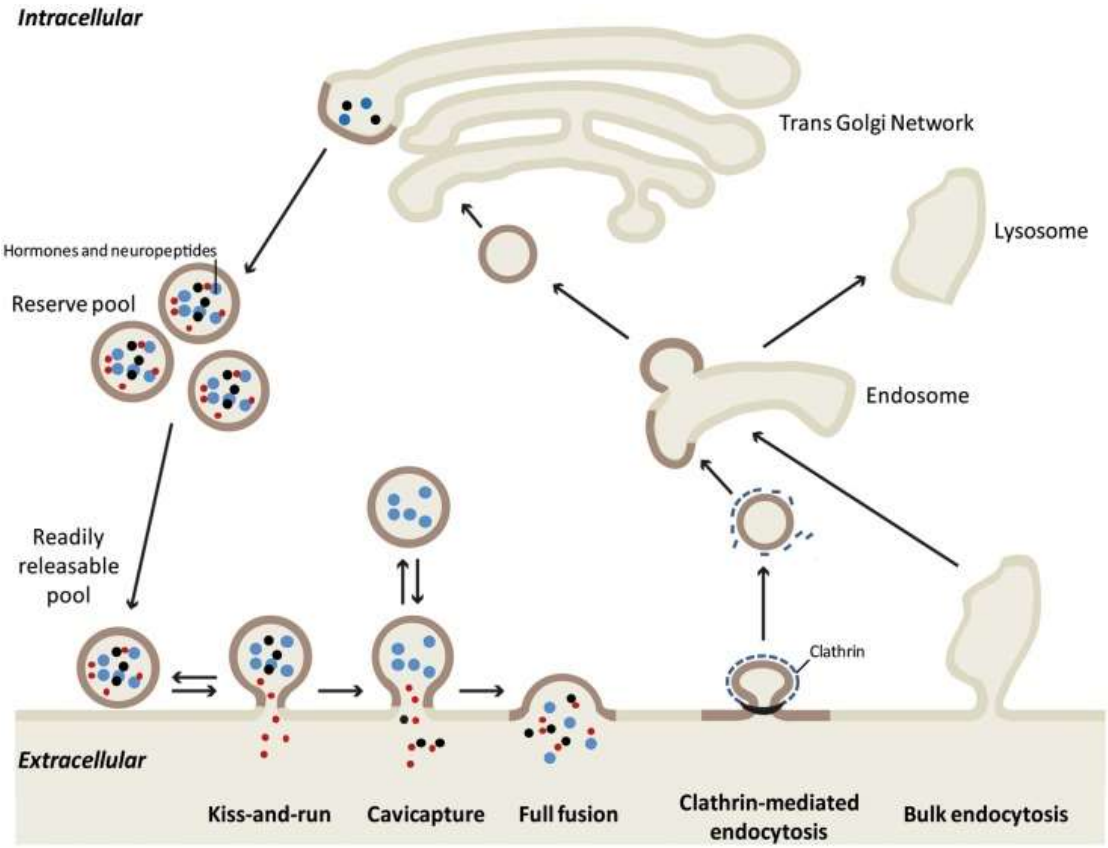


Figure 2.3: Schematic representation of modes of exocytosis (Reference; Tae Chul Moon et al., 2014)

Furthermore NSF, SM (sec1/Munc18) family, Munc 13-14, secretory carrier membrane proteins are also involved in the exocytosis process. Munc family also helps extensively in cytoskeletal reorganization with dissolution of actin-myosin complex and depolymerization of cortical actin. Coronin 1A and coronin 1B regulates this process. At first there is fusion of granules which is followed by fusion of fused granules with plasma membrane to form degranulation channel leading towards degranulation. This type of degranulation is called as compound exocytosis. Generally, the activation of mast cell by strong stimuli leads to compound exocytosis. Compound exocytosis is also called as multi vesicular exocytosis. Along with compound exocytosis there is also another type of exocytosis called as piecemeal exocytosis in which granule compounds are lost or bud off without evidence of any fusion as shown in figure 2.3. Ultra structural analysis has shown that the buds of small cytoplasmic granules move toward plasma membrane, gradually leading to emptying of vesicular content without any observable fusion event. After exocytosis mast cell can regranulate within 72 hours (Blank, 2011; Wernersson et al., 2014).

2.6 Mast cells as unique innate immune cells

Mast cells are the unique innate immune cells as they are capable to activate other cells of the immune system. Mast cells can discriminate self and non-self products by using canonical innate immune pathway. MCs are also capable of sensing danger or unusual signals from the surrounding tissue (epithelial, endothelial) and tissue-resident immune cells. Once they detect signals, they alert host while directly involving in clearance of unusual signals (Galli et al., 2011). Mast cell membranes have a large array of receptors including traditional PRRs and other receptors that directly sense unusual signals. Because of different external stimuli multiple pathways are activated in mast cells which simultaneously release discrete types of mediators. MCs increase inflammatory response and also activate neighboring immune cells. They are unique than other immune cells because they release significant amount of preformed mediators while other innate immune cells only start to change their transcriptional programs. Exocytosed granules can migrate long distances and are involve in an immune response. Abundant presence of mast cells on mucosal surface, epithelial layer and near blood vessels makes them uniquely capable to detect early infection and colonization events. They promote leukocyte recruitment to the site of infection by systemic dispersal of mast cell products (Galli et al., 2011). The actions of mast cells in early infection are beneficial but its excessive action can have a detrimental impact on health during chronic or overwhelming infection (St John et al., 2013). The same characteristics of mast cell that provide innate as well as adaptive immunity can also sometimes lead to severe pathological condition like allergy, Crohn's disease, cardiovascular disease, mastocytosis, autoimmune disease or any other when there is improper regulation of their function (da Silva et al., 2014a; Rao & Brown, 2008).

2.6.1 MCs are unique immune cells in respect to their origin

Bone marrow-derived hematopoietic stem cells are self-renewing progenitor cells. Self-renewing capacity is lost when they further develop into multipotent progenitors (MPPs). These MPPs divide into either common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs). The common myeloid progenitors further divide into

megakaryocyte/erythrocyte progenitors (MEPs) and granulocyte/monocyte progenitors (GMPs) during their development (Kondo et al., 1997). Chen and co-workers found a committed mast cell progenitor (MCp) within common myeloid progenitor population (Chen et al., 2005).

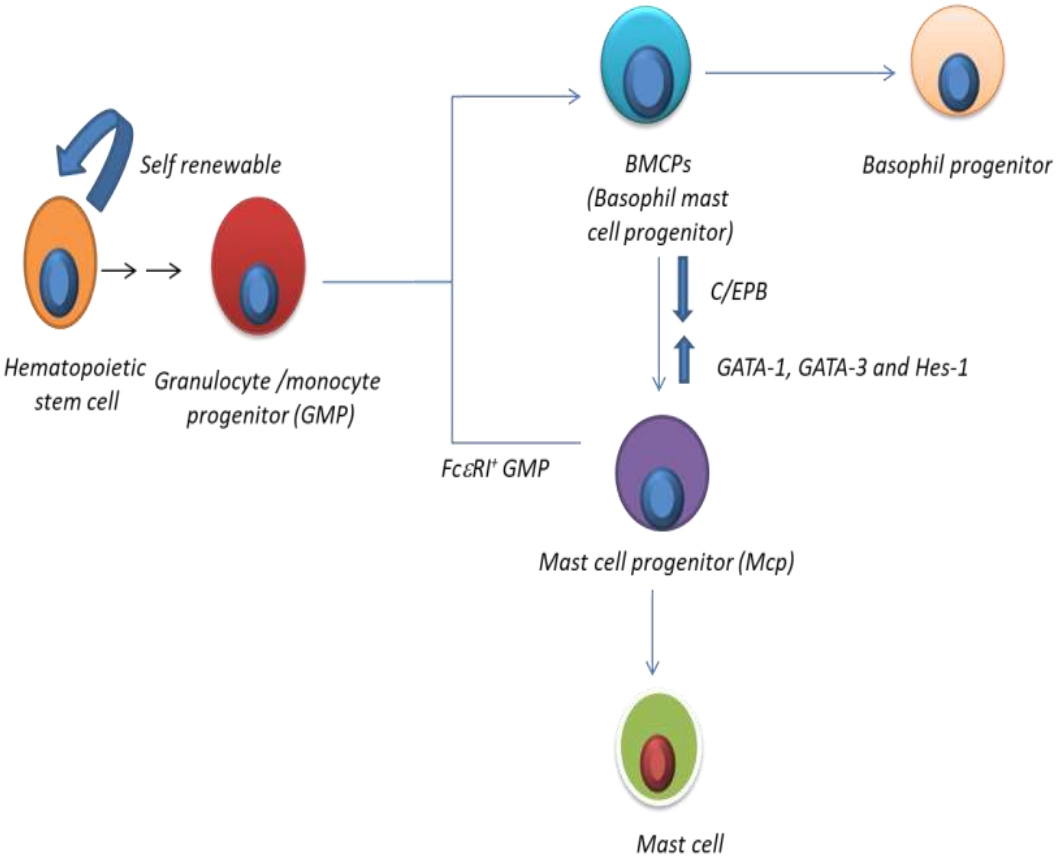


Figure 2.4: Schematic representation of origin of mast cell from hematopoietic stem cell (Adapted from; Dahlin et al., 2015)***.***

Many studies on mast cells suggest that the basophil and the mast cell lineage are closely related. Thus the committed MCp originate from bipotent progenitors with both mast cell and basophil capacity within the GMP population. Complex networks of transcriptional factors are involved during hematopoiesis in the differentiation of GMP progenitors into mast cell. CCAAT/enhancer binding protein α (C/EBPα) mainly control differentiation along with other transcription factors like Micropthalmia-associated transcriptional factor (MITF), GATA –binding protein (1, 2 and 3), Hairy and enhancer of split-1 (hes-1) as shown in figure 2.4. GMPs expressing C/EBPα activate GATA-2 factor to differentiate toward eosinophil progenitors. Committed MITF is up-regulated along with down-regulation of C/EBPα to form mast cell progenitor. Mast cells' progenitors circulate throughout the body and reside in different tissues where they mature. The homing of mast cell to tissue is a regulated process. Mature mast cells in tissues generally express c-kit receptor and high affinity for IgE receptor (FcεRI). Mature mast cells can be identified by histochemical staining but immature mast cells cannot be

identified by any histochemical staining techniques because of the absence of granules (Dahlin et al., 2015).

2.6.2 Life span of mast cells

MCs are ubiquitous components of the connective tissues of vertebrate animals. MCs are especially abundant in the skin of animals and rodent. Mast cells are high in number in the area that is exposed to external environment. In 1969 Baeckeland found mast cells in pieces of mesentery maintained in vitro for up to 3 weeks, but Kiernan in 1974 found mast cell on skin survived only for 4 days. Exact lifespan of mast cells is not still known. Various studies are ongoing to find out the exact lifespan of mast cells. Experiment on cutaneous mast cells on young rats found that mast cells density is higher in the area which is more susceptible to adverse environmental condition and higher the density of mast cells higher will be its lifespan. The half-lives of differentiated mast cell are 4-9 days in the skin of the back. But the half-life of the mast cell in the external ear of the same model was 7-20 days. On the basis of different studies, scientist have suggested that mast cells that are produced in early life have much shorter life spans than mast cells that are produced in the mature state in adult rats (Centre, 1979). Fischer and the group performed experiment on rat mast cell by incorporating thorium into the mast cell granules. The thorium incorporated mast cell decreases to less than 1% by 10 months. Their research concluded that mast cells are long lived and have the capacity to regranulate after degranulation and retain granules (Fischer et al., 1997).

2.7 Heterogeneity in mast cells

MCs demonstrate heterogeneity with respect to cell ultrastructure, receptor expression, mediator content, mediator release, immunologic, non-immunologic activation, pathological responses and even in pharmacologic responsiveness (Welle, 1997). Mast cell development starts from hematopoietic stem cells (HSC) lineage in bone marrow. HSC further proceed towards myeloid lineage through common myeloid progenitor (CMP) and granulocyte/macrophage progenitor (GMP). Mast cell committed mast cell progenitor (MCP) developed from either GMP or directly from HSC. Immature mast cell progenitor circulates in the bloodstream and migrates into targeted organs and tissues where their maturation occurs. Common basophil/mast cell progenitors (BMCP) can develop both basophil and mast cell. The transcriptional factors and various cytokines determine the fate of BMCP.

As the microenvironment of every organ as well as the tissue is different to each other, mast cell types that are found in different organs and locations are also different. The maturation of mast cells is directly influenced by the cytokines and growth factors. The heterogeneity in mast cells is due to the unstable microenvironment surrounding it in physiological as well as pathological condition as shown in figure 2.5. Likewise, the genetic and epigenetic regulation also affects the phenotype of mast cell (T C Moon et al., 2009). Similar to origin and maturation, mast cells also exhibit heterogeneity in their responses to various stimuli (Paudel et al, 2017).

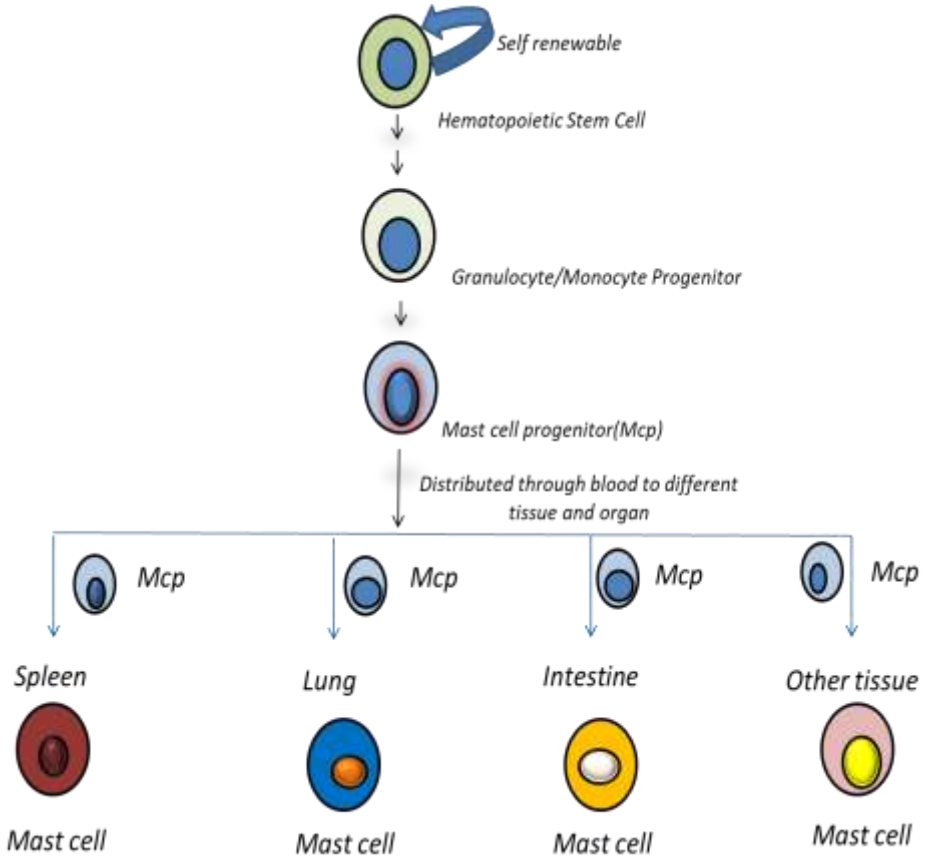


Figure 2.5: Model showing mast cell development and heterogeneity (Adapted from; T C Moon et al., 2009)**.**

2.7.1 Mast cells in rodent

Alcian blue/safranin histochemical staining of mast cells in the rodents help to distinguish subtypes of mast cells. Atypical, T cell-dependent mucosal mast cells (MMCs) and T cell-independent connective tissue mast cells (CTMCs) are two subtypes of MCs present in rodents. MMCs are especially predominant in mucosal surface of the gastrointestinal tract and lamina propia of respiratory tract whereas CTMCs are predominant in submucosal layer of the intestine, peritoneum as well as in the skin. Interleukins like IL-3, IL-4, IL-9, and IL-10 plays a crucial role in the proliferation of MMCs. Likewise, the proliferation of CTMCs is mediated by fibroblast growth factors like Stem cell factor (SCF). According to granularity, MMCs contains chondroitinsulfate and lesser histamine whereas CTMCs contains a large amount of heparin, histamine, and prostaglandin (Welle, 1997).

Table 2.2: Characteristics of rat mucosal mast cells and connective tissue mast cells

Characteristics	Mucosal mast cell(MMCs)	Connective tissue mast cell(CTMCs)	References
Staining with alcain blue/safranin	• Blue	• Red or blue and red	(Welle , 1997)
Appearance	• Small • variable shaped and sized granules	• Large • Uniform shaped and sized granules.	(Welle , 1997)
Behavior	• Migratory	• Non migratory	(Welle , 1997)
Dependent on	• T-cell and fibroblast	• Fibroblast(SCF)	(Welle , 1997)
Lifespan	• Shorter lifespan i.e. half-life less than 40 days	• Longer than MMCs i.e. half-life greater than 6 months	(Welle , 1997)
Histamine content	• Low	• High	(Welle , 1997)
Biogenic amine	• Histamine	• Histamine • Serotonin	(Welle , 1997)
Neutral proteases	• RMCP-2 • MMCP-5 • Carboxypeptidases	• RMCP-1 • Carboxypeptidase	(Welle , 1997)
Proteoglycan	• Chondroitin sulfates di-B, A, E	• Heparin • Chondroitinsulfate E	(Welle , 1997)

2.7.2 Mast cells in humans

Human mast cells are not possible to classify on the basis of staining. Like in rodent mast cells, human mast cells also contain protein components such as neutral proteinase and enzymes that are metabolically active at neutral pH. Mast cell specific proteases, chymase and tryptase are major criteria for differentiation of mast cells in human. Two types of mast cells i.e. mast cells containing only tryptase (MC_T) and mast cells containing both tryptase and chymase (MC_{TC}) are present. MC_{TC} also contain carboxypeptidases. Cathepsins G- like proteases are present in MC_{TC} but absent in MC_T. Serglycin proteoglycans in the human mast cells contain both heparin and chondroitin sulfate in 2:1 ratio. Besides heterogeneity in proteases content, human mast cells are also heterogeneous in the morphology of secretory granules and cytokines profile. Histamine content is slightly higher in MC_{TC}. Proteoglycans like heparin and

chondroitinsulfates A, E are present in both cell types. Secretory granules of MC_{TC} are of grating and lattice substructures.

On the other hand, secretory granules of MC_T are discrete scrolls types. MC_{TC} is dependent on T cells while MC_T is not T-cell dependent. IL-4 is predominant in MC_{TC} cells whereas IL-4, IL-5, IL-6 prevails in MC_T cell types. MC_T is predominantly found in the pulmonary mucosal surface and in mucosa of intestine. MC_{TC} types are found in the skin, lymph nodes, lung and the sub mucosa of gut. Recently in an asthmatic patient suffering with eosinophilic esophagitis, new types of mast cells were found in airway epithelium. This new type of mast cells express tryptase and carboxypeptidase A3, but not chymase (Welle, 1997). Mast cells in human also differ according to their location. The mast cells in uterine region, cardiac region, lungs and skin are somehow different from each other. Some are different in their morphology while some with respect to mediator release or activation by allergen. For example, substance P and morphine can selectively induce skin mast cells to release histamine but cannot induce cardiac and lung mast cells. Likewise, compound 48/80 can induce secretion of histamine in cardiac and skin mast cells but not in lung mast cells (Welle, 1997).MCs are heterogeneous distributed in different species. More interestingly, they are heterogeneous even within organs of same species.

2.8 MCs regranulation after degranulation

Mast cell degranulation is a very tough and complex process as it undergoes extensive morphological changes. Xiang and co-workers found that the mast cells can be morphologically recovered after degranulation. They activated mast cell repeatedly and performed protein release assay and observed cytokine transcript expression after each activation. Their findings concluded that mast cells can be granulated after degranulation (Xiang et al., 2001). When mast cells release mediators, they undergo distinctive changes in their morphology. After this mast cells undergo a series of recovery events to become normal mast cells. The time required for regranulation after degranulation depends on the extent of degranulation. Human lung mast cells that release almost all of their contents undergo pro-aggressive enlargement of Golgi apparatus followed by regeneration of large array of small cytoplasmic granules, small membrane bound vesicles and densely packed membrane bound granules. Eventually, newly formed mature cytoplasmic granules packed with mediators and other cell components are formed. Changes in the nuclear blast and cytoplasmic mass expansion accompany the process of mast cell Regranulation. The recovery pattern also differs on the basis of types of the mast cell. Some mast cells show a combination of channel recovery and remodeling of newly synthesized contents of granule within which content condensation synthesizes new granules (Dvorak et al., 1988). The unique capacity of mast cells to undergo several cycles of degranulation and regranulation for recovery is an important characteristic of these cells in the initiation and perpetuation of an inflammation and allergic reaction (Xiang et al., 2001).

2.9 Mast cell based therapies

Studies on mast cells have shown that the mast cells are the main player in the allergic disease. There are many other mast cell related disorders that are prevalent worldwide.

Nearly 20% (Ye et al., 2017) of population in world is affected by allergic diseases which majorly include atopic dermatitis, rhinitis, asthma, anaphylaxis and food allergy and about 10% of them with severe asthma that cannot be controlled with current therapeutic approaches (Cruse et al., 2016). In biological system, B cells stimulated by APCs produce antigen-specific IgE, which binds to high affinity surface receptors (FcεRI) on surface of mast cells. Cross-linking of antigen and IgE bound to FcεRI is necessary to activate mast cells. Activated mast cells release allergic mediators. These mediators then cause pathophysiological allergic disorders (Cildir et al., 2017). Many researchers are ongoing to find potent therapeutics to control abnormal activation and proliferation of mast cell in several mast cells related diseases like allergic asthma, chronic rhinitis, rhinosinusitis, conjunctivitis, dermatitis, food allergies, nasal polyps, aspirin-exacerbated respiratory disease (AERD) and chronic urticarial (Cildir et al., 2017). In the progression of these diseases, activity of other immune cells are also enhances. Because of this nearly 19.6% of the populations are affected (Cildir et al., 2017). Strategies targeting various intracellular or extracellular mast cell mediators are clinically used in patients. However, all the strategies that are in use are not safe in some circumstances like some show sedative effects; some are not well tolerated by patient. Likewise some treatments are only partially effective, and are not considered safe to use long term in children (Wang et al., 2014). Many researches are currently ongoing with the aim of discovery of potent therapeutic treatment in the mast cell related disorders.

2.9.1 MCs therapies in use

Numbers of compounds are currently in used for treatment of allergic reactions. Omalizumab (humanized monoclonal antibody) is a Food and Drug Administration approved drug that is widely being used to treat asthma and idiopathic urticaria. Omalizumab binds to free IgE in circulation preventing crosslinking of FcεRI receptor and IgE thus inhibiting MC activation. Omalizumab also attenuates the activity of other immune cells that express FcεRI, such as basophils and dendritic cell (Avila, 2007). Corticosteroids like topical budesonide, fluticasone, and oral prednisolone are being used to control symptoms of allergic disorders, arthritis, lupus, psoriasis or breathing disorders. However, some patients are resistant to corticosteroids over long term use during the course of their disease and even have numerous well-known side effects. Similarly, Antihistamines are used to get relief from watery eyes, itchy eyes/nose, runny nose and sneezing. Furthermore, use of anti–IL-5 monoclonal antibody (e.g. Mepolizumab) against severe asthma is very effective (Avila, 2007; Cildir et al., 2017). Mast cell Tyrosine kinase inhibitors (imatinib) and MC Tryptase inhibitors (nafamostat mesylate and tranilast) based therapies are widely accepted for the treatment of cancer (Article, 2016).

2.9.2 MCs based therapies under clinical trials

Many successful findings in mouse models are under clinical testing to ensure their safety and efficacy in humans. Upstream molecules that control the expression of proinflammatory downstream mediators can be targeted so that this down regulation decreases the expression thus decreasing detrimental effects of proinflammatory mediators to the host. DNAzymes are single-stranded oligonucleotides that combine the extraordinary specificity of antisense base-pairing with an inherent RNA-cleaving enzymatic activity. GATA-3 specific DNAzymes (SB010) is effective in clinical trials with

allergic asthma patients. Similarly, DNAzymes can also be used to target upstream regulators that control mast cell related diseases including mastocytosis and mast cell activation disease (Cildir et al., 2017). In chronic inflammation anti–IL-4 and anti–IL-13 monoclonal antibody (Dupilumab) show high efficacy profiles in comparison to treatment by corticosteroids alone (Bagnasco et al., 2016). Furthermore, Phosphoinositide 3-kinase d (PI3Kd) also contributes to mast cell mediator release and phase II studies of a PI3Kd inhibitor (GSK2269557) are currently ongoing in asthma and chronic obstructive pulmonary disease(COPD) (Rommel, 2010).

TLR activated MCs agonist shows anti-tumor effects in melanoma and in tumors. TLR-2/6 agonist MALP-2 in combination with gemcitabine is showing better efficacy and safety in phase I and II trial. Thus TLR activated MCs agonists could be potent therapeutics against tumor and neo-angiogenesis (Article, 2016). There are many sialic acid binding Ig-like lectin (Siglec) receptors in immune cells with different expression patterns. They are also called as inhibitory receptors. In human mast cells and eosinophil, Siglec 8 is specifically expressed. Human mast cells that are pretreated with an antibody activating Siglec 8 inhibit mast cell degranulation. So Siglec 8 could be novel therapeutics. The clinical trials of antibodies that activate Siglec 8 (AK001 and AK002) are ongoing in mast cell disorder patients (Cildir et al., 2017).

2.9.3 Preliminary research involving MCs based therapies

Advances in science have led to the discovery of many therapeutics. Many researches on mast cells biology are giving common direction to prepare a safe therapeutic agent against allergic inflammation. Proinflammatory cytokines have a direct role in allergic inflammation. Targeting upstream molecules that control the activation of proinflammatory downstream mediators could be a successful therapeutic approach. MAPKs, PI3K and kinase Akt are essential regulators of pro-inflammatory cytokines. Polydatin (natural component from Polygonum cuspidatum) modulates allergic inflammation by suppressing the expression of proinflammatory cytokines and reduces the downstream effectors of FcεRI by down regulating MAPKs, PI3K and kinase Akt. Thus, PD could be novel therapeutic approach for allergic disorders by inhibiting degranulation of mast cell and deducing the expression of proinflammatory cytokines (Ye et al., 2017).

Similarly, clinical trials using specific DNAzyme are ongoing to treat allergic asthma patients. So similar approaches of using DNAzyme might be used to treat other mast cell related diseases (Cildir et al., 2017). Further in the field of cancer, MCs targeting agents such as MC tryptase inhibitors (nafamostat mesylate) or c-Kit-R tyrosine kinase inhibitors (imatinib, masitinib) have antiangiogenic as well as antiresorptive strategies so can be used for the treatment of gastric cancer (Article, 2016).

In addition, antibodies that activate Siglec 8 (AK001 and AK002) are under clinical trial to treat polyposis and mastocytosis patients. Other than Siglec 8, Siglec 7 also deduces FcεRI dependent mast cell activation. Antibodies that activate Siglec 7 could also be a reliable and safer therapeutic target to treat mast cell related disorders in future. Use of antisense oligonucleotides mediated exon skipping has been successfully applied to manipulate *MS4A2* splicing, targeting FcεRIβ and down-regulation of FcεRI. However,

even though exon skipping technique is very promising, it is still in early stages of developing therapeutic option. Furthermore, MRGPRX2 receptor can also be another promising target for allergic diseases. MRGPRX2 is novel GPCR known as MAS-related G protein coupled receptor-X2 and is expressed on connective tissue mast cells. This receptor participates in peptide-drug induced pseudo allergic reaction. So MRGPRX2 receptor can be targeted with monoclonal antibodies or inhibitors to alleviate such reactions (Cildir et al., 2017).

Calcium signaling is a crucial for FcεRI-dependent mast cell activation and degranulation. So targeting channels regulating Ca^{2+} signals either directly or indirectly could be another potential therapeutic target. Activation of mast cells by TLR ligands and IgE enhances production of cytokines so targeting of both TLR receptors and FcεRI might be more effective than targeting FcεRI alone. Thymic stromal lymphopoietin (TSLP) is implicated in the pathophysiology of allergic respiratory diseases (Arthur & Bradding, 2016). So anti-TSLP antibody could be another reliable therapeutic that attenuate early as well as late-phase responses to allergen challenge in patients with mild allergic asthma. Cysteinyl leukotriene receptor (CysL) receptor antagonists such as montelukast, prostaglandin D2 inhibitors could be clinically beneficial. Levels of MC proteases are elevated in several allergic inflammations so MC proteases could attractive therapeutic targets for allergic diseases (Arthur et al., 2016).

2.10 Future directions

Mast cells have beneficial as well as detrimental roles in health and disease. Advanced research facilities and considerable progress in mast cell research have found out some new ideas regarding mast cell. These findings reinforced our understanding that mast cells have multiple functions and have great impact on health. Because of availability of genomic tools and animal model, our knowledge about function and plasticity of mast cells is increasing. Although researches are going on to control abnormal activation and proliferation of mast cells, there are several challenges to be addressed while studying these in disease settings. After getting significant result in *in vitro*, experiment will be carried out *in vivo* models especially in mouse models. However, the mast cell of mouse and human are different from each other. Hence, it is imperative to make a humanized mouse model which could be another valuable tool to study human mast cells and their regulation in detail. Furthermore, using genome editing technology (CRISPR-Cas9), single-cell sequencing can be used to identify novel molecular targets in mast cells. Single cell sequencing techniques uncover various tissue resident mast cells and then could be applied to characterize human mast cells properly so that the types of mast cells involved in various pathophysiologies could also be studied. The role of noncoding RNA is still unknown in activation and development of mast cells. Many studies have concluded that noncoding RNA have a role in development and activation of mast cells and also in controlling inflammatory responses. By using CRISPR technique the exact role of noncoding RNA and detailed mechanisms of its involvement in mast cell development and activation can be exploited. The role of noncoding RNA in the interaction of mast cell with other immune cells can also be studied (Cildir et al., 2017).

As we are aware that mast cells generate many mediators including cytokines, chemokines, hormones and other soluble lipids as well as non-lipid mediators. Thus

targeting an upstream regulator rather than a single mediator or cytokines will undoubtedly be a more reliable and attractive target against allergic diseases. Nowadays studies on microbiome have shown that human physiology is directly influenced by micro biome and this has a role in many diseases. Mast cells are tissue based cells that reside in connective and epithelial layer of skin and intestines so the effects of skin and intestinal microbiota on the function of mast cells should be studied (Cildir et al., 2017). Further, additional efforts can be done to define the complex interactions of mast cell with other cells in their environment. This will potentially lead towards the development of novel and safer clinical approaches for controlling allergic inflammation as well as many other mast cell related disorders (Krystel-Whittemore et al., 2016).

Chapter 3
Materials and Methods

3.1 Reagents

RPMI-1640 Medium, Phenol red negative, HEPES and Dinitrophenyl-Bovine Serum Albumin (DNP-BSA) were purchased from Sigma Aldrich (MO, USA). Essential Medium Eagle with Earle's salts, Iscove's Modified Dulbecco's Medium, Dulbecco's Modified Essential Medium, Trypsin, Chloroform, Isopropanol, ethanol, Ethylene Diamine Tetra Acetic Acid (EDTA), Trizol (TRI reagent), Diethyl pyro carbonate (DEPC), Dimethyl sulfooxide (DMSO), *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, agarose and primers were purchased from Sigma Aldrich (India). Fetal Bovine Serum (FBS) were purchased from Gibco, Life Technologies (Grand Island, NY, USA). Anti-mouse CD16/32 Fc block and mouse anti IgE-PE was purchased from BioLegend, USA. Reagents such as RNase inhibitor, Oligo(dt)18, dNTPs mix, Taq polymerase, Taq buffer, M-MuLV reverse transcriptase enzyme and its buffer and Nuclease free water (GeNei) were purchased from NEB (UK). 100bp DNA ladder was purchased from NEB (UK) and GeneDireX (Taiwan). All disposable plastic culture wares were supplied from Costar (NY, USA).

3.2 Maintenance of cell lines

The Rat Basophilic Leukemia (RBL-2H3) mast cell lines were purchased from ATCC, USA. Cells were maintained in RBL complete medium containing equal parts of Minimum Essential Medium Eagle with Earle's salts and Iscove's Modified Dulbecco's Medium supplemented with 25mM HEPES (N-[2-100 hydroxyethyl]piperazine-N0-[2-ethanesulfonic acid]) 50µg/ml gentamicin sulfate, and 20% heat-inactivated Fetal Bovine Serum (FBS). Cells were also maintained in DMEM (Dulbecco's Modified Essential Medium) media supplementing with 25Mm HEPES, 50 µg/ml Gentamicin sulfate, Glutamine and sodium pyruvate and 10% FBS in a humidified atmosphere containing 5% CO2 at 37^{o}C at Forma SERIES II WATER JACKET, Thermo Scientific incubator. This cell line was maintained as adherent and sub cultured by trypsinization. Viability of cells were determined by using trypan blue exclusion method using formula Percent (%) Viability = (Live cells/Total number of cells) X 100.

3.3 Growth Curve of RBL-2H3 cell line

25000 cells per well were plated in 1ml media in 48 well plate and incubated at 37^{o}C incubator supplied with 5% CO_2. Cells were harvested after 12, 24, 36, 48, 60, 72, 84, and 96 hrs. Cells were harvested at each time point and counted under hemocytometer using Nikon ECLIPSE TS 100 light microscope. Harvesting should be done very carefully with repeated flushing so that even single cell will not be left on the plate. Doubling time was calculated using the formula from obtained data.

$$Doubling\ time = \frac{duration * \log 2}{\log(final\ concentration) - \log(initial\ concentration)}$$

3.4 Comparison of growth kinetics of RBL-2H3 cells in different media

Growth curve of RBL-2H3 cells were generated in RBL complete media as well as in Dulbecco's Eagle Modified Medium. Morphology of cells and their growth kinetics was compared between two medium under same conditions.

3.5 Induction for secretion in RBL-2H3 cells by IgE crosslinking (multiple allergen challenge)

$1.5x10^7$ cells were seeded in 500 µL DMEM media in 48 well plates in 3 sets. After 3 hour cells were sensitized with DNP-BSA specific IgE (TIB-142 sup) in 1:100 ratios, overnight. Next day, cells were washed two times with warm RPMI PR^- media. Then 100µL of 100 ng/ml DNP-BSA (diluted in RPMI PR^- media) was added and incubated at 37°C in CO_2 incubator for 45 min. After 45 min supernatant was collected. The cells were further washed with ice cold RPMI PR^- media two times to stop the reaction. Lysates were collected from set 1 for primary challenge. In other sets, fresh complete media was added and left undisturbed for 6 hour. After 6 hour the cells were harvested and seeded for next challenge. Cells were sensitized and activated multiple times in a similar way as primary challenge. After each challenge, supernatants and lysates were collected and stored at -80°C until assayed.

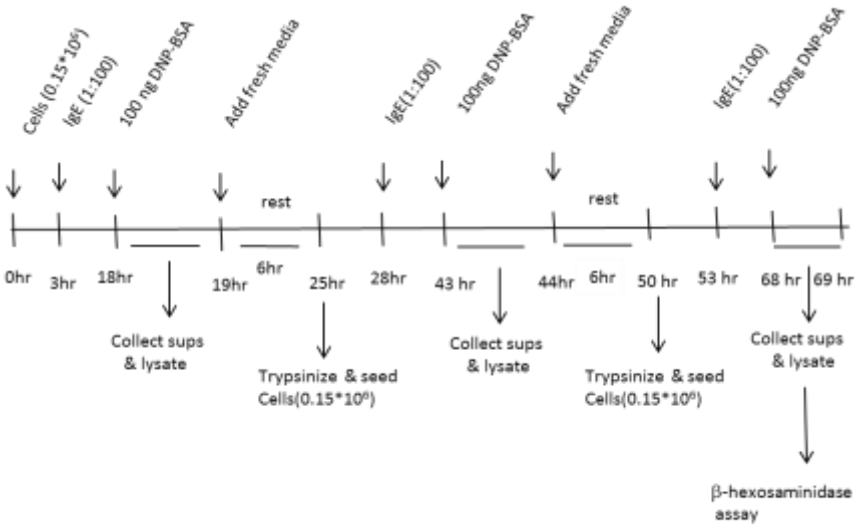


Figure 3.1 : Timeline of induction of secretion in RBL cells by IgE crosslinking.

3.6 RBL-2H3 secretion assay

RBL-2H3 secretion was assayed by checking the secretion of lysosomal hydrolase β-hexosaminidase. So β-hexosaminidase assay was performed. 20 µl of the supernatants

and cell lysates were incubated with 50 µl of the substrate solution (27.35 mg/ml of *p*-nitro phenyl-*N*-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) for 90 min at 37 °C. The reaction was terminated by adding 100 µl of stop solution (0.2 M NaOH/0.2 M glycine/0.2M Nacl). Absorbance was read at 405 nm in spectramax multiplate reader, and the amount of secretion was expressed as the percentage of total β-hexosaminidase activity present in cells (Vaidyanathan et al., 2001).

3.7 Flow cytometric analysis of cell surface receptors on RBL-2H3 cells on multiple sensitizations

To analyze expression of FcεRI receptor on the surface, $0.2x10^6$ RBL-2H3 cells were sensitized multiple times and the cells were collected after each 24 hour of sensitization. Cells($0.2x10^6$) were suspended in 20 µl phosphate buffer saline (PBS) followed by incubation with anti-mouse CD16/32 Fc block (1 µg/10^6 cells) for 15 minute at 4^oC prior to staining. The Fc block binds to the Fc receptors on the cell surface thus avoiding non-specific binding of antibody of interest. Cells were then incubated with mouse anti IgE-PE and their isotypes control antibody for 30 min at 4^oC. Cells were then washed twice with PBS and fixed in 200 µl of 2% paraformaldehyde (PFA). Cells were analyzed on BD FACS caliber by using Cell Quest software by acquiring at least 10,000 events.

3.8 Treatment of mast cells with different antigens

RBL-2H3 cells (2.5 million) were seeded in DMEM media at 37°C in CO_2 incubator for overnight. Next day cells were trypisinized and collected. RBL cell (2.5 million) were sensitized with DNP-specific IgE (1:100) and cultured overnight followed by trypsinization and collection of cell pellet. 2.5 million RBL cells sensitized with DNP-BSA were cultured overnight. Next day 100ng of DNP-BSA (diluted in RPMI PR^- media) was added and incubated for 2 hour at 37°C in CO_2 incubator. After 2 hour the cells were washed with cold PBS to stop the reaction. Then onwards the cells were collected by trypsinization. Similarly 2.5 million cells cultured overnight in 2 set for adherence. Next day cells were treated with 15µg/ml C 48/80 and DPT (@ MOI 1:10) for 1 hour and 6 hour respectively followed by collection of cell pellet by trypsinization. Furthermore 2 set of 2.5 million RBL cells were seeded overnight for adherence. Next day 15µg/ml Poly I:C and sonicated *Escherichia coli* (MOI 1:100) was added in the media and incubated overnight. Then next day the cells were collected by trypsinization. All the cells were then further processed for RNA isolation.

3.9 Treatment of RBL-2H3 with pathogens

RBL-2H3 (2.5 million) cells were cultured overnight in DMEM media for adherence. Then next day incubated with *Leishmania donovani* and *Leishmania tropica* promastigote (@ MOI 1:10) for 24 hour in CO_2 incubator(Naqvi et al., 2017) and incubated with sonicated BCG (@ MOI 1:100)(Paudel, Puri, & Nehru, 2017) for 12 hour in CO_2 incubator. After incubation the adhered cells were washed with PBS and then the cells were harvested by using trypsin. Then the cells were centrifuged at 1000 rpm for 5 minute to collect the pellet. Then it was further processed for analysis.

3.10 Isolation of RNA

RNA extraction is complicated due to presence of RNase enzyme in the environment. RNase is very active and almost present everywhere so before starting RNA isolation all the equipment and working area is treated with Di-ethyl pyro carbonate (DEPC). RNA was isolated from resting, sensitized RBL, activated RBL, RBL treated with poly I:C, RBL co-cultured with sonicated Bacillus Calmette-Guerin (BCG), RBL co-cultured with *Escherichia coli* (E.coli), RBL co-cultured with DPT (Diptheria, pertussis and tetanus), RBL treated with compound 48/80 (C48/80), RBL co-cultured with *Leishmania donovani* (L.d) and *Leishmania tropica* (L.t). 1 ml Trizol (TRI) reagent was used for 5 million cells and mixed properly followed by addition of 200µL chloroform. It was mixed properly by inverting and incubated at room temperature for 15 minute. After incubation it was centrifuged at 12,000g for 15 min at 4^{o}C at eppendorf Centrifuge 580 R. After centrifugation upper aqueous phase, middle and lower phenol-chloroform layer containing RNA, DNA and protein respectively. Upper aqueous layer was carefully transferred to a fresh DEPC treated eppendorf tube and 0.5ml isopropanol was added and mixed to precipitate RNA and incubated at room temperature for 15 minute. Then it was centrifuged at 12000g for 15 min at 4^{o}C. White gel like pellet was obtained which was washed two times with 75% ethanol by centrifugation at 7500g for 5 min at 4^{o}C. After centrifugation it was air dried. After complete dry, pellet was mixed with Tris/borate/EDTA (TBE) buffer. For proper mixing, tubes were incubated at 70^{o}C for 10 min. Then the RNA was stored in -80°C and was further used for cDNA synthesis.

3.10.1 Determination of yield and quality of RNA

The yield and the integrity (quality) of the RNA were determined by measuring absorbance at 260nm using Nanodrop ND 2000 spectrophotometer. At first reference baseline was set by a using TBE buffer and ratio of the readings at A260/A280 and A260/A230 was measured. A260/A280 ratio around 2.0 and A260/230 ratio greater than 1.8 denotes the purity of RNA. Absorbance values below 2 indicate phenol, protein or other contaminants.

3.10.2 Formaldehyde Agarose gel electrophoresis of total RNA

RNA have tendency to form secondary structures so it forms smearing upon electrophoresis. To get distinct bands RNA has to be denatured before electrophoresis. Heating denaturing the RNA sample prior to electrophoresis is insufficient, as secondary structures will simply reform unless a denaturing system is used, so denaturing chemical agents like formaldehyde, ethiduim bromide to the agarose gel prevents from reforming of secondary structure. Formaldehyde also serves as RNase inhibitory agent maintaining RNA integrity during separation. 1.2% Formaldehyde Agarose gel was prepared in 1X formaldehyde buffer by adding 1.2% agarose. Ethiduim bromide (EtBr) (0.5µg/ml) was added after cooling to 40-50^{o}C. Sample was loaded (4 volume of RNA + 1 volume of 5X RNA loading dye) in the gel tank having 1X formaldehyde buffer. Electrophoresis was carried out at 60V for 2-3 hrs and visualized under ALPHA IMAGE GEL documentation system.

3.11 Reverse transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR is a commonly used technique for the detection of RNA expression. It qualitatively detects gene expression through complementary DNA (cDNA) transcripts from RNA. Reverse transcription was performed using Moloney murine leukemia virus (M-MuLV) reverse transcriptase (200U/µl) and an oligo (dT) 18 (50µM) primer in a total reaction mixture of 20 µl. The amount of RNA template used was determined on the basis of different standardization conditions.

Table 3.1: Reaction mixture for RT-PCR

Components	**20 µl Reaction**
Nuclease Free water	Calculated accordingly(to 20 µl)
RNA template	Variable
Oligo(dT)-18	1.0 µl
Heated for 5mins at 65^{o}C and kept at room temperature for 2mins and then on ice	
5X M-MuLV RT buffer	4.5 µl
dNTPs mix	2.0 µl
M-MuLV RT enzyme 200µ/µl	1.0 µl
Total	20.0 µl

Synthesis of cDNA from RNA template was done in eppendorf mastercycler pro PCR thermocycler started with heating reaction mixture to 37^{o}C for 60 min, followed by heating at 70°C for 10 min in order to terminate the reaction. Finally reaction product was held at 4°C.

3.11.1 Agarose gel electrophoresis for cDNA

1.2% of agarose gel was prepared in 0.5x running buffer (5x TBE buffer+ Milli Q water) by heating and after cooling to 40-50^{o}C, 0.5µg/ml of EtBr was added and casted on tray. After gel was set it was transferred to gel tank containing 0.5X in TBE running buffer. cDNA samples (1 volume of 6X DNA loading dye+5 volume of cDNA) were loaded along with DNA ladder (100bp) .The gel electrophoresis was carried out at 90V ,for 1-2 hrs and visualized under ALPHAIMAGE gel documentation system.

3.12 Primer designing

Primers were designed to observe the heterogeneity in expression of selected cytokines in mast cell treated with different antigen, pathogen and with pathogen derived antigen. Primers were designed for GAPDH, macrophage inflammatory protein-1α (MIP-1α), Tumor Necrosis Factor α (TNF- α), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-6 (IL-6), Interleukin-13 (IL-13) gene of *Rattus Rattus* using Primer-Blast

(https://www.ncbi.nlm.nih.gov/tools/primer-blast/) software by setting following parameters: amplicon size 110bp-300bp for reverse transcriptase PCR, primer length 19-22 base long, 45-60% GC content and with no self-complementarity. Primer blast is a robust and fully implemented primer design tool that designs target specific PCR primers. Bioinformatics tools for sequence editing such as Oligo Calc (http://biotools.nubic.northwestern.edu/OligoCalc.html) and MultAlin (https://multialin.toulouse.infra.fr/multialin/) were used to further validate the parameters of the primers designed. Oligo Calc is used for calculating the physical properties of DNA and RNA oligonucleotides including melting temperature, molecular weight, % GC content and absorbance coefficient for a given oligonucleotide sequence. MultAlin is a multiple-sequence alignment tool for protein and nucleic acid sequences. . The list of primers with their primer length, melting temperature, GC content along with their product length is listed in Table.

Table 3.2: RT-PCR primers used for studying cytokine release after treating with different Triggers

S.N	Name of cytokines	Primer Sequence Forward primer(FP) Reverse primer(RP)	Primer length (b)	Melting Temperature (Tm°C)	GC%	Product length (Bp)
1	IL-4	FP CTCCATGCACCGAGATGTT RP ACTGCAAGTATTTCCCTCGTAG	19 22	63.9 61.1	52.6 45.4	291
2	IL-5	FP GAGGATGCTTCTGTGCTTGA RP CCCTCGGACAGTTTGATTCTT	20 21	63.2 64.2	50 47.6	235
3	IL-6	FP CCGTTTCTACCTGGAGTTTGT RP GTTTGCCGAGTAGACCTCATAG	21 22	61.9 61.6	47.6 50	273
4	IL-13	FP CTGGAATCCCTGACCAACATC RP TGAGGTCCACAGCTGAGAT	21 19	65.5 61.1	52.3 52.6	224
5	MIP-1α	FP TGCTGCTTCTCCTATGGACG RP GCCGGTTTCTCTTGGTCAGG	20 20	65.4 68.1	55 60	112
6	TNF-α	FP CGTGTTCATCCGTTCTCTACC RP GCAATCCAGGCCACTACTT	21 19	63.4 61.7	52.3 52.6	209
7	GAPDH	FP GCGAGATCCCGCTAACATCA RP CTCGTGGTTCACACCCATCA	20 20	67.4 67.2	55 55	178

3.13 Semi-quantitative reverse transcriptase PCR

The cDNA obtained was amplified by conventional PCR using different primers for cytokines as listed in Table in a total reaction mixture of 25µl. The amplification started with heating the sample to 95^{o}C for 2 minute, followed by number of cycles (30-40) consisting of denaturation for 15 sec at 95^{o}C, annealing at 50-60^{o}C for 15 sec and extension at 68^{o}C for 45 sec. The last cycle was followed by an additional extension for 5mins at 68^{o}C and final hold at 4^{o}C.

Table 3.3: Reaction mixture for semi-quantitative PCR

Components	**25 µl Reaction**	**Stock Concentration**
Nuclease Free Water	Calculated accordingly (to 25µl)	-
Taq Buffer (20mM $MgCl_2$)	4.0 µl	10X
dNTPs Mix	2.0 µl	800µM
Forward Primer	1.0 µl	100µM
Reverse Primer	1.0 µl	100µM
Template(cDNA)	2.0 µl	-
Taq polymerase	0.125 µl	5U/µl

3.13.1 Agarose gel electrophoresis of amplified cDNA

1.2% Agarose gel containing 0.5µg/ml ethidium bromide was prepared in 0.5X TBE buffer. 6X DNA Gel loading dye (5µl) was added to the PCR products (25µl) and mixed well and loaded to the respective well in the gel along with DNA ladder (100bp,100µg/ml) and run for 1- 2 hrs at 90V. The gel was visualized under alpha image gel documentation system.

3.13.2 Quantitation of band intensity

Band intensity of PCR products was measured by using gel alpha ease FC gel quantitation software since band intensity is directly proportional to amount of gene present in the gel.

Chapter 4
Results

4.1 Growth kinetics of RBL-2H3 mast cell line in different media

Cells cultured in two different media (DMEM and RBL) to determine the growth kinetics in laboratory condition, generated the growth curve. Two different parameters i.e. Cell morphology and doubling time were compared. The cells were adhered and have fibroblast like morphology. Few round shaped cells were floating in the media which may represent dividing cells. The morphology of cells looks similar in both media. Growth curve was plotted on the basis of viable cell count. RBL-2H3 cells cultured in RBL media have lag phase of less than 24hrs, exponential phase after 24hrs and decline phase after 72hrs whereas RBL-2H3 cells cultured in DMEM media have lag phase less than 24 hour, exponential phase after 24 hrs and decline phase after 60 hrs. The doubling time of RBL-2H3 cells was determined to be 18.9 hour± SEM and 15.12 hour±SEM in DMEM and RBL media respectively by using formula described in materials and methods.

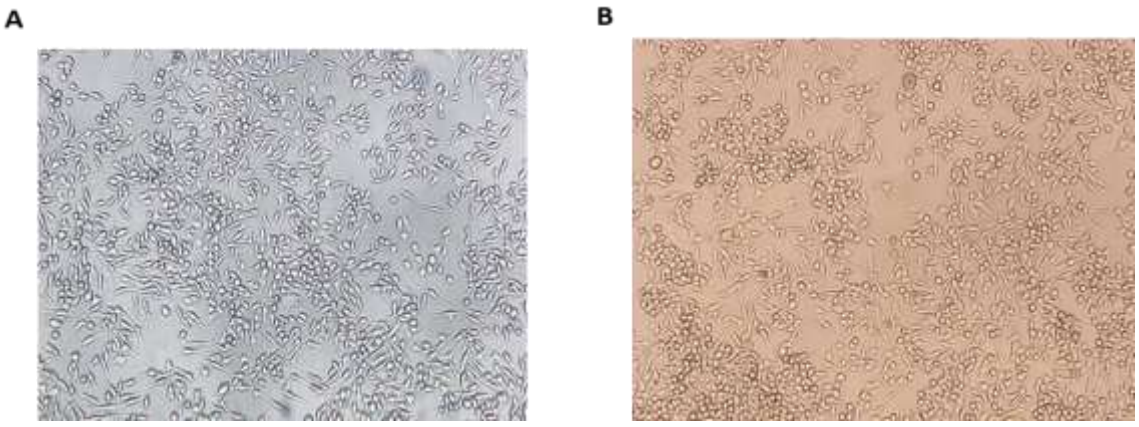
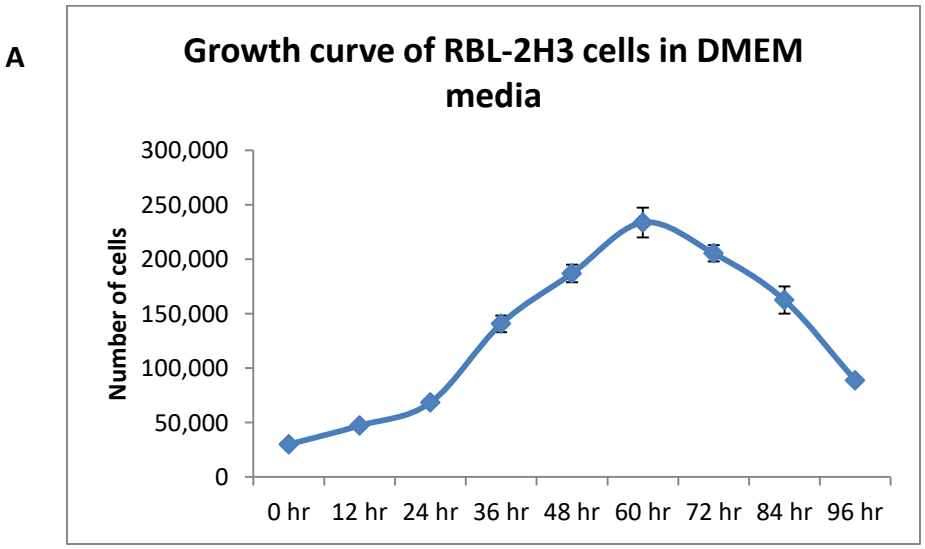


Figure 4.1: Morphology of RBL-2H3 cells: 0.15*10^6 cells were seeded in 48 well plates and observed under light microscope after 24 hour. **(A)** The cells cultured in DMEM media. **(B)** Cells cultured in RBL media under 10 X magnifications.



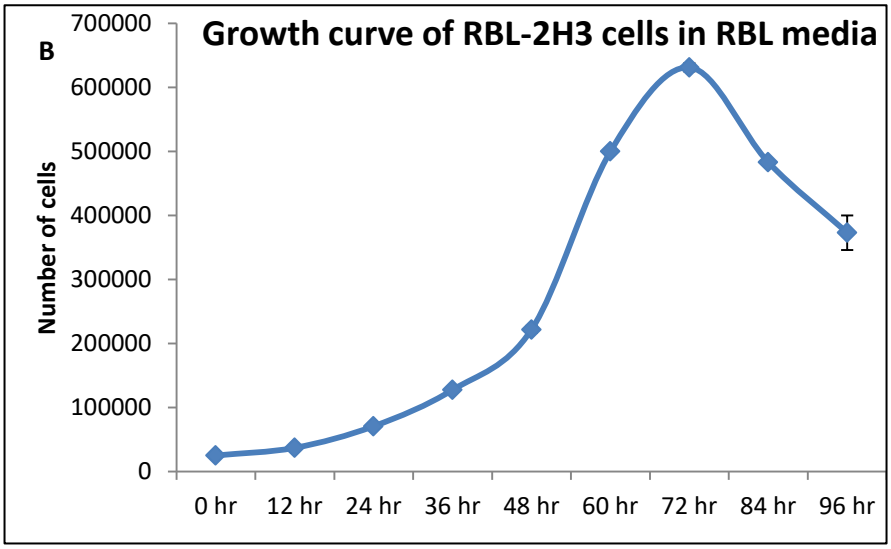
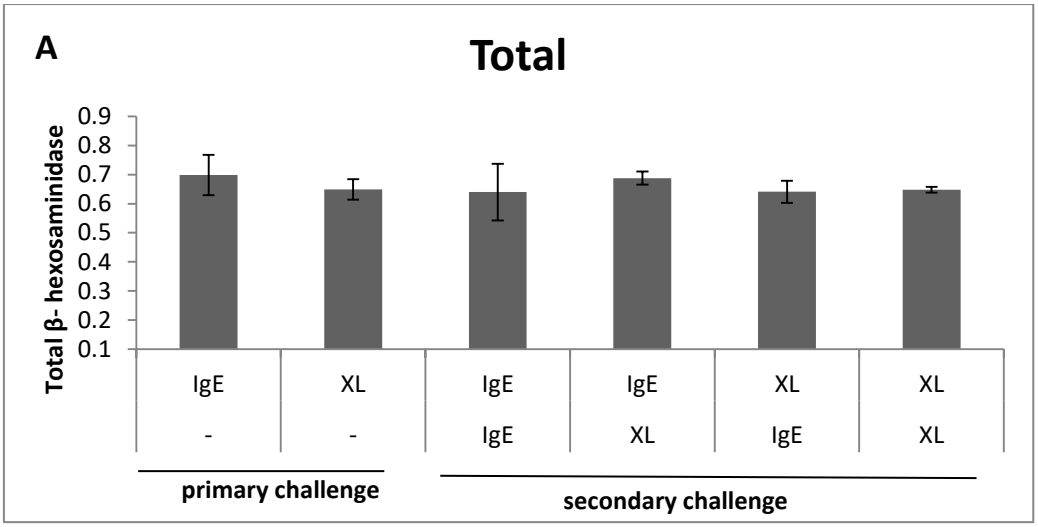


Figure 4.2: Growth kinetics of RBL-2H3 cells: (A) Growth curve of RBL-2H3 cells in RBL media plotted on the basis of viable cell recoveries in which each time point represents mean±SEM. **(B)** Growth curve of RBL-2H3 cells in DMEM media plotted on the basis of viable cell recoveries in which each time point represents mean±SEM of values obtained from duplicate assays.

4.2 Effect of multiple antigenic challenges on mast cell mediator release.

The supernatants and lysates that were collected were assayed for β-hexosaminidase enzyme release. Data was plotted as percent secretion and total β-hexosaminidase. β-hexosaminidase release was 44.22% and 36.11% out of total β-hexosaminidase during primary and secondary challenge, respectively. Similarly when RBL cells were challenge up to tertiary their secretion was 54 %, 47% and 40% out of total β-hexosaminidase in primary challenge, secondary challenge and tertiary challenge respectively. Statistical analysis was done by using students T-test.



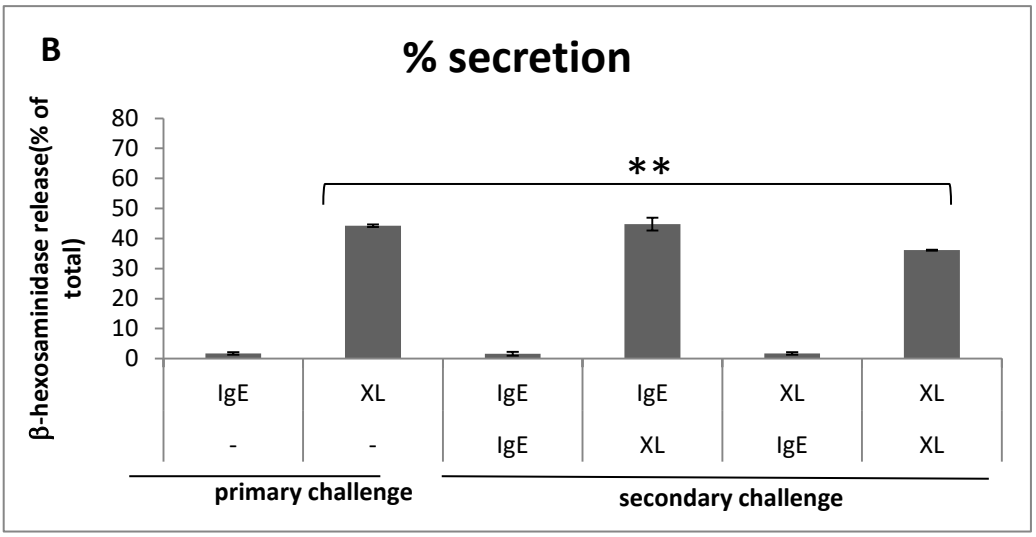
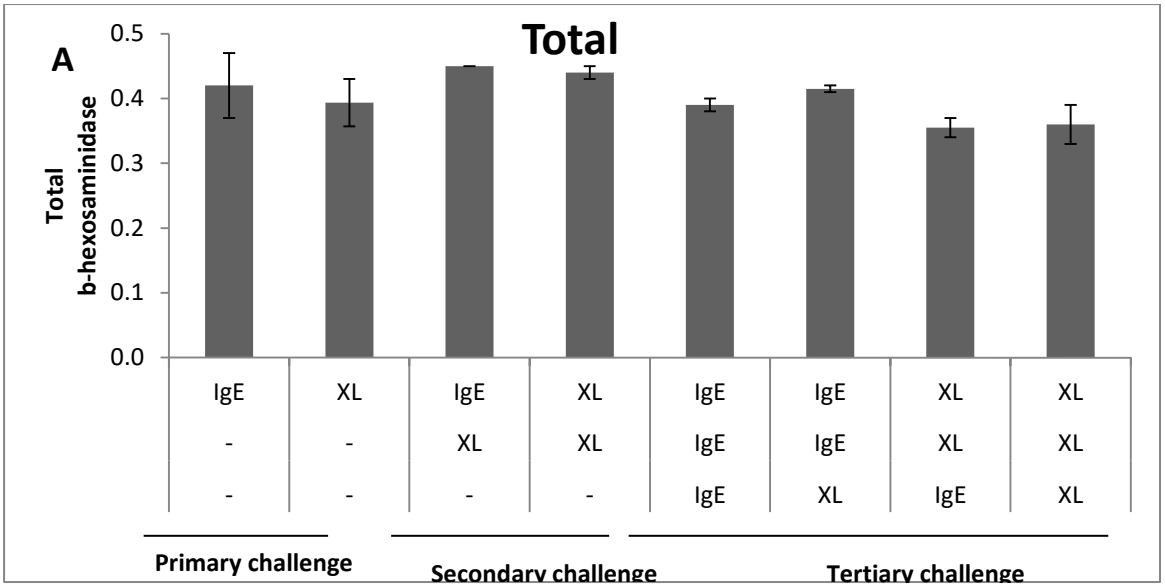


Figure 4.3.1: Mast cells response to multiple challenges (till secondary). RBL cells were sensitized overnight with anti DNP-BSA specific IgE, which is then activated with 100ng DNP-BSA for 45 minutes. After 24 hrs cells were again sensitized and activated for secondary challenge. XL indicates cross-linked/activated RBL cells whereas IgE indicate sensitization only. Total beta hexosaminidase and percent secretion is plotted in figure A and B respectively. Bar represents mean±SEM of values obtained from two independent experiments. *p<0.05,**p<0.001.



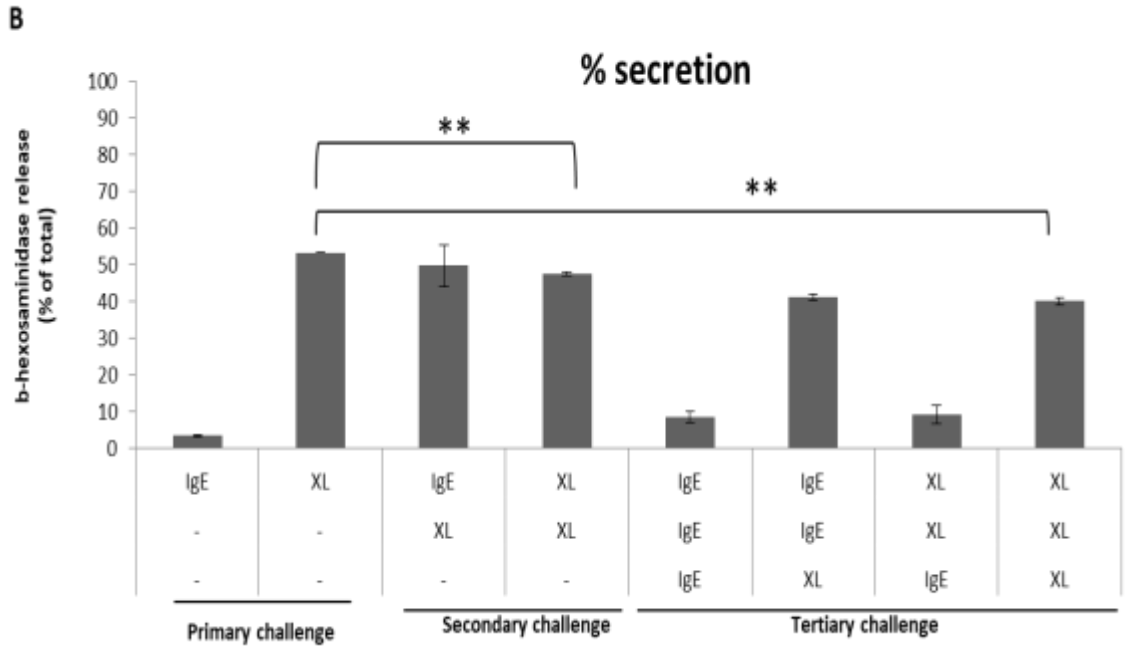


Figure 4.3.2: Mast cells response to multiple challenges. RBL cells were sensitized overnight with anti DNP-BSA specific IgE, which is then activated with 100ng DNP-BSA for 45 minute. After 24 hrs cells were again sensitized and activated for secondary challenge. After 24 hour of secondary challenge again cells activated for tertiary challenge. XL indicates cross-linked/activated RBL cells whereas IgE indicate sensitization only. Total beta hexosaminidase and percent secretion is plotted in figure A and B respectively. Bar represents mean±SEM of values obtained from two independent experiments. *p<0.05,**p<0.001.

4.3 Flow cytometric analysis of cell surface receptors on RBL-2H3 cells on multiple sensitization

The Mean Fluorescence Intensity (MFI) of isotype control was 4.275±0.825. IgE sensitized cells have 197±3.89, 193±8.89 and 199±4.06 MFI on primary, secondary and tertiary sensitization respectively. As compared to isotype MFI of sensitized cells are very high. However, there is no significant difference in receptors expression profile in multiple sensitized cells as shown in figure 4.4.

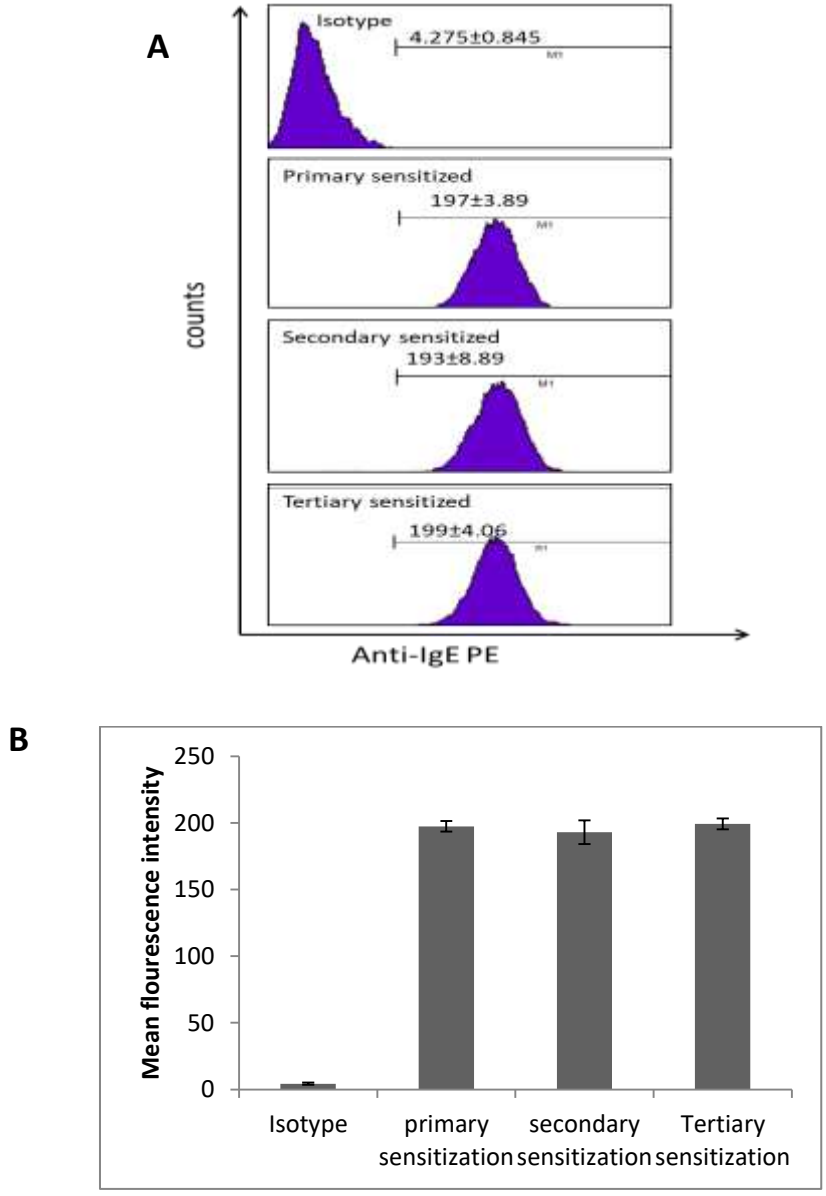


Figure 4.4: Surface receptor expression after each sensitization. RBL cells were sensitized multiple times. After each sensitization cells were harvested and stained for analyzing FcεRI receptor expression with anti IgE-PE and analyzed by FACS. **(A)** MFI of respective histogram. **(B)** Graphical representation of MFI. Bar represents mean±SEM of two independent experiments.

4.4 Isolation of RNA from RBL-2H3 cells after treatment with different triggers

RNA was isolated from RBL-2H3 cells that are treated with different antigens, pathogens and pathogenic products by using Trizol method.

4.4.1 Quantitation of RNA

The amount and purity of RNA were determined by measuring the absorbance at 260nm using Nanodrop (ND 2000) spectrometer. Amount of RNA isolated from mast cells after treatment with different triggers were variable. The Purity ratio with respect to contaminants, such as protein and organic content were found within the range.

Table 4.1: Yield and purity of RNA isolated from different samples using Nanodrop (ND-2000) spectrophotometer

Cell line ($2.5*10^6$)	**Treatment**	**Amount of RNA per million cells (µg/µl)**	**Purity ratio (260/280)**	**Purity ratio (260/230)**
RBL-2H3	-	1.4	2.1	1.9
RBL-2H3	IgE	1.4	2.0	1.1
RBL-2H3	DNP-BSA	1.9	2.0	1.2
RBL-2H3	Poly I:C	2.5	2.0	1.7
RBL-2H3	*E.coli*	2.7	2.0	0.8
RBL-2H3	BCG	2.7	2.0	1.6
RBL-2H3	DPT	0.7	2.0	0.4
RBL-2H3	C48/80	0.7	2.1	1.1
RBL-2H3	*Leishmania donovani (L.d)*	0.4	2.0	1.7
RBL-2H3	*Leishmania tropica (L.t)*	0.6	2.0	1.8

4.4.2 Formaldehyde agarose gel electrophoresis of RNA for determining the quality and integrity of RNA.

The quality of RNA isolated from RBL-2H3 cells after treatment with different triggers was determined by using 1.2% formaldehyde agarose gel. Formaldehyde was used for denaturing RNA ensuring that RNA will remain single stranded. Sharp 28S and 18S rRNA band along with 5S smear was observed. The band intensity of 28S rRNA band and 18S rRNA band is nearly 2:1 ratio indicating that the isolated RNA is intact. Isolated RNA is free of genomic DNA as indicated by the absence of smear in wells area.

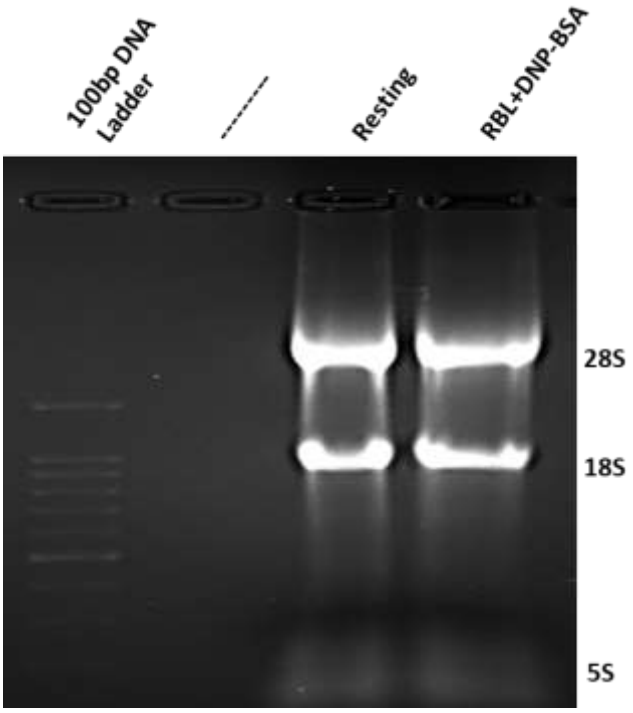


Figure 4.5: Formaldehyde agarose gel electrophoresis of RNA. Total RNA was isolated from resting and activated RBL-2H3 cells and quality of isolated RNA was determined by using 1.2% Formaldehyde agarose gel. Presence of Sharp 28S and 18S rRNA band along with 5S smear indicates isolated RNA is of good quality for further analysis.

4.4.3 Gel electrophoresis of cDNA isolated from RBL-2H3 cell after different treatment

As the quality and integrity of RNA was good, respective cDNA was synthesized using Moloney murine leukemia virus (M-MuLV) reverse transcriptases and an oligo (dT) 18 primer.

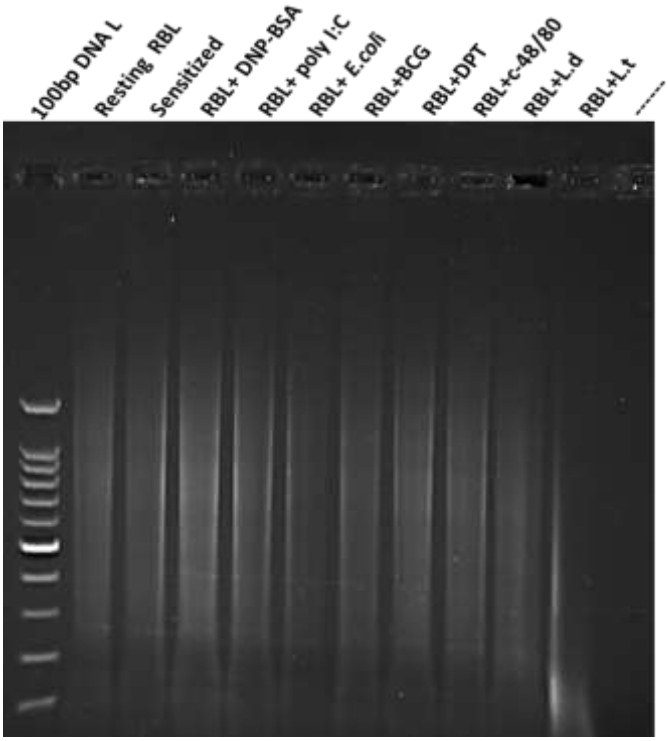


Figure 4.6: 1.2% agarose gel electrophoresis (with0.5µg/µl EtBr staining) of cDNA obtained by reverse transcription of total RNA using oligo dT primer along with 100bp ladder. Presence of smearing indicates RNA is converted to cDNA.

4.4.4 Standardization of Quantitative RT-PCR for amplification of IL-4 from RBL-2H3

RT-PCR standardization was performed for IL-4 cytokine for temperature gradient (50^{o}C-60 ^{o}C), number of cycle (25-40) and amount of template (15ng-600ng) by using RNA isolated from resting RBL cells. The PCR products were run on 1.2% Agarose gel and thus obtained bands were quantified using alpha ease gel quantification software. After quantification, ideal conditions were found to be 52^{o}C as annealing temperature, 30 numbers of cycles and 300ng of the RNA amount as shown in figure 4.7.

4.4.5 Standardization of Quantitative RT-PCR for amplification of IL-5 from RBL-2H3

RBL was activated by treating the cells with 100ng DNP-BSA for 2 hour. RT-PCR was performed for IL-5 cytokine for temperature gradient (55^{o}C-65 ^{o}C), number of cycle (25-40) and amount of template (15ng-600ng) using RNA isolated from activated RBL cells. The PCR products were run on 1.2% Agarose gel and thus obtained bands were quantified using alpha ease gel quantification software. Non-specific band of 400bp. Specific band is of 235 basepair. So there is single band at 64.1^{o}C, so it is selected as annealing temperature. 300ng of RNA and 35 number of cycle was found to be ideal condition as shown in figure 4.8.

4.4.6 Standardization of Quantitative RT-PCR for amplification of IL-6 from RBL-2H3

RT-PCR standardization was performed for IL-6 cytokine for temperature gradient (50^{o}C-60 ^{o}C), number of cycle (25-40) and amount of template (15ng-600ng) using RNA isolated from resting RBL cells. The PCR products were run on 1.2% Agarose gel and thus obtained bands were quantified using alpha ease gel quantification software. After quantification, ideal conditions were found to be 56^{o}C as annealing temperature, 35 numbers of cycles and 150ng of the RNA amount as shown in figure 4.9.

4.4.7 Standardization of Quantitative RT-PCR for amplification of IL-13 from RBL-2H3

RT-PCR was performed for IL-13 cytokine for temperature gradient (50^{o}C-60^{o}C), number of cycle (25-40) and amount of template (15ng-600ng) using RNA isolated from resting RBL cells. The PCR products were run on 1.2% Agarose gel and thus obtained bands were quantified using alpha ease gel quantification software. After quantification, ideal conditions were found to be 56^{o}C as annealing temperature, 35 numbers of cycles and 150ng of the RNA amount as shown in figure 4.10.

4.4.8 Standardization of Quantitative RT-PCR for amplification of Tumor Necrosis Factor-α (TNF–α) from RBL-2H3

RT-PCR standardization was performed for TNF-α cytokine for temperature gradient (50^{o}C-60^{o}C), number of cycle (25-40) and amount of template (15ng-600ng) using RNA isolated from resting RBL cells. The PCR products were run on 1.2% Agarose gel and thus obtained bands were quantified using alpha ease gel quantification software. After quantification, ideal conditions were found to be 56^{o}C as annealing temperature, 35 numbers of cycles and 150ng of the RNA amount as shown in figure 4.11.

4.4.9 Standardization of Quantitative RT-PCR for amplification of Macrophage Inflammatory Protein-1α from RBL-2H3

RBL was activated by treating the cells with 100ng DNP-BSA for 2 hour. RT-PCR was performed for MIP-1α for temperature gradient (56^{o}C-64.9^{o}C), number of cycle (25-40) and amount of template (15ng-600ng) using RNA isolated from activated RBL cells. The PCR products were run on 1.2% Agarose gel and thus obtained bands were quantified using alpha ease gel quantification software. After quantification, ideal conditions were found to be 57.5^{o}C as annealing temperature, 35 numbers of cycles and 300ng of the RNA amount as shown in figure 4.12.

4.4.10 Standardization of Quantitative RT-PCR for amplification of GAPDH from RBL-2H3

RT-PCR was performed for housekeeping gene GAPDH for temperature gradient (50^{o}C-60^{o}C), number of cycle (25-40) and amount of template (15ng-600ng) using RNA isolated from resting RBL cells. The PCR products were run on 1.2% Agarose gel and thus obtained bands were quantified using alpha ease gel quantification software. After quantification, ideal conditions were found to be 54^{o}C as annealing temperature, 30 numbers of cycles and 75ng of the RNA amount as shown in figure 4.13.

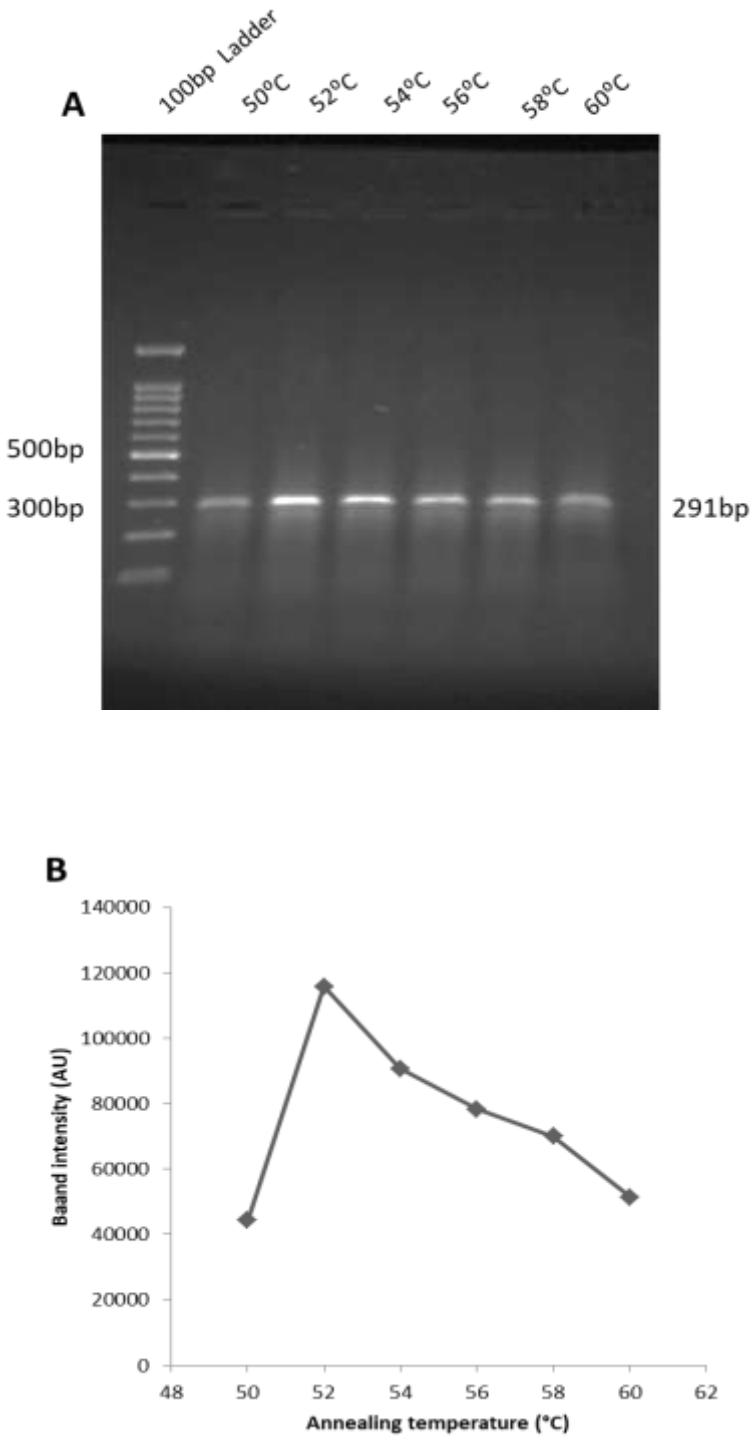


Figure 4.7.1: Standardization of annealing temperature for IL-4 RT-PCR. PCR reaction was performed by starting with 300ng RNA from resting RBL-2H3 for 35 cycles at various temperatures. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION (ALPHA IMAGER) as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

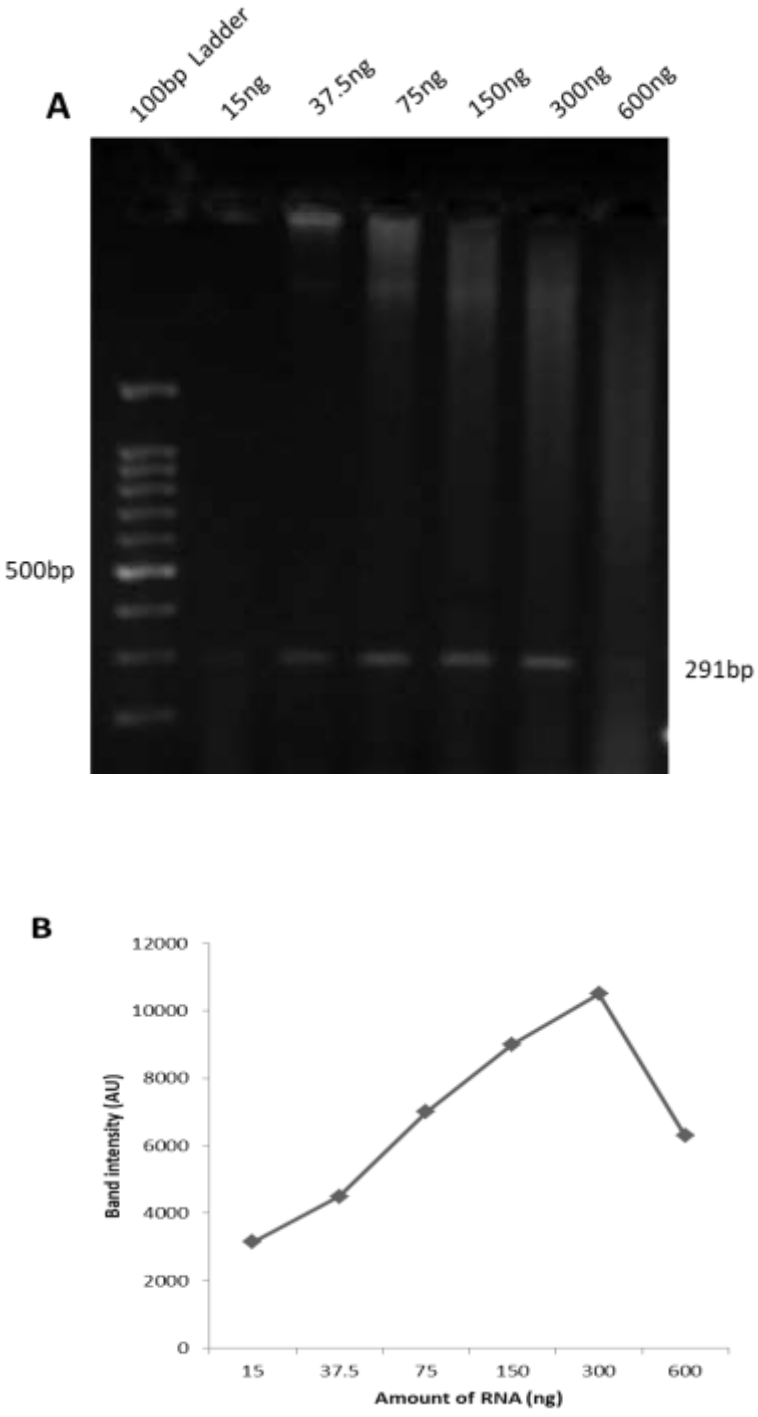


Figure 4.7.2: Standardization of amount of RNA template for IL-4 RT-PCR. PCR reaction was performed using different amount of RNA from resting RBL-2H3 for 35 numbers of cycles at 52°C. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION SYSTEM as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

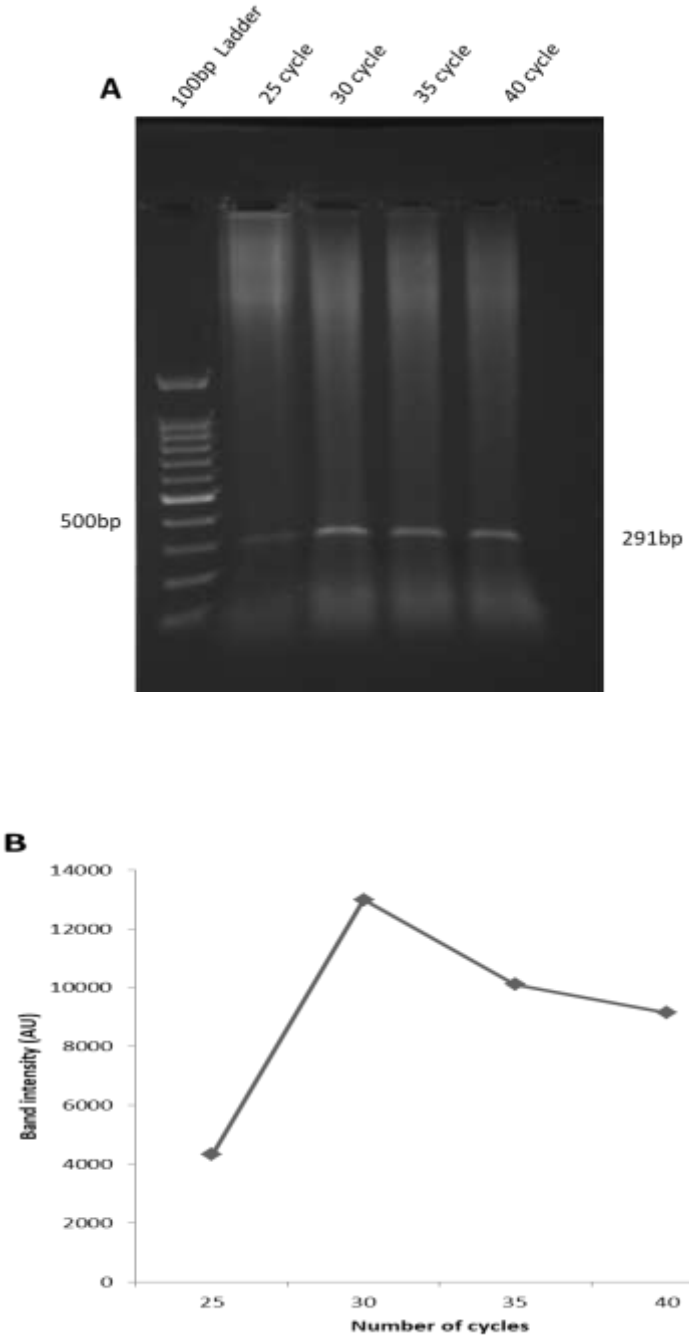


Figure 4.7.3: Standardization of number of thermocycles for IL-4 RT-PCR. PCR reaction was performed with 300ng RNA from resting RBL-2H3 for different number cycles at 52°C. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION SYSTEM as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

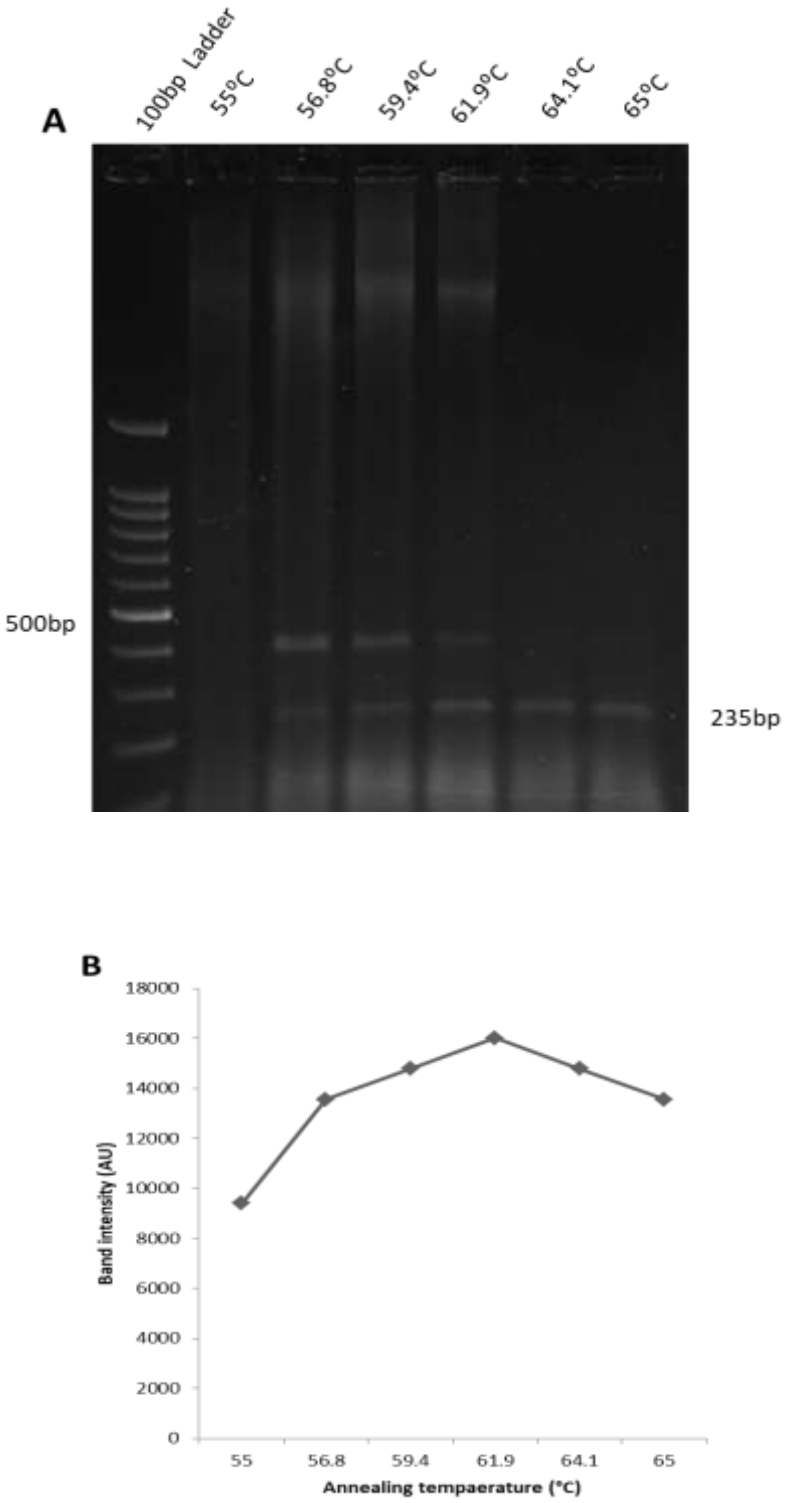


Figure 4.8.1: Standardization of annealing temperature for IL-5 RT-PCR. PCR reaction was performed by starting with 300ng RNA from activated RBL-2H3 for 35 cycles at various temperatures. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B. 235 bp band is specific band.

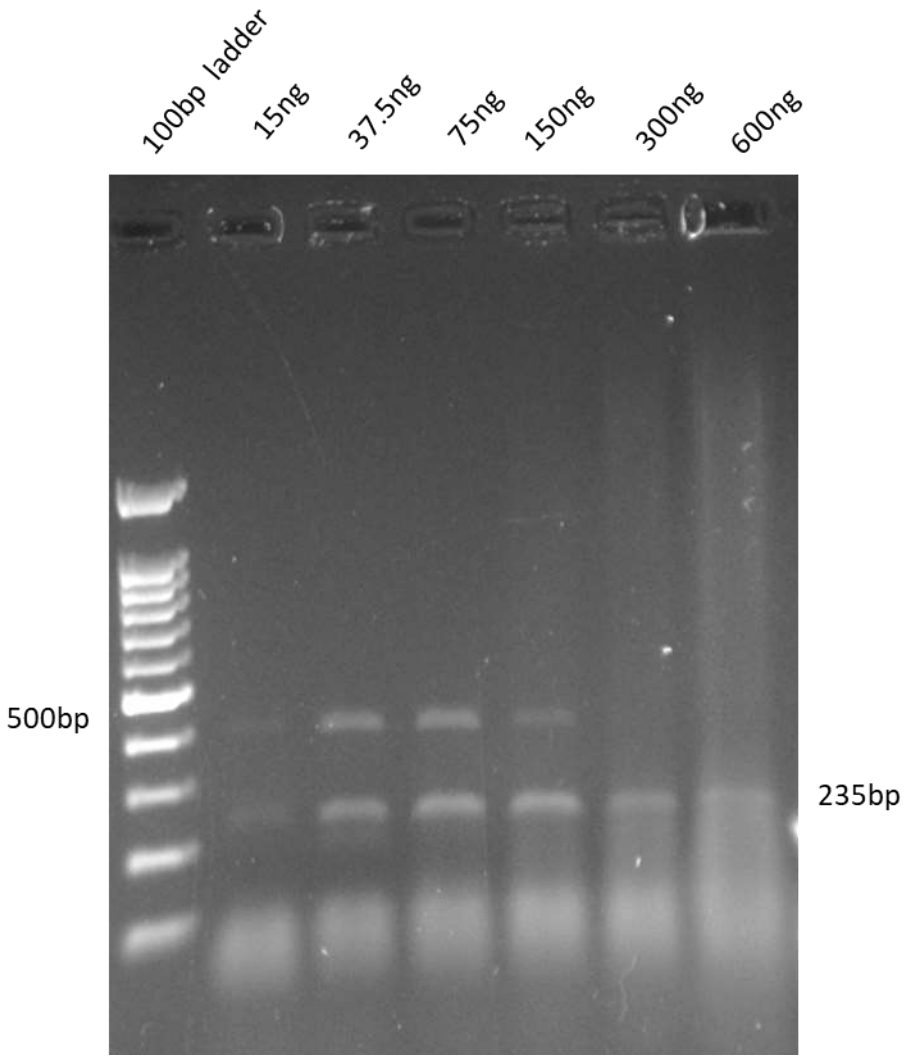


Figure 4.8.2: Standardization of amount of RNA template for IL-5 RT-PCR. PCR reaction was performed using different amount of RNA from activated (2 hr) RBL-2H3 for 35 numbers of cycles at 64°C. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION SYSTEM as shown in figure. Specific band is observed only in 300 ng of RNA concentration so it is selected for experiment.

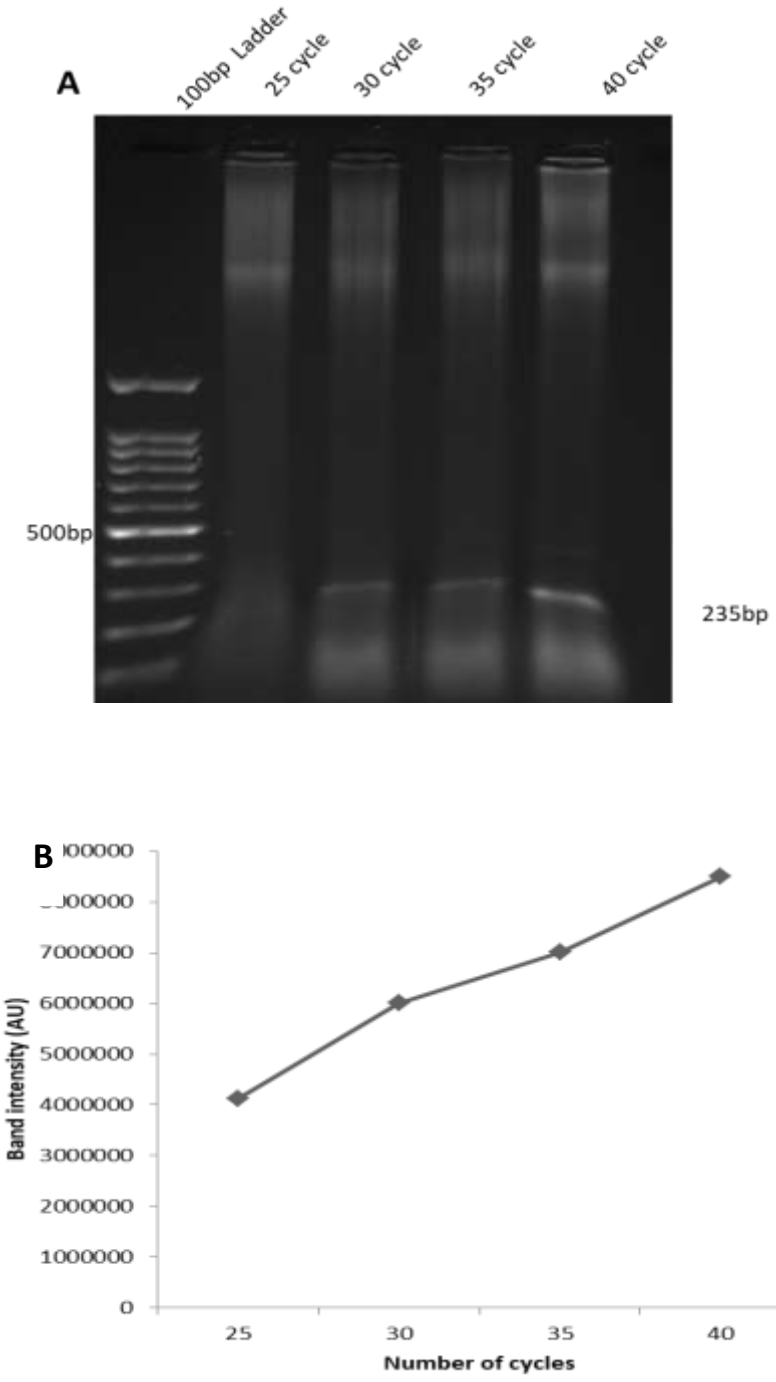


Figure 4.8.3: Standardization of number of thermocycles for IL-5 RT-PCR. PCR reaction was performed with 300ng RNA from activated (2 hr) RBL-2H3 for different number cycles at 64°C. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION SYSTEM as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

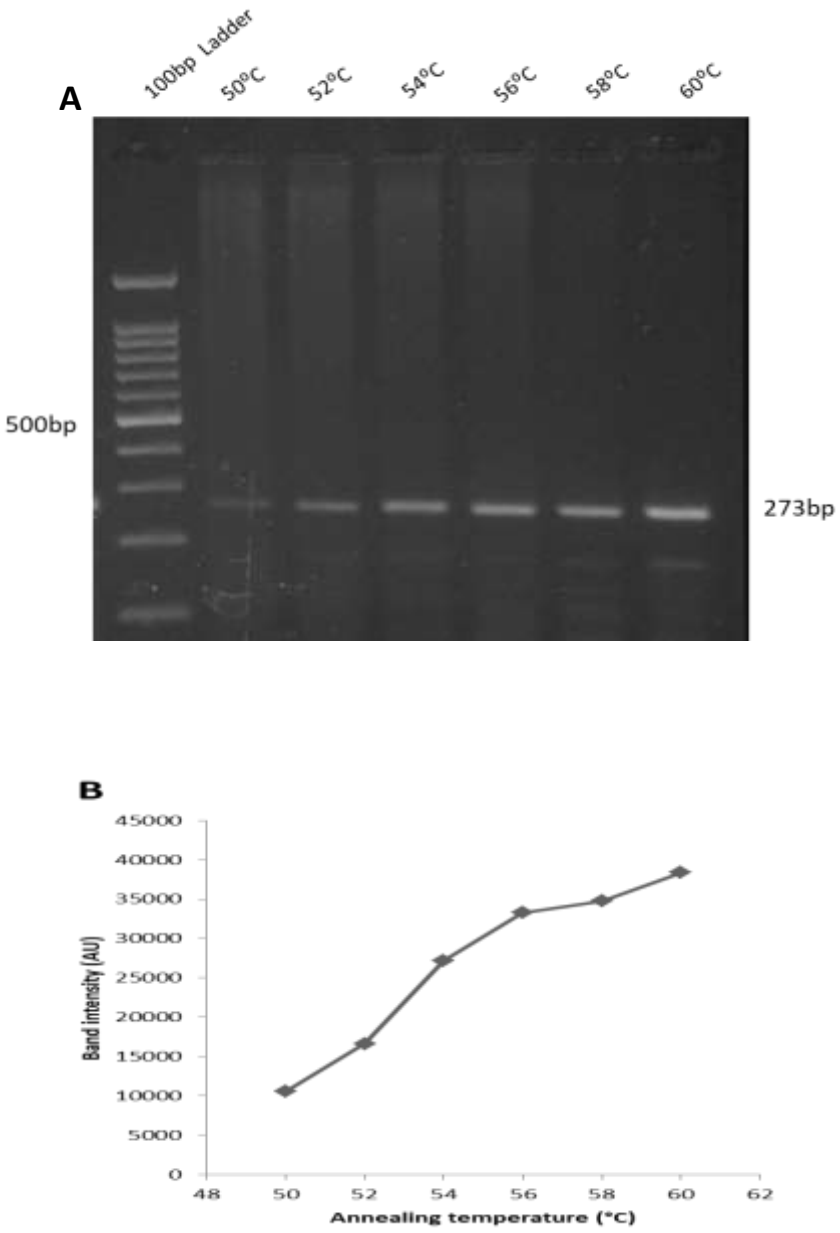


Figure 4.9.1: Standardization of annealing temperature for IL-6 RT-PCR. PCR reaction was performed by starting with 300ng RNA from resting RBL-2H3 for 35 cycles at various temperatures. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

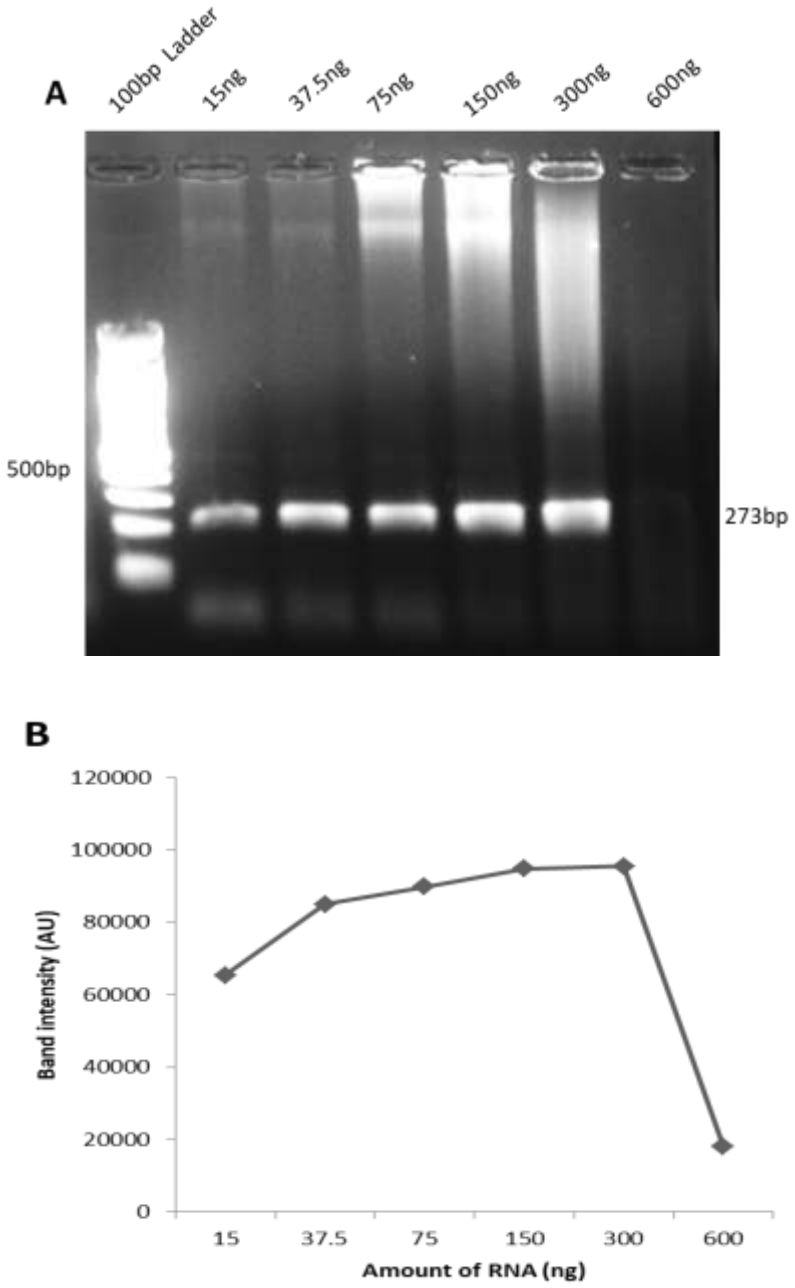


Figure 4.9.2: Standardization of amount of RNA template for IL-6 RT-PCR. PCR reaction was performed using different amount of RNA from resting RBL-2H3 for 35 numbers of cycles at 56°C. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION SYSTEM as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

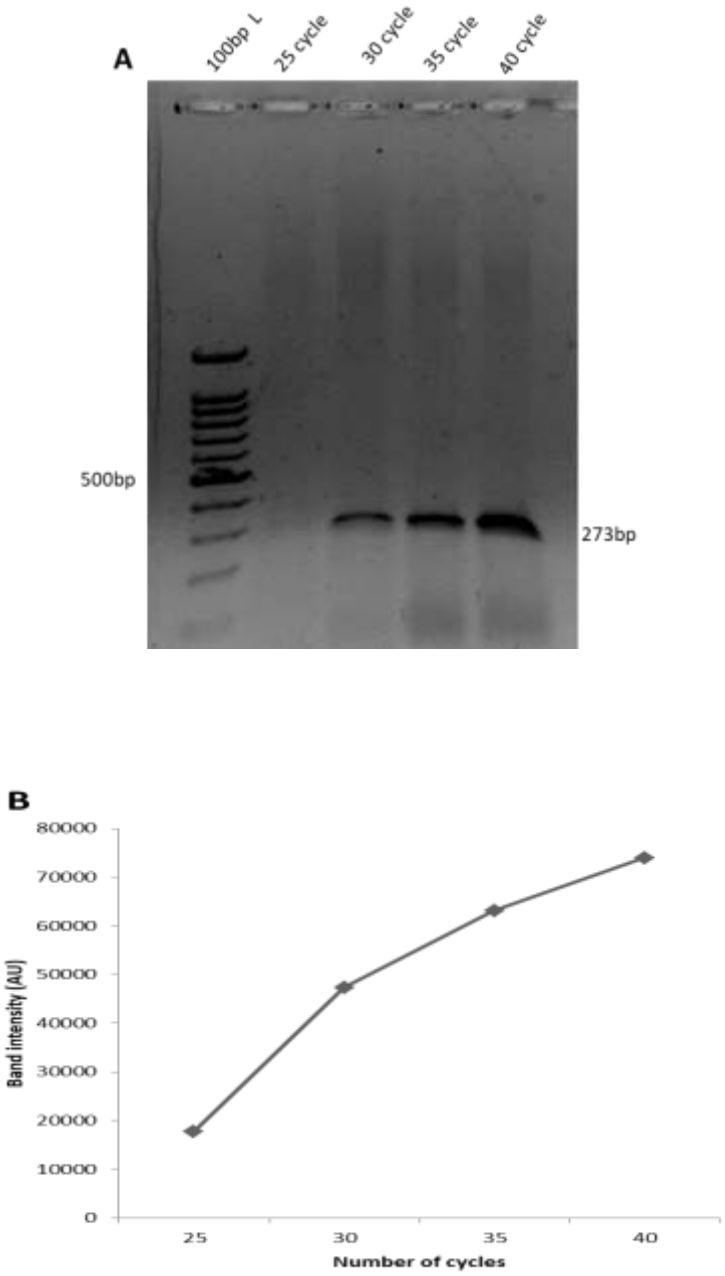


Figure 4.9.3: Standardization of number of thermocycles for IL-6 RT-PCR. PCR reaction was performed with 150ng RNA from resting RBL-2H3 for different number cycles at 56°C. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION SYSTEM as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

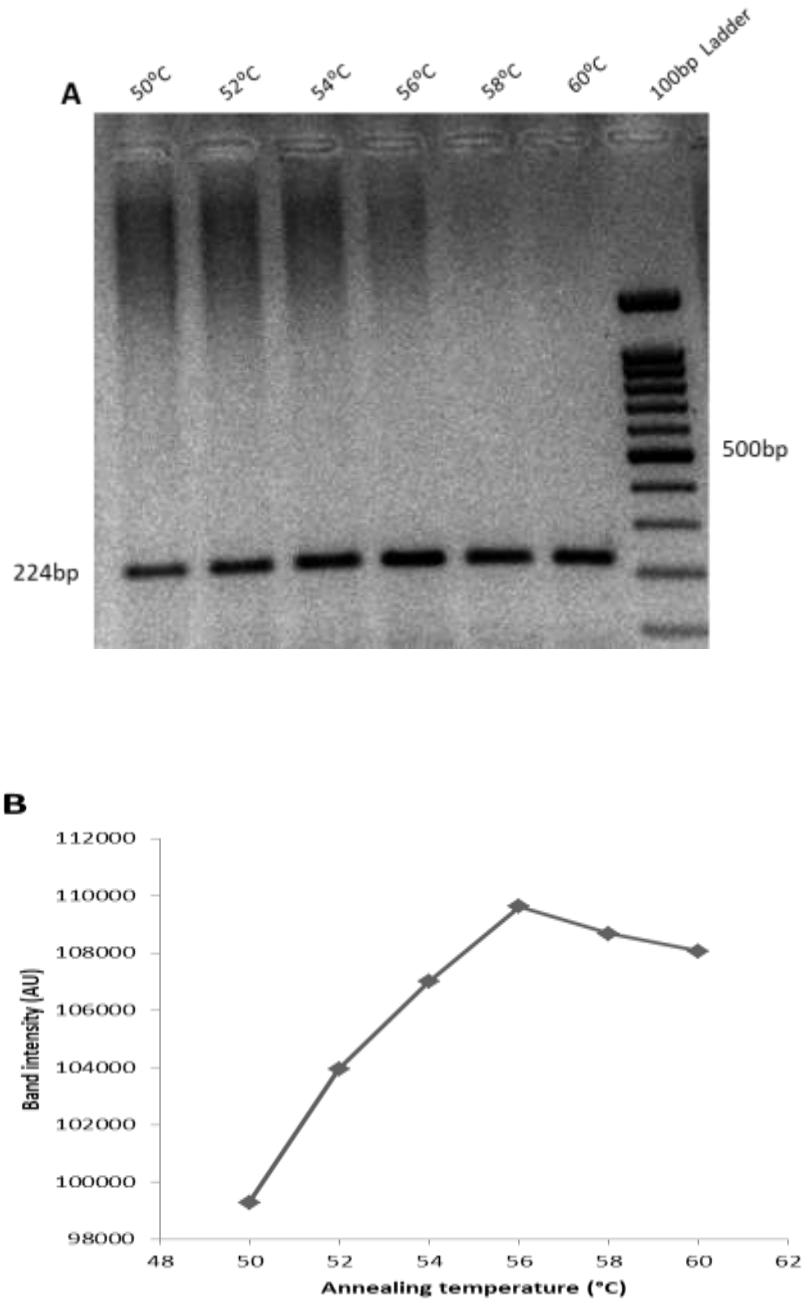


Figure 4.10.1: Standardization of annealing temperature for IL-13 RT-PCR. PCR reaction was performed by starting with 300ng RNA from resting RBL-2H3 for 35 cycles at various temperatures. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

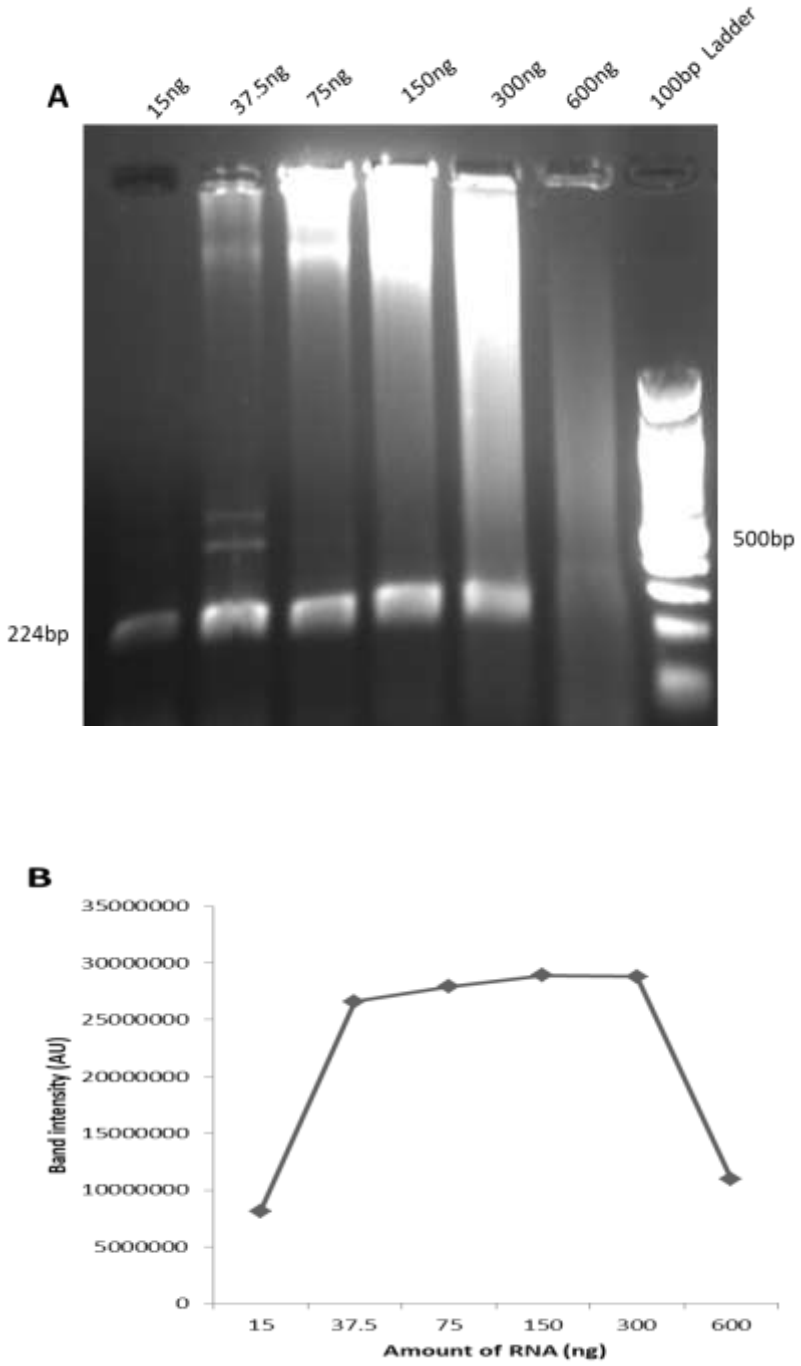


Figure 4.10.2: Standardization of amount of RNA template for IL-13 RT-PCR. PCR reaction was performed using different amount of RNA from resting RBL-2H3 for 35 numbers of cycles at 56°C. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION SYSTEM as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

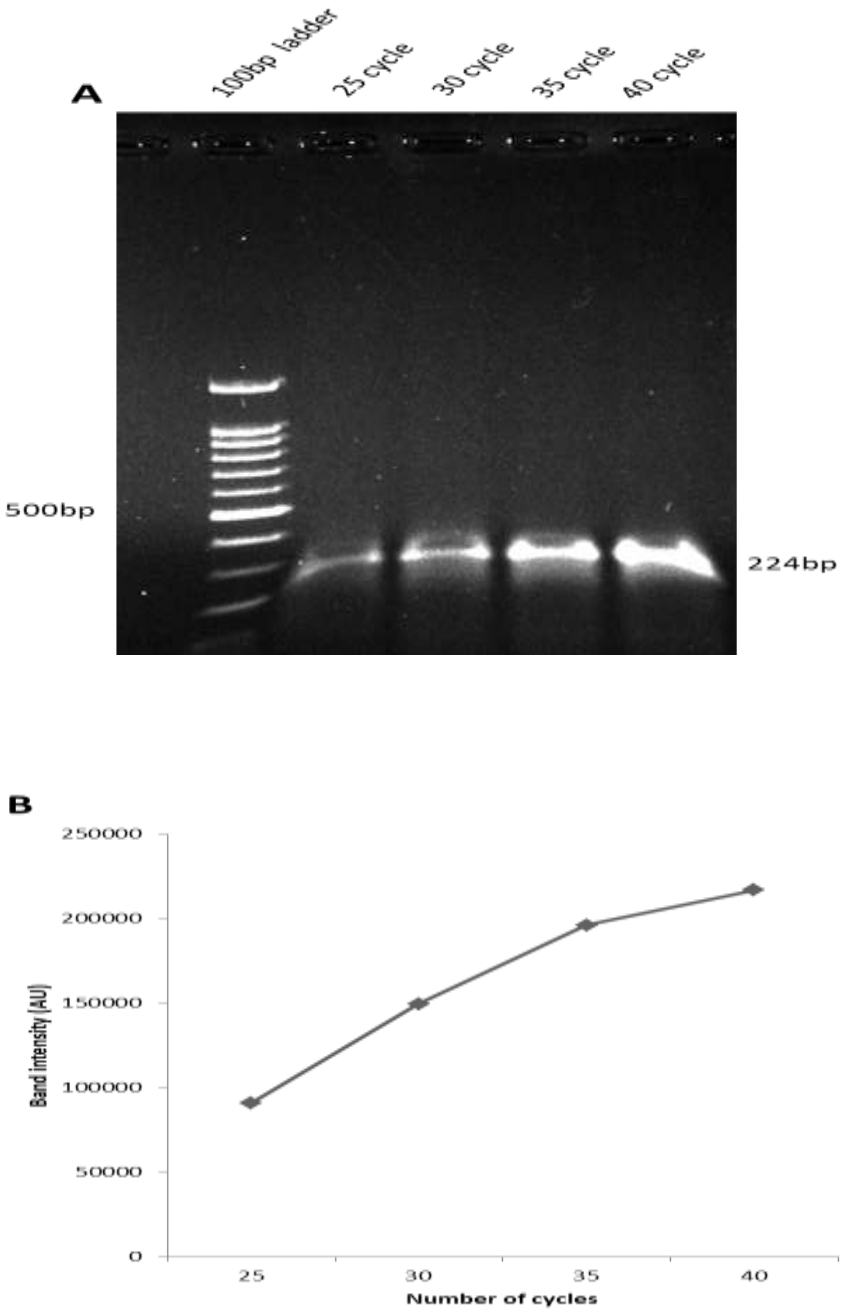


Figure 4.10.3: Standardization of number of thermocycles for IL-13 RT-PCR. PCR reaction was performed with 150ng RNA from resting RBL-2H3 for different number cycles at 56°C. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION SYSTEM as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

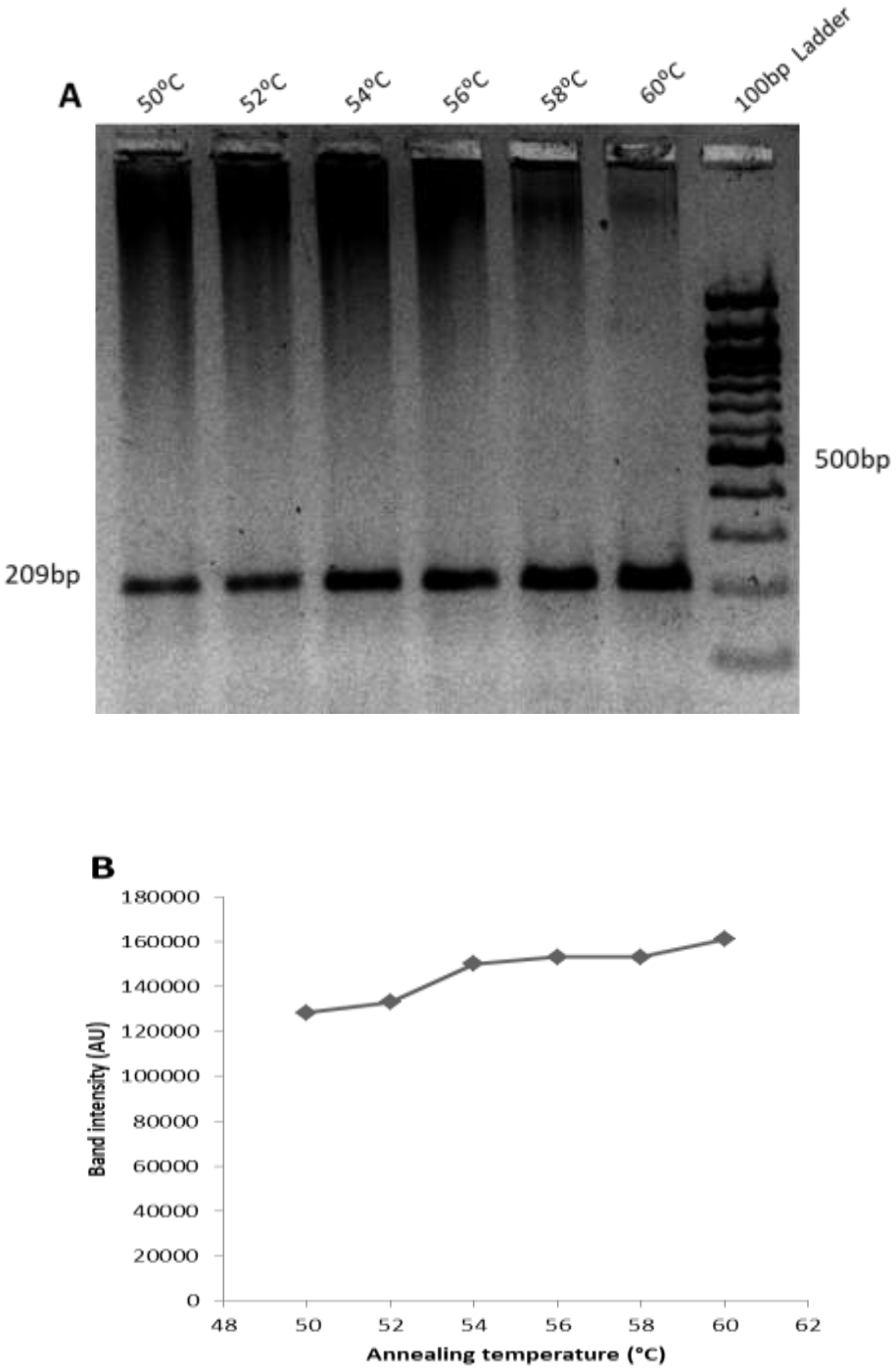


Figure 4.11.1: Standardization of annealing temperature for TNF-α RT-PCR. PCR reaction was performed by starting with 300ng RNA from resting RBL-2H3 for 35 cycles at various temperatures. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

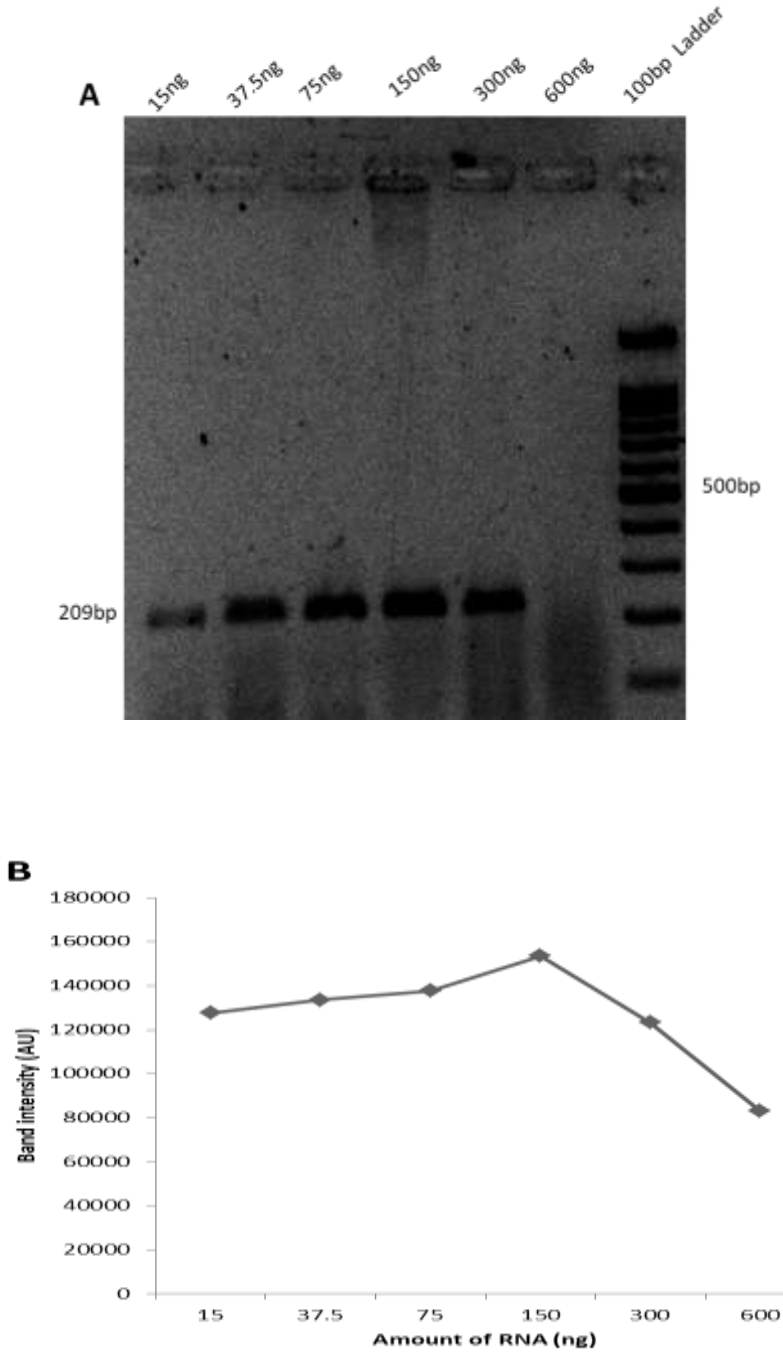


Figure 4.11.2: Standardization of amount of RNA template for TNF-α RT-PCR. PCR reaction was performed using different amount of RNA from resting RBL-2H3 for 35 numbers of cycles at 56°C. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION SYSTEM as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

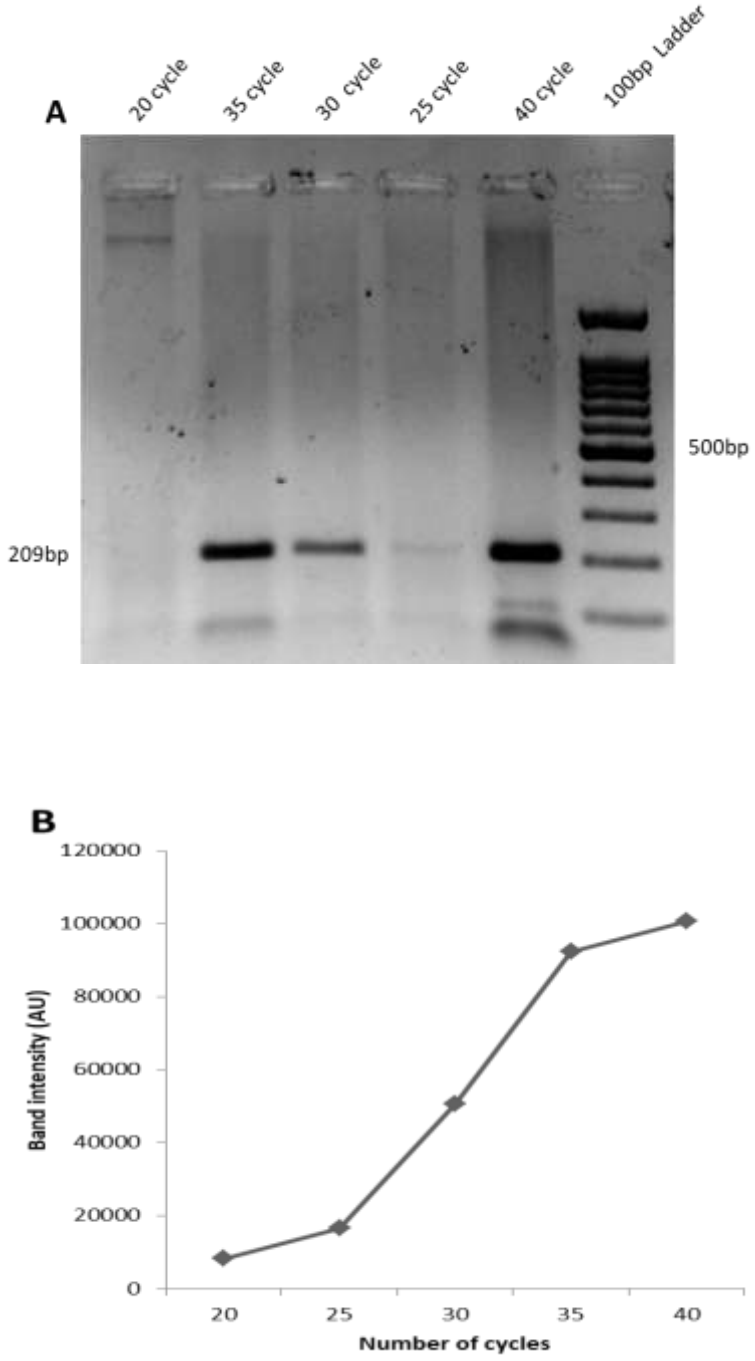


Figure 4.11.3: Standardization of number of thermocycles for TNF-a RT-PCR. PCR reaction was performed with 150ng RNA from resting RBL-2H3 for different number cycles at 56°C. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION SYSTEM as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

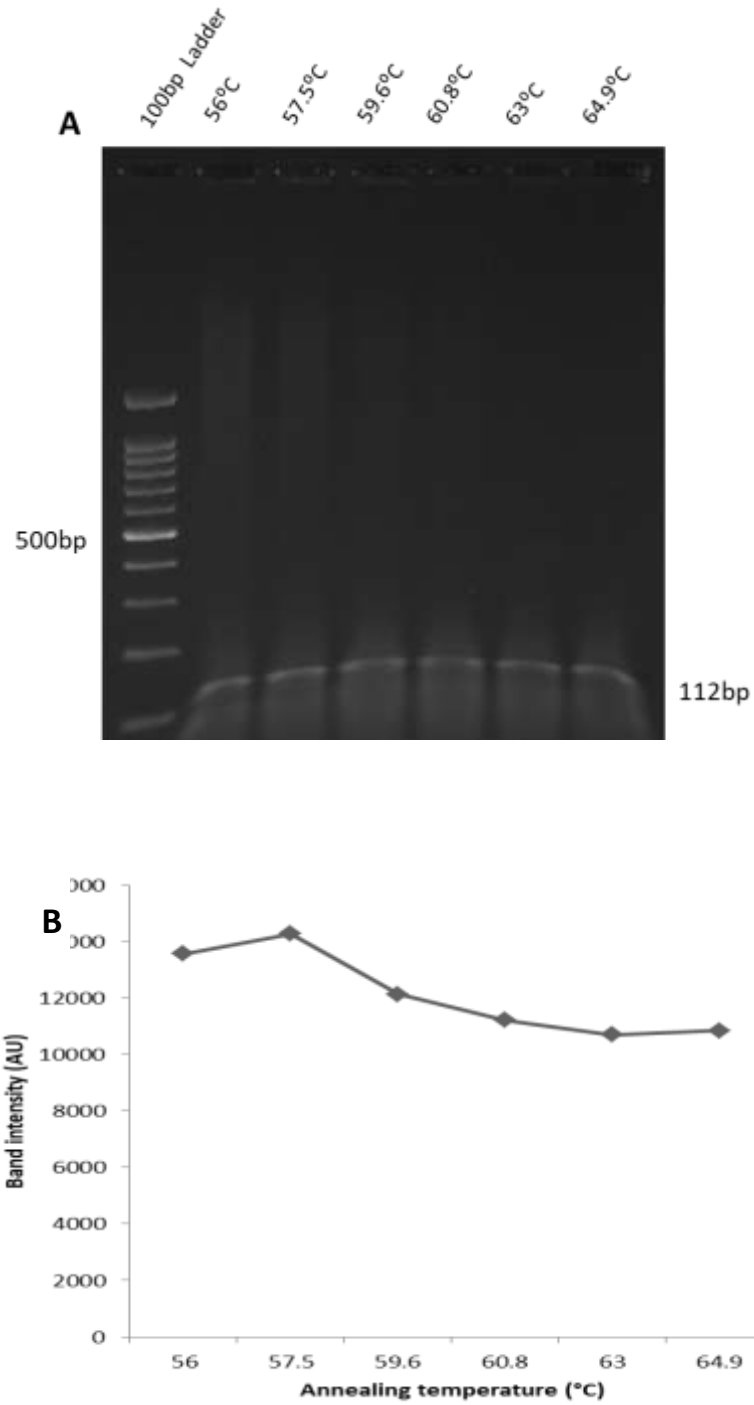


Figure 4.12.1: Standardization of annealing temperature for MIP-1α RT-PCR. PCR reaction was performed by starting with 300ng RNA from activated RBL-2H3 for 35 cycles at various temperatures. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

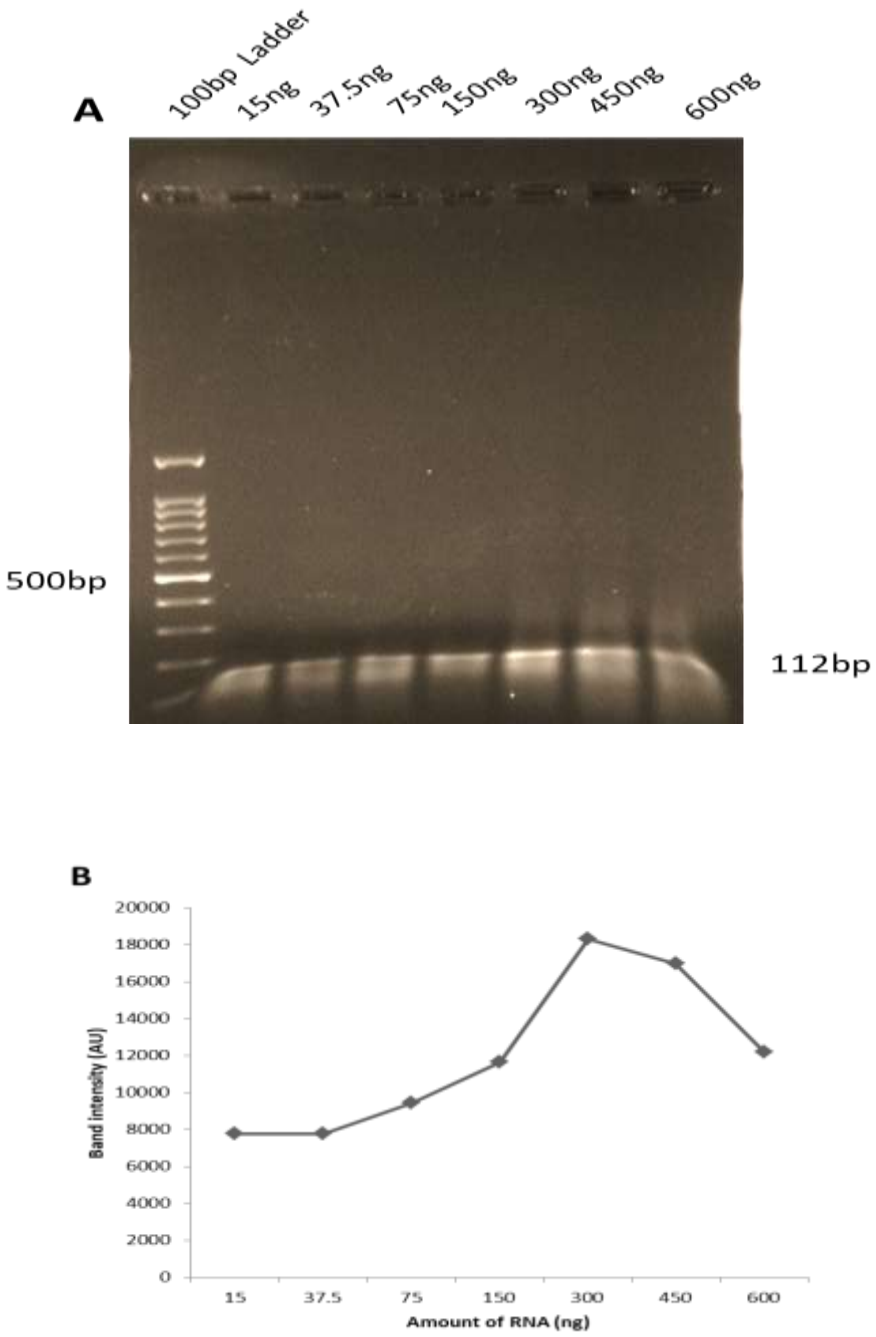


Figure 4.12.2: Standardization of amount of RNA template for MIP-1α RT-PCR. PCR reaction was performed using different amount of RNA from activated (2 HR) RBL-2H3 for 35 numbers of cycles at 57.5°C. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION SYSTEM as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

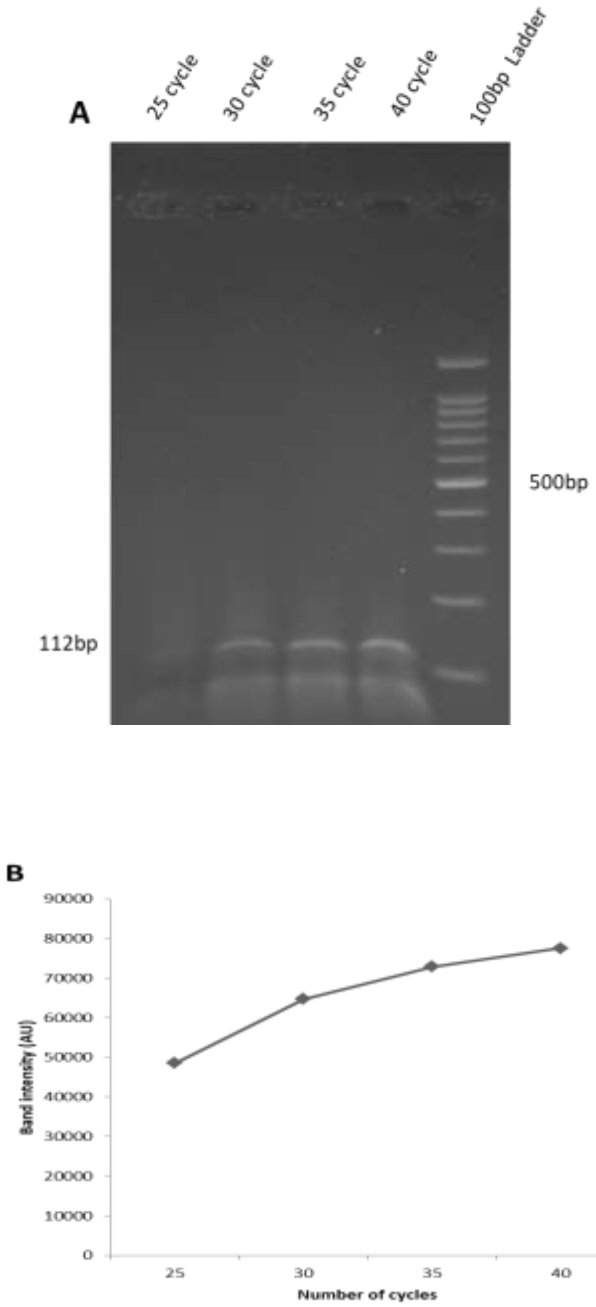


Figure 4.12.3: Standardization of number of thermocycles for MIP-1α RT-PCR. PCR reaction was performed with 300ng RNA from activated (2 hr) RBL-2H3 for different number cycles at 57.5°C. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION SYSTEM as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

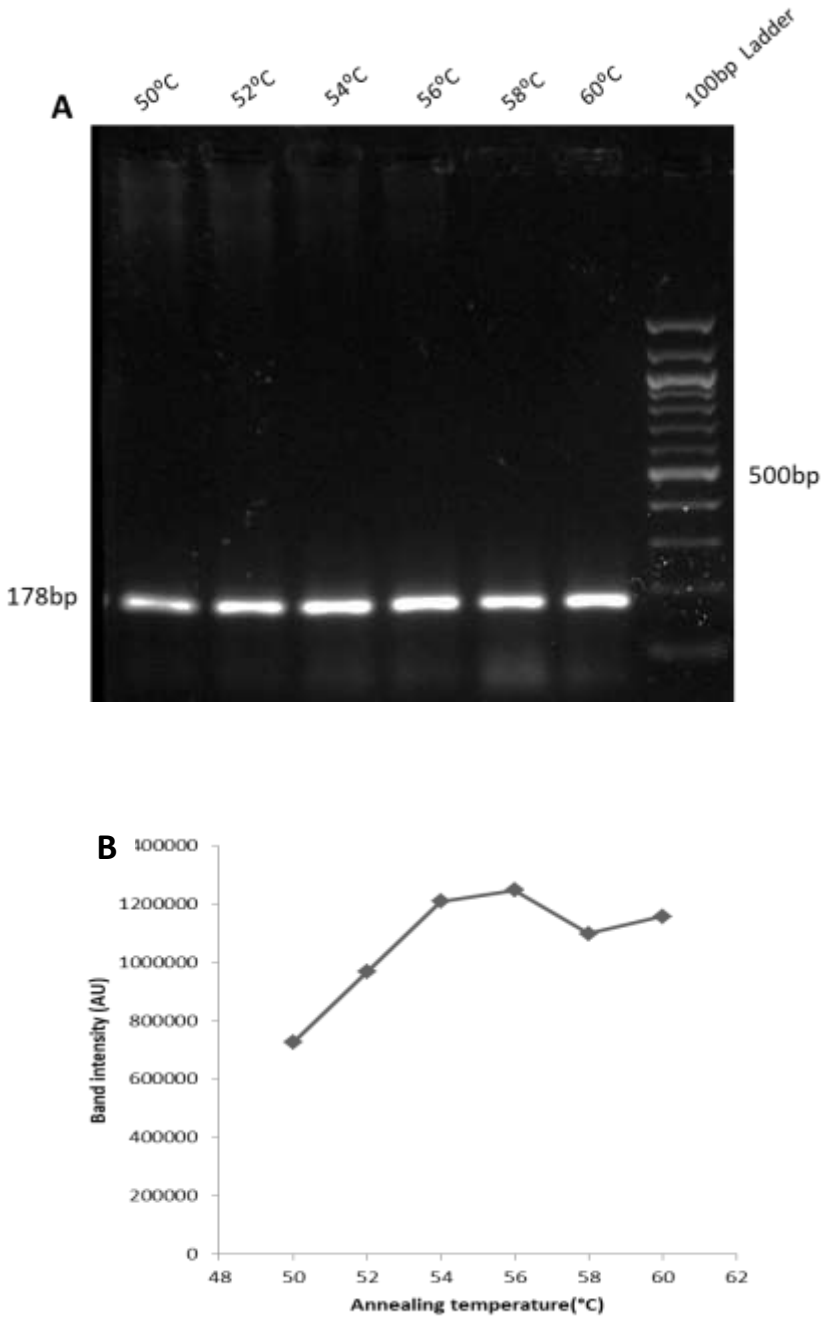


Figure 4.13.1: Standardization of annealing temperature for GAPDH RT-PCR. PCR reaction was performed by starting with 300ng RNA from resting RBL-2H3 for 35 cycles at various temperatures. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

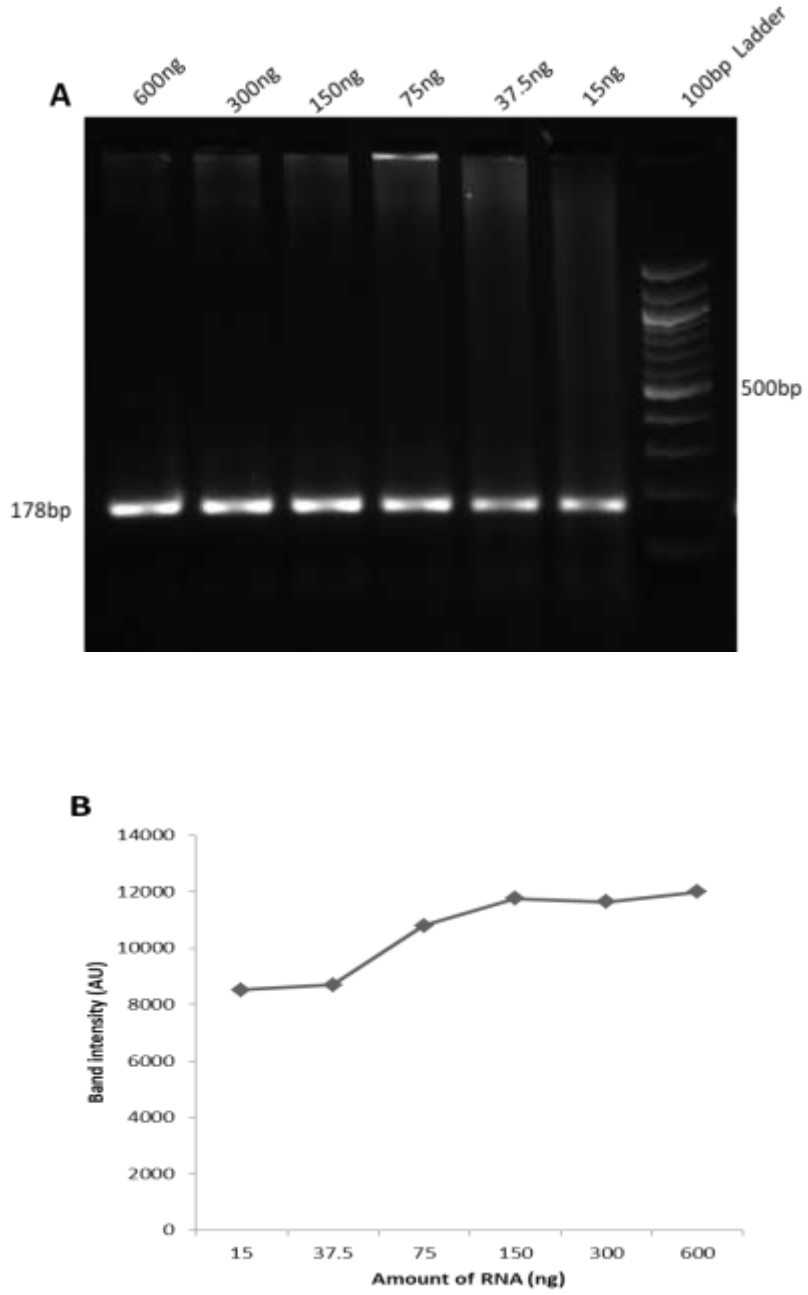


Figure 4.13.2: Standardization of amount of RNA template for GAPDH RT-PCR. PCR reaction was performed using different amount of RNA from resting RBL-2H3 for 35 numbers of cycles at 54°C. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION SYSTEM as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

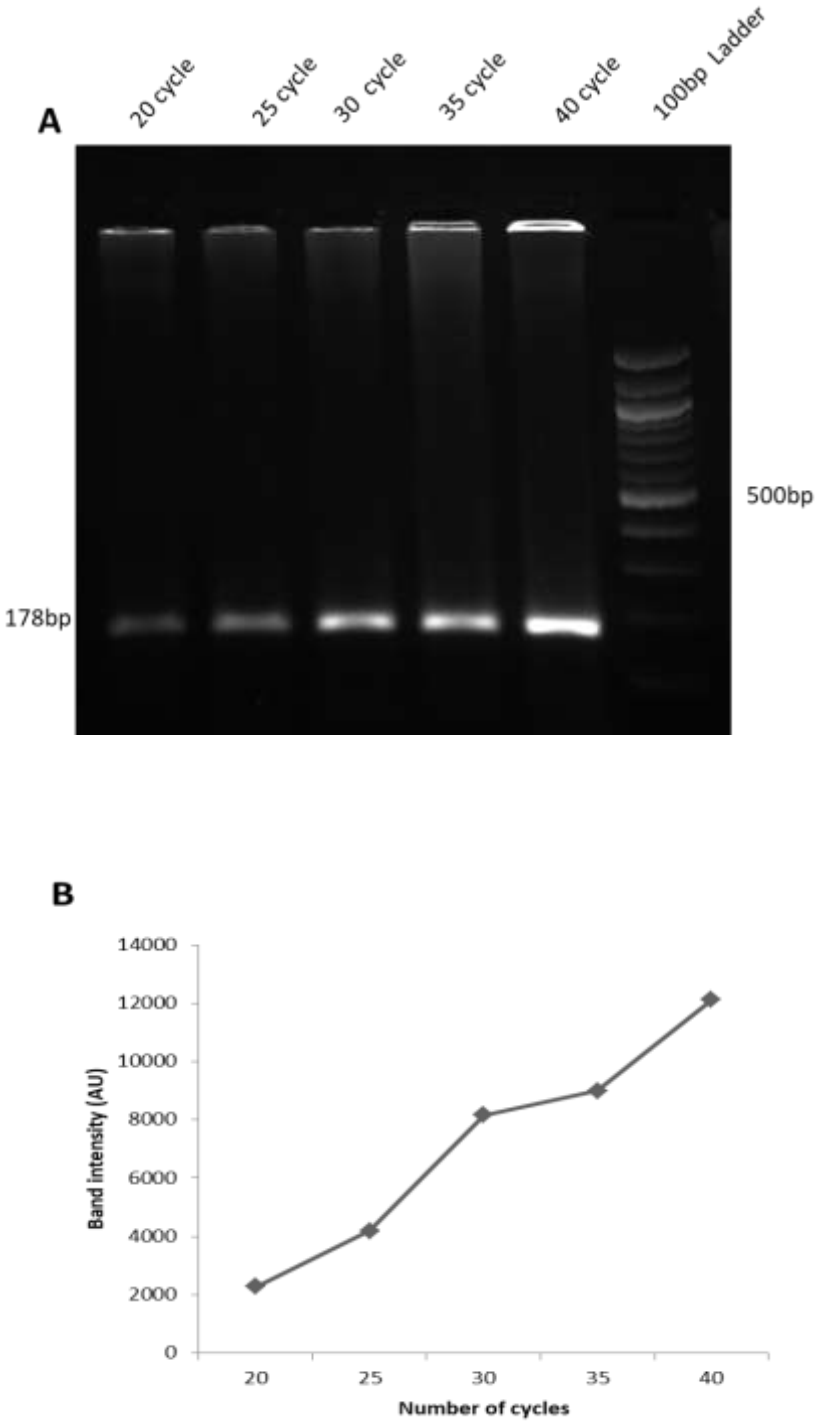


Figure 4.13.3: Standardization of number of thermocycles for GAPDH RT-PCR. PCR reaction was performed with 75ng RNA from resting RBL-2H3 for different number cycles at 54°C. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION SYSTEM as shown in figure A. Thus obtained band intensity were quantified using alpha ease gel quantitation software as shown in figure B.

Table 4.2 Standardized PCR conditions for amplification of different genes

S.N.	Name of cytokines	Annealing Temperature(°C)	Concentration of RNA(ng)	Number of Cycles(cycles)
1	IL-4	52	300	30
2	IL-5	64	300	35
3	IL-6	56	150	35
4	IL-13	56	150	35
5	TNF-α	56	150	35
6	MIP-1α	57.5	300	35
Housekeeping gene				
7	GAPDH	54	75	30

4.5 Heterogeneity in mast cell response after treating mast cell with different antigens, pathogen and pathogen derived products using standardized conditions

PCR condition for all the cytokines, chemokines and housekeeping gene was standardized for PCR amplification. The expression of IL-4, IL-5, IL-6, IL-13, TNF-α, MIP-1α and GAPDH was compared in mRNA level after treatment with different triggers. GAPDH is used as internal control; whose expression is almost similar in all cells after different treatments as shown in figure 4.14. IL-4 is expressed only in rest RBL cells, RBL cells treated with DNP-BSA and in RBL cells treated with DPT as shown in figure 4.15. The intensity of IL-4 expression is higher in cells treated with DNP-BSA than DPT and rest RBL. IL-5 is moderately expressed in cells treated with DPT as shown in figure 4.16.

Similarly IL-6 is expressed during all treatments. In comparison to rest RBL cells the cells treated with DNP-BSA have highest expression followed by cells treated with DPT, BCG and Poly I:C. IL-6 expression is similar as compared to rest in all other remaining treatments as shown in figure 4.17. IL-13 is expressed only in rest and DNP-BSA treated cells. The expression of IL-13 increases as compare to rest RBL cells in DNP-BSA treated cells as shown in figure 4.18. In addition the expression level of TNF-α is also different in all treatments. TNF-α is expressed in all treatments except in cells co-cultured with *Leishmania tropica*. TNF-α expression is highest in cells treated with DNP-BSA. In comparison to resting cells TNF-α expression level decreases in all other treatments as shown in figure 4.19. Furthermore MIP-1α is expressed in all the cells with different treatment. In comparison to rest RBL cells its expression is slightly higher in cells co-cultures with *Leishmania donovani* and cells treated with poly I:C. In all other treatments the expression level is same for MIP-1α as shown in figure 4.20.

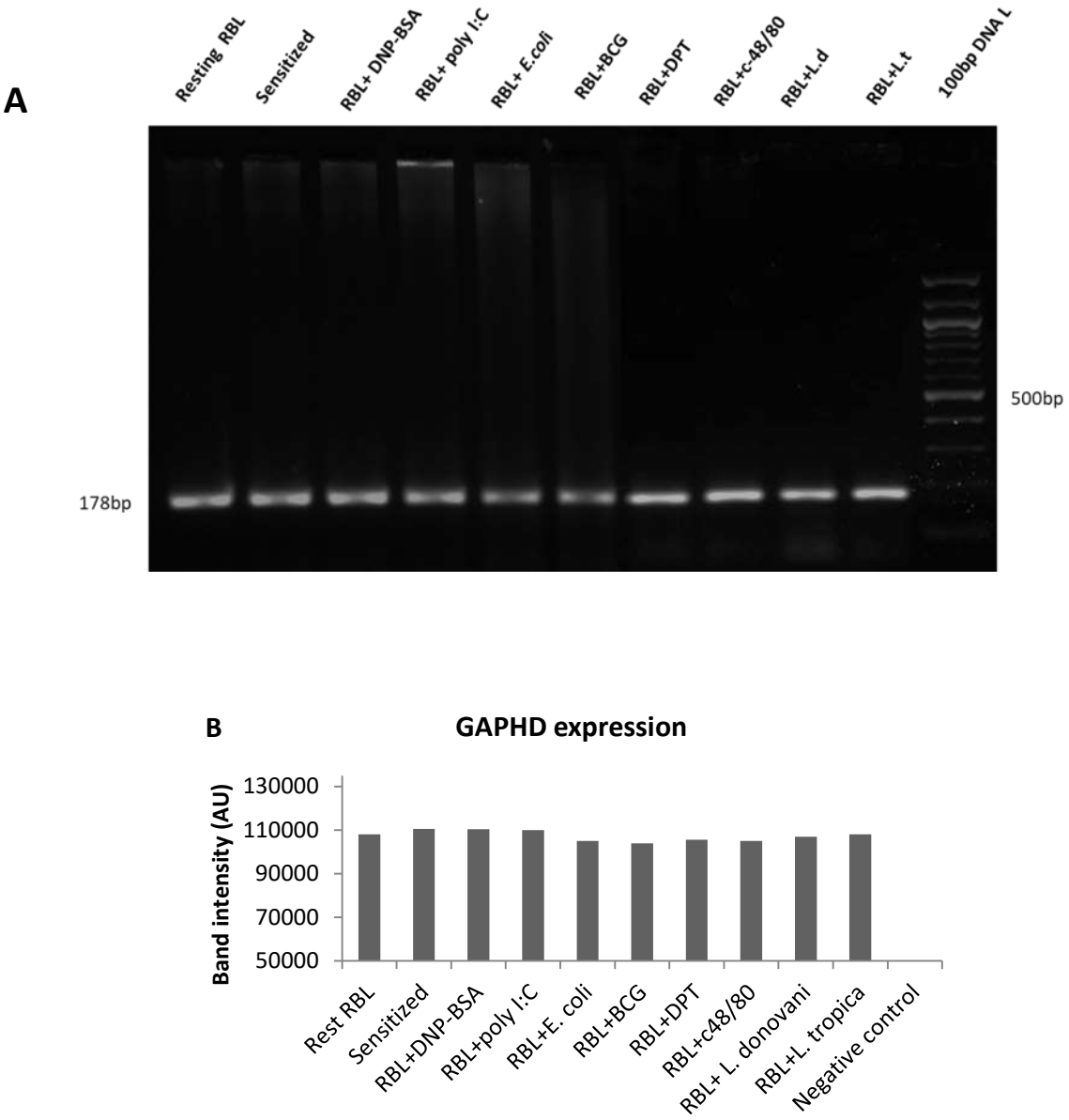


Figure 4.14: GAPDH expression profile by RBL-2H3 cells after treated with different treatments (A) 1.2% agarose gel electrophoresis (with EtBr staining) of GAPDH expression by RBL co-cultured with *L. tropica* and *L. donovani*, RBL cells treated with C48/80, BCG, sonicated *E.coli*, poly I:C, DNP-BSA, sensitized with IgE and resting RBL-2H3 cells respectively from lane 1 to lane 10 along with 100bp DNA ladder. PCR was performed using 75 ng of RNA from different treatment at 54^{o}C for 30 cycles. The obtained bands were quantified by using alpha ease gel quantitation software as shown in figure B.

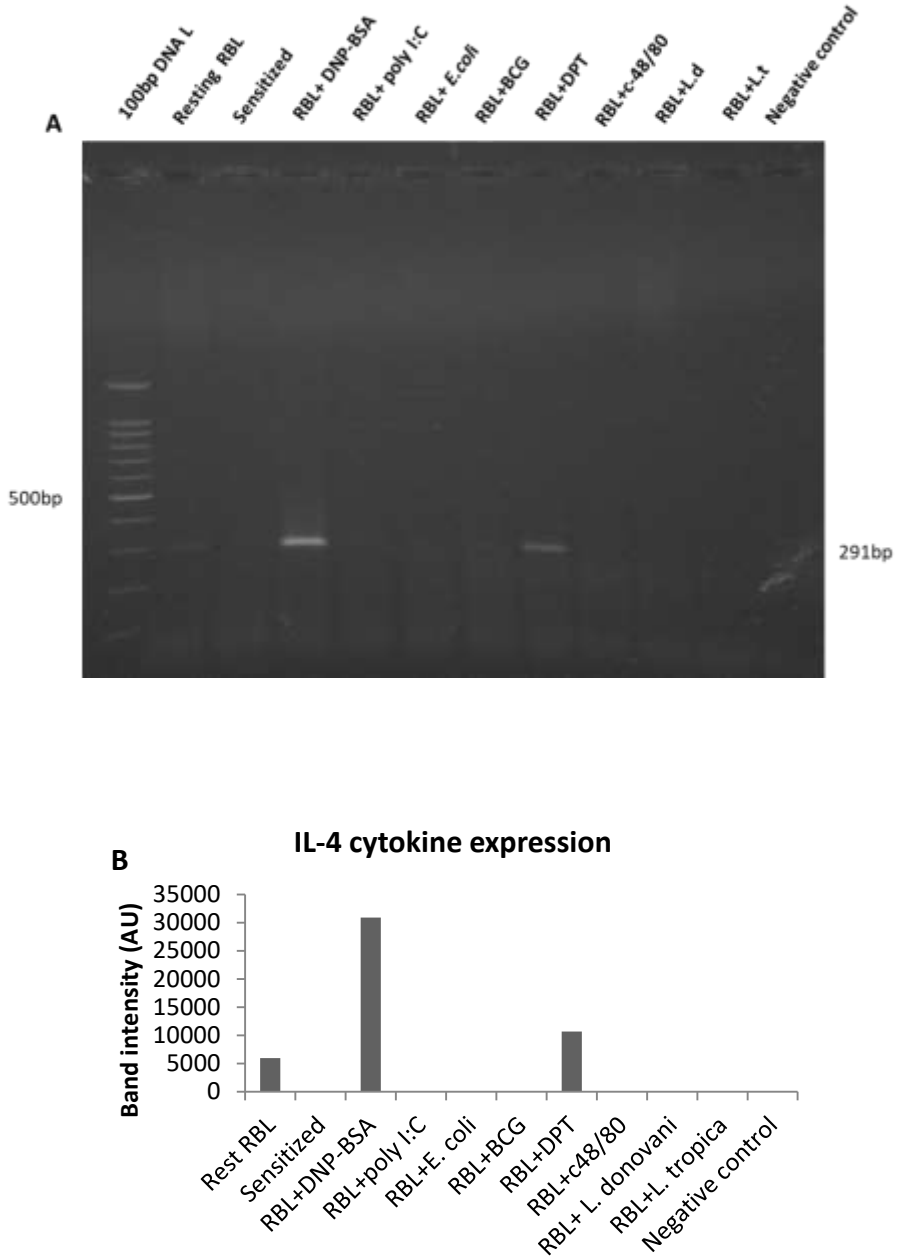


Figure 4.15: IL-4 expression by RBL-2H3 cells after treated with different treatments. (A) 1.2% agarose gel electrophoresis (with EtBr staining) of IL-4 expression by resting RBL-2H3 cells, sensitized RBL, RBL treated with DNP-BSA, poly I:C, *E.coli*, BCG, DPT, C48/80, co-culture with *L. donovani* and *L.tropica* and negative control respectively from lane 2 to lane 12 along with 100bp DNA ladder. PCR was performed using 300 ng of RNA from different treatment at 52^{o}C for 30 cycles. The obtained bands were quantified by using alpha ease gel quantitation software as shown in figure B.

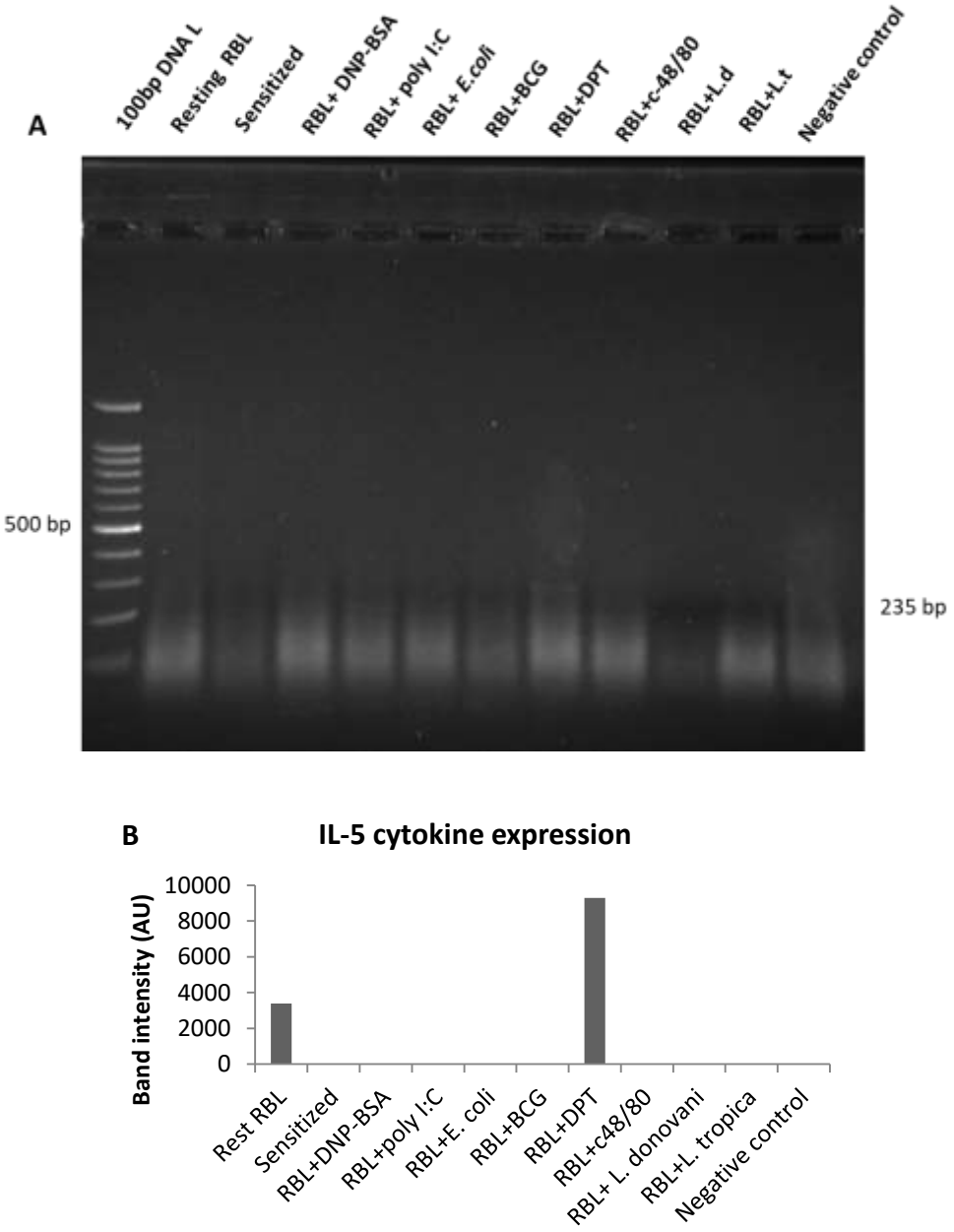


Figure 4.16: IL-5 expression by RBL-2H3 cells after treated with different treatments. (A) 1.2% agarose gel electrophoresis (with EtBr staining) of IL-5 expression by resting RBL-2H3 cells, sensitized RBL, RBL treated with DNP-BSA, poly I:C, *E.coli*, BCG, DPT,C48/80, co-culture with *L. donovani* and *L.tropica* and negative control respectively from lane 2 to lane 12 along with 100bp DNA ladder. PCR was performed using 300 ng of RNA from different treatment at 64^{o} c for 35 cycles. The obtained bands were quantified by using alpha ease gel quantitation software as shown in figure B.

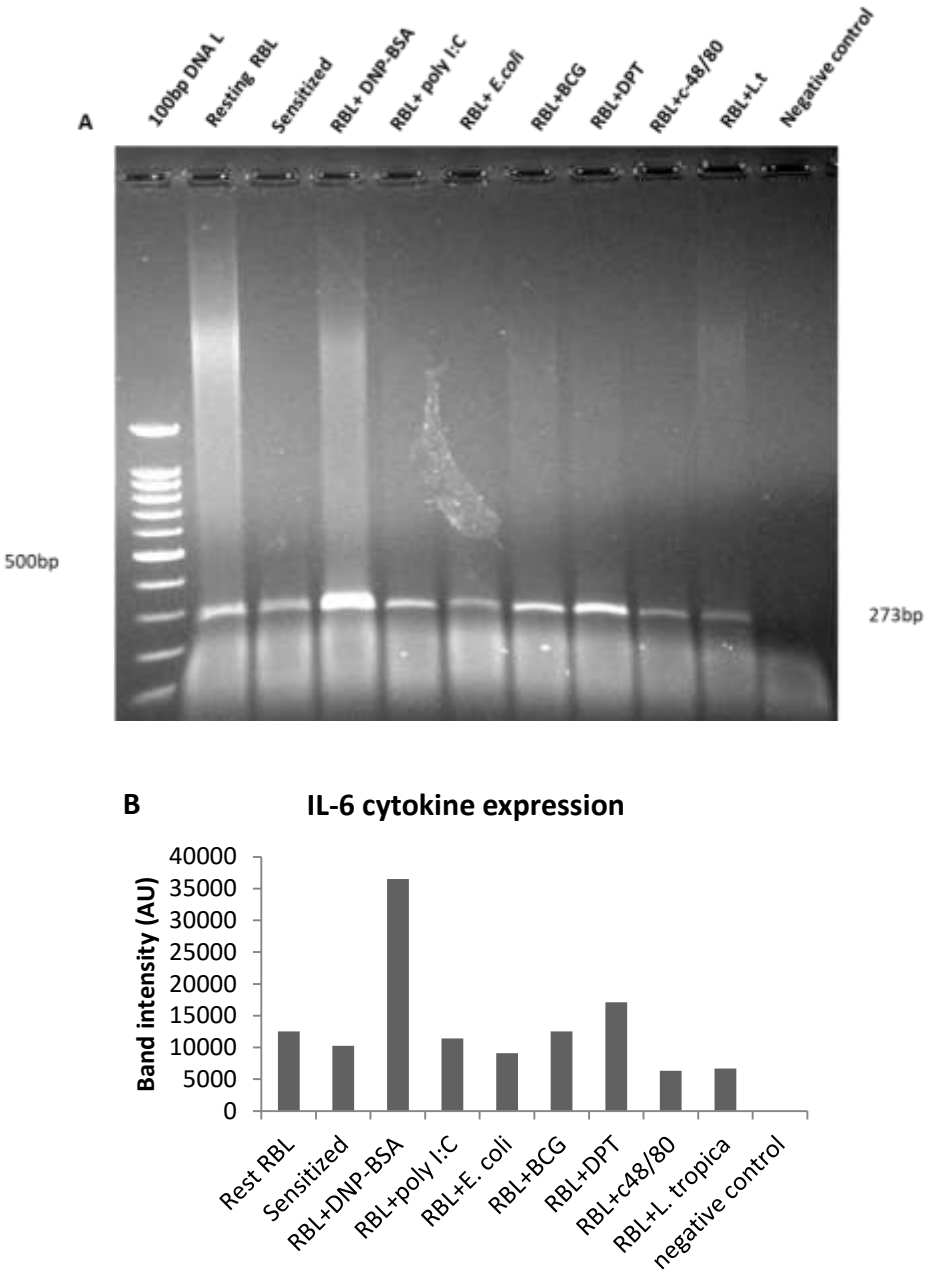


Figure 4.17: IL-6 expression by RBL-2H3 cells after treated with different treatments. (A) 1.2% agarose gel electrophoresis (with EtBr staining) of IL-6 expression by resting RBL-2H3 cells, sensitized RBL, RBL treated with DNP-BSA, poly I:C, *E.coli*, BCG, DPT,C48/80, co-culture with *L.tropica* and negative control respectively from lane 2 to lane 11 along with 100bp DNA ladder. PCR was performed using 150 ng of RNA from different treatment at 56^{o} c for 35 cycles. The obtained bands were quantified by using alpha ease gel quantitation software as shown in figure B.

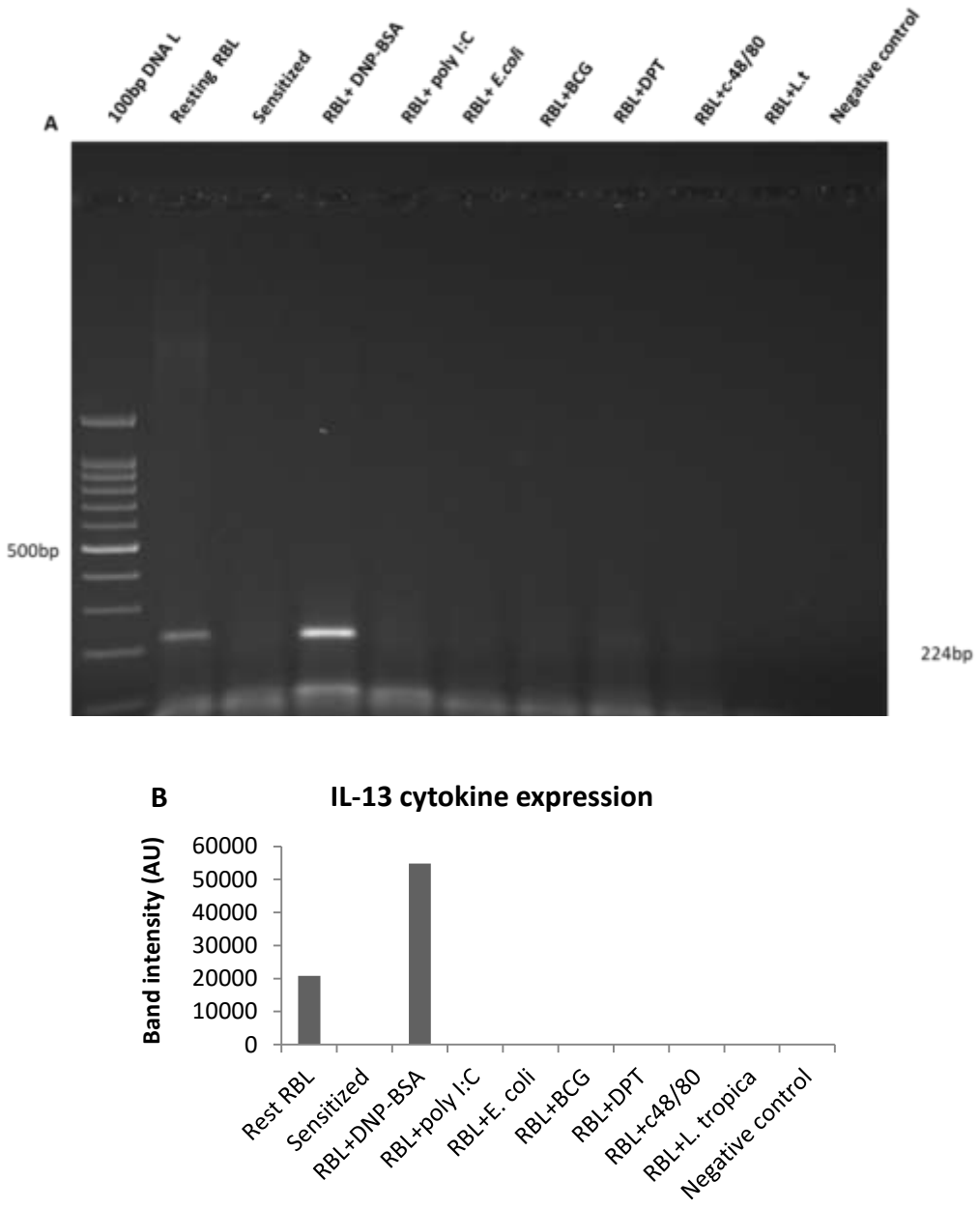


Figure 4.18: IL-13 expression by RBL-2H3 cells after treated with different treatments. (A) 1.2% agarose gel electrophoresis (with EtBr staining) of IL-13 expression by resting RBL-2H3 cells, sensitized RBL, RBL treated with DNP-BSA, poly I:C, *E.coli*, BCG, DPT,C48/80, co-culture *L.tropica* and negative control respectively from lane 2 to lane 11 along with 100bp DNA ladder. PCR was performed using 150 ng of RNA from different treatment at 56^{o} c for 35 cycles. The obtained bands were quantified by using alpha ease gel quantitation software as shown in figure B.

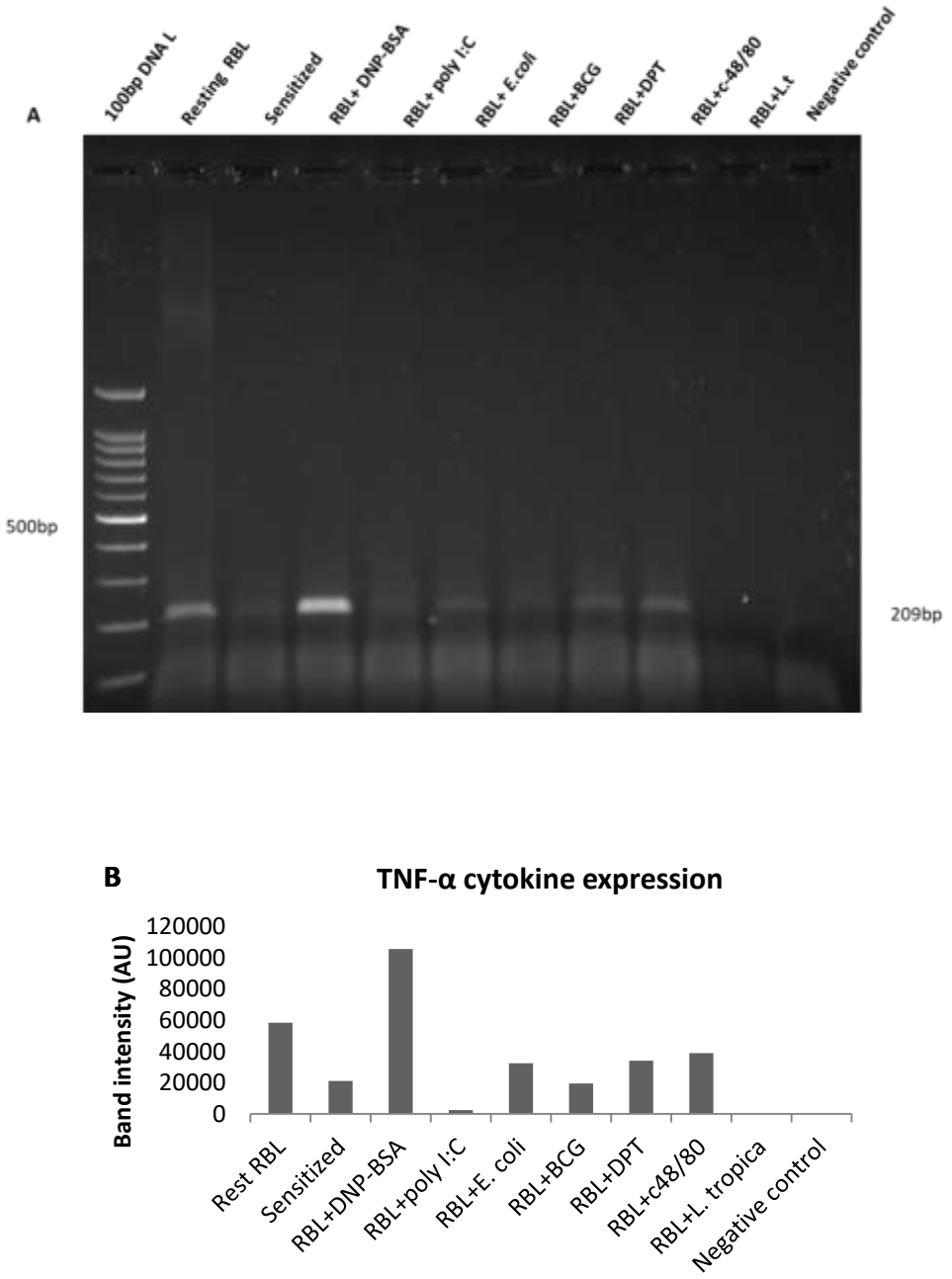


Figure 4.19: TNF-α expression by RBL-2H3 cells after treated with different treatments. (A) 1.2% agarose gel electrophoresis (with EtBr staining) of TNF-α expression by resting RBL-2H3 cells, sensitized RBL, RBL treated with DNP-BSA, poly I:C, *E.coli*, BCG, DPT,C48/80, co-culture with *L. donovani* and *L. tropica* and negative control respectively from lane 2 to lane 11 along with 100bp DNA ladder. PCR was performed using 150 ng of RNA from different treatment at 56^{o} c for 35 cycles. The obtained bands were quantified by using alpha ease gel quantitation software as shown in figure B.

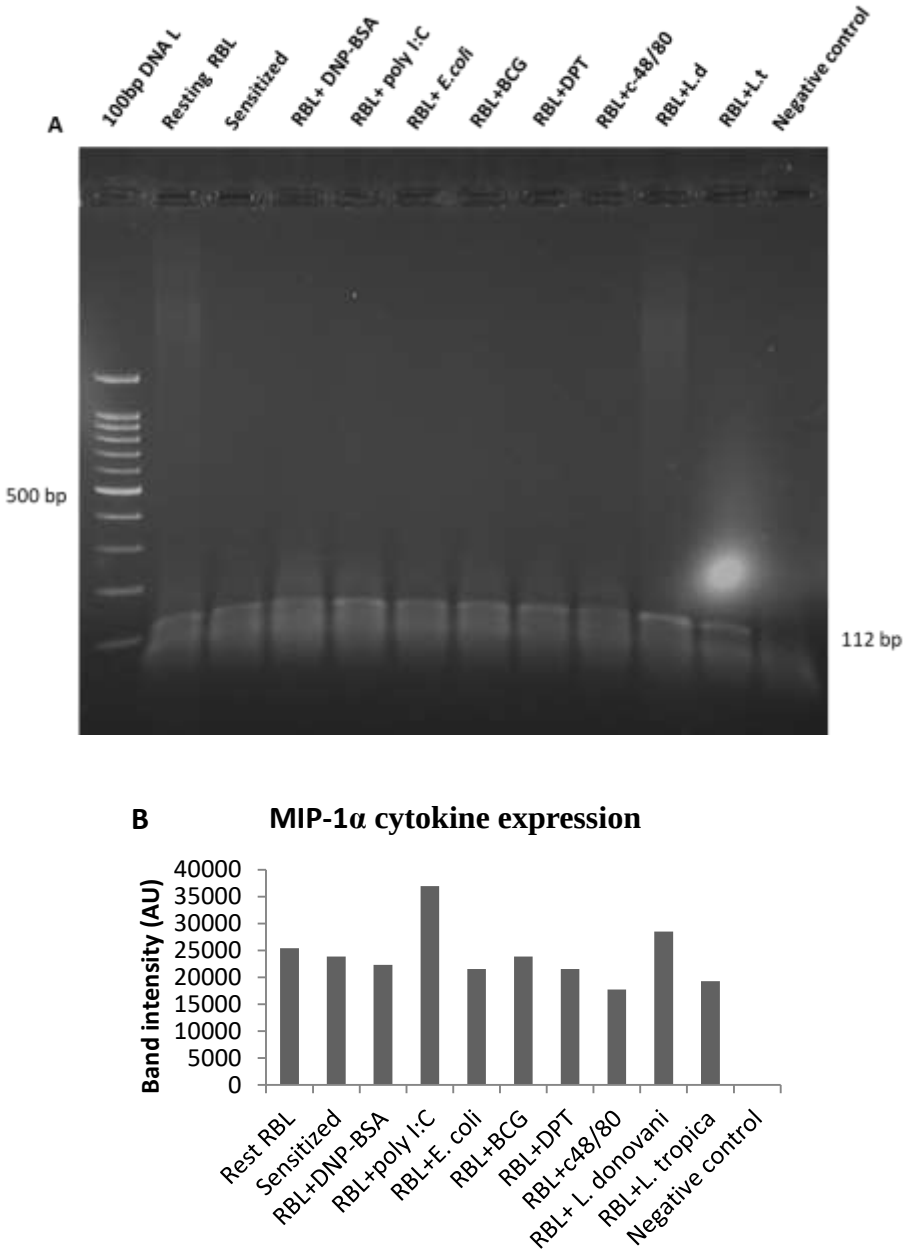


Figure 4.20: MIP-1α expression by RBL-2H3 cells after treated with different treatments. (A) 1.2% agarose gel electrophoresis (with EtBr staining) of MIP-1α expression by resting RBL-2H3 cells, sensitized RBL, RBL treated with DNP-BSA, poly I:C, *E.coli*, BCG, DPT,C48/80, co-culture with *L. donovani* and *L. tropica* and negative control respectively from lane 2 to lane 12 along with 100bp DNA ladder. PCR was performed using 300 ng of RNA from different treatment at 57.5^{o} c for 35 cycles. The obtained bands were quantified by using alpha ease gel quantitation software as shown in figure B.

Chapter 5
Discussion

Mast cells are essential immune cells of myeloid lineage. They are localized at the junctions where the external environment meets the internal host environment. The activation and degranulation phenomenon of mast cells significantly regulates many aspects of physiological and pathophysiological conditions (Marshall, 2004). Mast cells regulate vasodilation, vascular homeostasis, innate and adaptive immune responses, angiogenesis, and venom detoxification as a normal physiological function. Whereas they are also involved in pathophysiological conditions of many diseases including allergy, asthma, anaphylaxis, gastrointestinal allergic disorders and cardiovascular diseases (Krystel-Whittemore et al., 2016). We aimed to explore the response of mast cells in terms of mediator release when activated by different antigens or pathogenic challenges.

For this purpose, we used RBL-2H3 cells line. RBL-2H3 cell line is continuous rat cell line which originated from leukemic cells isolated from rats that were treated with chemical carcinogen β-chlorethylamine (Passante et al., 2009). RBL-2H3 cell line is extensively used in studying mast cells related disorders including allergy because of their strong response of the cells to IgE and its FcεRI receptor (Wang et al., 2012). They are highly stable and can also be genetically modified. It can also be humanized and a powerful tool for studying intracellular membrane trafficking, exocytosis, detection of allergens, safety of vaccines and diagnosis of allergic sensitization (Passante et al., 2009, Falcon et al., 2015). First of all the growth kinetics of RBL-2H3 cells in Rat Basophilic Leukemic (RBL) media and Dulbecco Modified Eagle Medium (DMEM) media was determined. We compared two parameters including doubling time and morphology of cells. The cells exhibited fibroblast like morphology and looked healthy in both media. The doubling time was found to be 15.12 hour and 18.9 hour in RBL and DMEM media respectively. Cells doubled 3 hour earlier in RBL media as RBL media is consisted of more nutrients and serum than DMEM media. But, we selected DMEM media and maintained cultures in DMEM media as suggested by ATCC.

Bone marrow derived cultured mouse mast cells can undergo single round of re-activation to release mediators such as β-hexosaminidase after replenishment of their granular content (Xiang et al., 2001). The consequences of mast cells responses to multiple challenges are not known yet. That's why we explored the response of mast cells in multiple allergenic challenges. Cross-linking of FcεRI bound IgE with multivalent antigens initiates the activation of MCs by promoting the aggregation of FcεRI mimicking the allergic challenge in *in vitro* condition (Naskar & Puri, 2017). Mast cells were first sensitized with anti DNP-BSA specific IgE followed by activation by DNP-BSA. The cells were challenged three times to induce MCs exocytosis. The extent of exocytosis was determined by analyzing the amount of lysosomal enzyme: β-hexosaminidase released. Study on secondary challenge after 48 hours in mouse mast cells shows that there is 31% reduction in β-hexosaminidase release when compared to primary challenge (Xiang et al., 2001). We challenged cells after 24 hour and got 12% and 14% reduction in percent secretion of β-hexosaminidase during secondary and tertiary challenge respectively when compared to primary. The difference in β-hexosaminidase release

was significant during both challenges; secondary and tertiary when compared to primary challenge. Total granular content remains constant after each challenge. Even after multiple activations, the cells were capable to secrete β-hexosaminidase which indicates that the mast cells undergo recovery after activation even in multiple challenges. This feature of mast cells can help in exploring the mast cells in terms of allergic disorders. Further we explored the effect of multiple allergen challenge on FcεRI receptor expression in mast cells after multiple sensitizations by FACS. Aggregation of FcεRI receptor after binding of IgE leads to activation of downstream signaling resulting in mast cell mediator release. So, we were interested to know whether the surface receptor expression is affected by multiple sensitizations. We found no difference in FcεRI receptor expression during any of the sensitization as shown by mean fluorescent intensities. These results conclude that the surface receptors expression of FcεRI receptor is not affected by the number of sensitizations.

Mast cells directly influence many biological processes including defense response against parasites, bacteria and viruses, wound healing, angiogenesis, autoimmune disorders, metabolic disorders as well as in cancer (Mukai et al., 2018; Naqvi et al., 2017). These function of mast cells are because of their ability to secrete large spectrum of cytokines (IL-4, IL-5, IL-6, IL-13, TNF-α) and chemokines (MIP-1α, MIP-2) (Mukai et al., 2018). During inflammation and allergic disorders, there is influx of leukocytes at the site of inflammation which is mediated by cytokines and chemokines secreted by mast cells (Sewell et al., 1998). We aimed to explore the release of cytokines and chemokine by mast cells after their treatment with different antigens, pathogens and pathogen derived products.

The quantitative RT-PCR based method relies on co-amplification of the cDNA of interest, with a housekeeping gene as control. Housekeeping genes such as glyceraldehyde-3 phosphate dehydrogenase (GAPDH) are commonly used as reference genes as they are constitutively expressed in the cells. RT-PCR technique an extensively used approach as it offers the advantage of being superior, sensitive, cost efficient, simple, highly specific and versatile. The protein-based methodologies are often time consuming, not adapted to low number of cells, and antibodies of newly characterized proteins are not always available. Conversely, the development of molecular biology techniques enables detection of gene expression at the RNA level with their quantification. So we decided to use quantitative RT-PCR as our method of choice to study expression analysis of pro-inflammatory cytokines and chemokine secreted by mast cells. We selected inflammatory cytokines including IL-4, IL-5, IL-6, IL-13, TNF-α; chemokine MIP-1α from literature survey.

TH2 cells secrete IL-4, IL-5, IL-6, IL-13 (Williams & Galli, 2000). IL-4 involves in development of Th2 cells and allergic reaction. These influences the differentiation ,growth and functions of mast cells itself as well as involves in development, proliferation and survival of monocyte, macrophage, B-cells and T-cells (Church, 1995; Moller et al., 1998). IL-5 influences immune response and is growth factor and chemo-attractant for eosinophils (Akatsu, 2011; Church, 1995; Mukai et al., 2018). Similarly IL-6 has a major role in inflammation and pathogenesis of asthma or allergic response. Though they are one of the main pro-inflammatory cytokines, it is also involved in

growth and survival of mast cell itself and is required for development and proliferation of neutrophils, eosinophils and basophils (Church, 1995; Gordon et al., 1990; Moller et al., 1998; Stanley et al., 2018). IL-13 is involved in type 2 immune response and its functions overlaps with IL-4. They are involved in host defense against parasitic infection (Mukai et al., 2018). TNF-α is another major pro-inflammatory cytokines that is involved in gastric inflammation and provide immunity against bacteria. It recruits leukocytes and neutrophils at the site of inflammation and is also involved in migration of dendritic cells to draining lymph nodes (Gordon et al., 1990; Stanley & Lacy, 2018). Chemokine MIP-1α helps mast cells migration towards macrophages, monocytes, eosinophils and basophils(Moller et al., 1998).

The PCR amplification conditions for IL-4, IL-6, IL-13, TNF- α and GAPDH were standardized in resting RBL cells while IL-5 and MIP-1α were standardized in activated RBL cells. Cytokine's expression by RBL cells during different triggers were analyzed; the cells were treated with different antigens; allergen (DNP-BSA), vaccine (DPT), pathogens (*Leishmania donovani* and *Leishmania tropica*), pathogenic derived product (Poly I:C, sonicated *Escherichia coli* and BCG) and chemical degranulator of mast cells (Compound 48/80). Resting and IgE-sensitized mast cells were taken as control. After different treatments, RNA was isolated, and cDNA was synthesized followed by PCR amplification of cytokines and chemokine mRNA. GAPDH expression was similar in all the cells after treatment. Its expression was not altered during treatment.

IL-4 mRNA expression was dependent on IgE-cross linking in the presence of SCF (Bradding et al., 1999). mRNAs for IL-4, IL-5, IL-6, IL-13 and MIP-1 α were expressed at low levels in unstimulated cells. Since TNF-α is prestored and constitutively expressed, mRNA level for TNF-α in unstimulated cells was little high as compared to other cytokines as shown in figure 5.1. After stimulation with antigen, mRNA levels for IL-4, IL-6, IL-13 and TNF-α were markedly increased. IL-4 mRNA was also induced in DPT treatment. In other treatments, whether it was decreased or not can further be explored. IL-5 has a prominent role in cellular infiltration of eosinophils during asthma and other disorders with eosinophilia (Sewell et al., 1998). During standardizations, IL-5 expression was induced after allergen stimulation but after other treatments a faint band was observed only in DPT treated samples.

In comparison to rest and IgE sensitized RBL, the expression of IL-6 mRNA increased in allergen plus IgE treated cells, DPT treated cells and BCG treated cells. IL-6 expression decreased in poly I:C, *Escherichia coli*, C48/80 and *Leishmania donovani* treatments. IL-13 was expressed only in rest and allergen activated cells. The expression of IL-13 increased as comparison to resting cells in allergen activated cells. Other triggers did not induce the IL-13 mRNA expression. TNF-α is prestored in mast cell as well as can be induced by allergen, C48/80 (Mukai et al., 2018). Here TNF-α mRNA was expressed in Resting RBL cells and its expression increased in cells treated with allergen. However, its expression slightly decreased in C48/80 treated cells and markedly decreased in other treatments in comparison to resting cells. MIP-1α is a potent activator of basophil, and also involved in migration of immune cells including T-cells, Basophil, Eosinophil, Neutrophil, dendritic cells, monocyte and macrophages (Alam, Forsythe, Stafford, Lett-Brown, & Grant, 1992). MIP-1α was expressed in all treated cells. As compared to resting

cells, its expression increased in RBL cells co-cultured with *Leishmania donovani* and poly I:C treated cells.

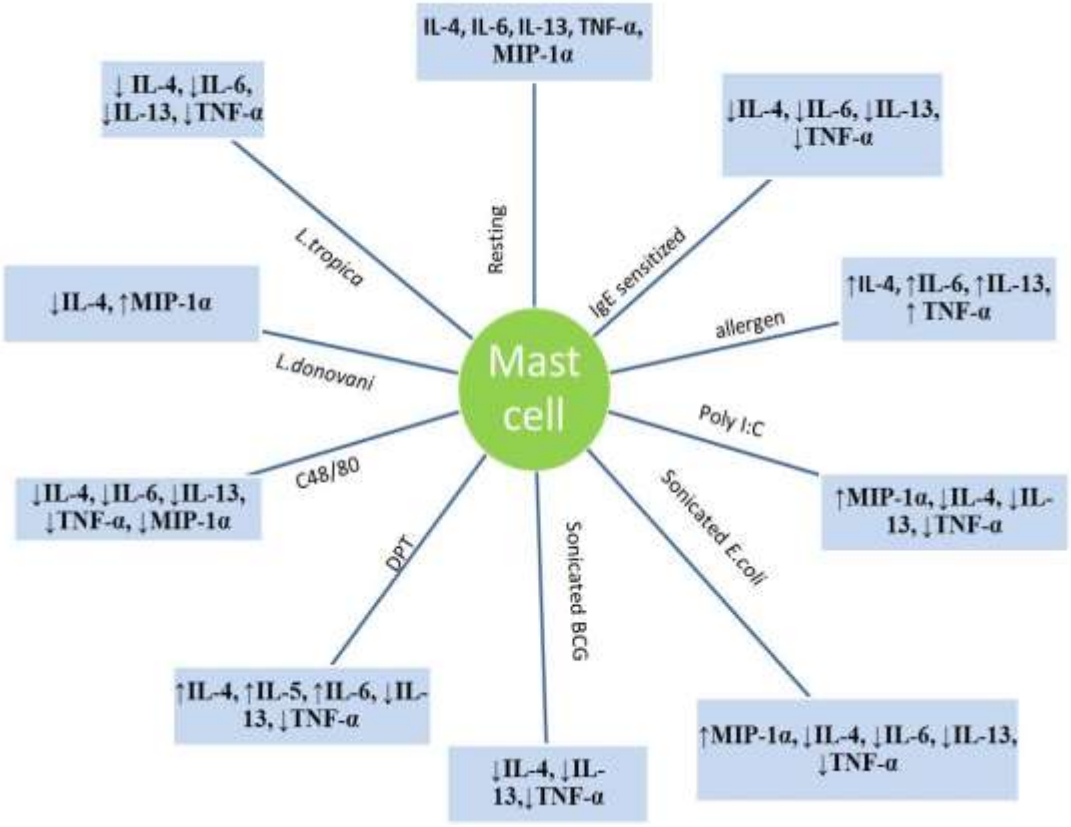


Figure 5.1: ***Schematic representation of up and down regulation of cytokines*** *in comparison to control (resting) that continuously express IL-4, IL-6, IL-13, TNF-α and MIP-1α after different treatments.*

Taking altogether, our preliminary results showed that the mRNA expression, of all the above cytokines and chemokines during various treatments or challenges, was differentially regulated in the RBL-2H3 cell line, an analog of mucosal mast cells. mRNA expression for all the cytokines we selected (except IL-5) were increased after allergen stimulation. Among all the treatments, DPT was found to be promising as mRNA levels were induced for some cytokines. This can further be explored for mast cell responses to DPT treatments. This shows that mast cells respond differently to different triggers. As mRNA levels of different cytokines are changing, this may lead to differential secretion of these cytokines as suited to combat that particular threat. Increase in mRNA level may be due to fresh transcription as well as due to increased stability of mRNA. These studies can further be useful in revealing mast cells responses during various infection or vaccine administrations and also during infection or vaccine development in atopic individuals during co-challenge experiments.

Chapter 6
Summary

Mast cells are innate immune cells that are present throughout the body, especially in the periphery where there is direct contact of host with external agents. They are the first cells along with macrophages and dendritic cells to encounter incoming challenges (Krystel-Whittemore et al., 2016). MCs perform their functions by altering the inflammatory environment after recognition of an infection and by attracting several other cells of immune system at the site of infection (Galli et al., 2005). They are capable of secreting a wide range of pharmacologically active mediators (Dahlin & Hallgren, 2015). MCs possess numbers of receptors for important regulatory compounds, such as growth factors, activators of secretion and receptors for adhesive molecule that enable the mast cells to attach to certain sites (Stone et al., 2010). Appropriate receptors and signaling pathways in MCs made them capable to defend incoming antigens or pathogens involving innate immune as well as adaptive immune response for successfully tackling the incoming threats. After activation, MCs start releasing mediators including different proteases, amines, cytokines and chemokines (Stone et al., 2010). These mediators help in kick-starting an inflammatory response. Mediators especially cytokines and chemokines change the immediate environment and also recruit and activate many other cells (Mukai et al., 2018). Regranulation after degranulation capacity and long life-span of mast cells make it more sustainable in host's responses for long time periods. This capacity is disadvantageous in allergic disease and also in chronic inflammatory conditions. Similarly the actions of mast cells in early infection are beneficial but its excessive action have detrimental impact on health during chronic or overwhelming infection (Rao & Brown, 2008).

The release of mediators in response to different stimuli is also poorly understood so we aimed to find out the response of mast cells in context of cytokine expression when mast cells are treated with different challenges. For this experiment, we select RBL-2H3 cell line as it has been used as model for studying MCs. First of all we choose best media for culture of RBL-2H3 cell. DMEM media was selected and cells were maintained in DMEM media. Mast cells response up to secondary challenges was studied(Xiang et al., 2001) but its response in tertiary challenge was not known. So to study the effect of multiple antigenic challenges on mast cells mediator release we set up an experiment. Mast cells were sensitized with anti DNP-BSA specific IgE antibody followed by primary challenge, secondary challenge and tertiary challenge with 100ng of DNP-BSA in every 24 hours interval. After each challenge, β-hexosaminidase assay was performed. β-hexosaminidase is lysosomal enzyme. The percent secretion was 54% in primary, 47% in secondary and 40% after tertiary challenge. There is significant reduction in secretion of β-hexosaminidase in each challenge. Though they are capable to secrete enzyme after multiple times activation, there is reduction in secretion which indicates that mast cell is not fully granulated in 24 hour of resting period. Thus we conclude that the mast cells can be replated even after three time activation. Further we aimed to study the expression of FcεRI receptor expression on the surface of mast cell after multiple sensitizations with IgE antibody. We found no significant difference in receptor expression profile in multiple sensitized cells.

Furthermore, we aimed to determine pro-inflammatory cytokine (IL-4, IL-5, IL-6, IL-13, TNF-α) and chemokine MIP-1α expression of mast cell after their treatments with different antigens, allergen, vaccine, chemical degranulator and pathogenic challenge. GAPDH was used as control gene. The cells were sensitized with IgE, sensitized cells treated with DNP-BSA, RBL cell treated with Poly I:C, sonicated BCG, sonicated *E. coli*, DPT, compound 48/80, *Leishmania donovani, Leishmania tropica* respectively in separate culture disc. After treatments RNA was isolated by using Trizol reagents. cDNA was synthesized from respective RNA by using oligo (dT) 18 primer. After this the primer were designed for selected pro-inflammatory cytokines and chemokine. PCR condition of all the primers were standardized for annealing temperature, amount of RNA and number of cycles. After standardization, cytokine expression was analyzed in all treatments. Up-regulation and down regulation of cytokines was compared with resting RBL cells. From our results, it was concluded that selected cytokines and chemokine during various challenge or treatment is differentially regulated in the RBL-2H3 cell line.

These finding could be further used to study detail mechanism of mast cell response against various infection and can also be used in vaccine development for allergic individual.

Chapter 7
Conclusion

In our study we found out that mast cells can be re-plated and challenged with antigen again and again. Our data on β– hexosaminidase release assay concludes that mast cells are capable of undergoing regranulation and degranulation at least up to 2 times. Total β- hexosaminidase being un-affected suggest that mast cells are able to completely replenish their granular content. There are reports only for regeneration of granules and activation not for modulation of these multiple mast cell responses. Modulating mast cell responses can help us better understanding the role of mast cells in pathophysiology of asthma and other allergic inflammatory disorders.

We studied the cytokine expression of mast cell after different treatments. We found that IL-4, IL-6, IL-13, TNF-α and MIP-1α is constitutively expressed in RBL-2H3 cell line. MIP-1α is expressed in all treatments with highest expression on RBL cells co-cultured with *Leishmania donovani* and Poly I:C treated cells. Mast cells can differentially be activated by various triggers to degranulate or release cytokine. By understanding the nature of stimuli and specific mast cell response to it, mast cells can selectively be modulated during inflammatory or infectious diseases.

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Appendices

- **RPMI medium (1 litre)**

25 mM HEPES; with 10% FBS	2.5g
1x 10^5 U/L gentamicine	120 mg
2 mM L-gultamine	0.3g
10 µM 2-mercaptoethanol	2 ml
Glucose	1g
RPMI	10.4g
Sodium bicarbonate	2.6g

pH (7.0 in HCL) Volume maintained by milliQ water and filtered

- **RPMI PR^- media**

RPMI PR^- media	10.4g (1 bottle)
Sodium bicarbonate	2.6g
Glucose	1g
HEPES	2.5g

pH (7) Volume maintained by milliQ water and filtered

- **RBL Media (1 litre)**

Iscoves medium	8.85g
MEM medium	4.8g
Glutamine	0.3g
$NaHCO_3$	2.6g
HEPES	1.25g
Gentamicin	120mg

pH (7.0-7.25) Volume maintained by milliQ water and filtered

- **DMEM media (1 liter)**

DMEM media (1 bottle)	13.5g
HEPES	2.5g
Sodium bicarbonate	3.7g
Glutamine	0.3g
Gentamycin	120mg
Sodium pyruvate	0.11g

pH (7) Volume maintained by milliQ water and filtered

- **5X TBE Buffer**

Tris Base	54g
Boric Acid	27.5g
0.5 EDTA (pH 8.0)	20ml (3.72g)

Make the final volume up to one litre and final pH 8.

- **6x Gel loading dye**

10mM Tris pH 8

0.03% bromophenol blue

60% glycerol

60mM EDTA

- **5x RNA loading Buffer**

Saturated aqueous bromophenol blue solution	16µl
500mM EDTA, pH 8	80µl
37% 12.3 M formaldehyde	720µl

Glycerol	12ml
Formamide	3084µl
10x formaldehyde agarose gel buffer	4ml

Final volume adjusted to 10ml by adding RNase free water.

- **TRIZOL Reagent**

4M Guanidine thiocyanate Phenol

0.8 M Sodium citrate

0.5% N-laurosyl-5arcosine

0.1 M B-mercaptoethanol

- **Lysis buffer (1ml)**

RPMI PR^- media	990µl
Triton X-20	10 µl

- **10x Formaldehyde Agarose gel buffer**

200mM MOPS	41.9g
50mM Sodium acetate	4g
10mM EDTA	3.722g

Final volume up to one litre pH adjusted to 7 by NaOH. Autoclave and store at 4^oC in dark.

- **1x Formaldehyde Agarose gel buffer (1 litre)**

10x Formaldehyde Agarose gel buffer	100ml
37% (12.3M) formaldehyde	20ml
RNase free water	880ml

- **Phosphate Buffer Saline (1 litre)**

NaCl	8g
$Na_2HPO_4.2H_2O$	1.44g
KCl	0.2g
KH_2PO_4	0.2g

pH was maintained to 7.3-7.4 with HCl.

- **Trypan Blue (2% Stock solution)**

Trypan Blue	2g
Sodium azide	0.2g
MilliQ water	100ml

Kept at 37^{o}C for 10mins and stored at 4^{o}C.

- **Trypan Blue (0.2% Working solution)**

2% Stock solution	3ml
PBS	27ml

Final volume 30ml, kept at 37^{o}C for 10mins and stored at 4^{o}C.

- **DEPC Treated water**

MilliQ water	2 L
Di-ethyl pyrocarbonate	2 ml

- **Citrate buffer**

0.1 M Sodium citrate, pH 4.5	2.94g
0.2% Triton X-100	
MilliQ water	100 ml

- **Stop solution**

0.2M Glycine	2ml

0.2M Nacl	400µl
0.2M NaoH	200 µl

pH 10

Final volume is maintained 10 ml in MilliQ water.