Interaction of rodent mast cells with intracellular pathogens

Thesis submitted to Jawaharlal Nehru University
for the award of the degree of

DOCTOR OF PHILOSOPHY

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2017

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Dated: 21 /07 /2017

CERTIFICATE

The research work embodied in this thesis entitled **"Interaction of rodent mast cells with intracellular pathogens"** has been carried out by Mrs. Nilofer Naqvi in the School of Life Sciences, Jawaharlal Nehru University, New Delhi, India.

The work is original and has not been submitted so far, in part or in full for any other degree or diploma of any other University.

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Acknowledgements

This thesis has become a reality because of the supervision of Dr.Niti Puri. Her moral support and encouragement are a part and parcel of this work. I want to thank Dr. Selvapandiyan Angamuthai for giving me an opportunity to work with Leishmania as well as teaching many aspects of paper writing. I want to s thank Prof. R.K. Saxena for allowing me to work on BCG. I want to thank Prof. Sneha Sudha Komath for giving me an opportunity to work with different mutants of Candida albicans.

I am grateful to the Dean Prof. S. K. Goswami for the research facilities provided at the School of Life Sciences. I am thankful to Advanced Instrumentation Facility (AIRF), JNU for excellent advanced facilities that helped me to fulfil my objectives. I am thankful to School of Life Sciences for excellent facilities in CIF. I am thankful to UGC for providing me fellowship. I am thankful to Shiny Ma'am and Sunita Ma'am for helping me in all the administrative work always with a smiling face.

I am thankful to Dr. Anwar Alam, Dr. Sreoshi Chaterjee, Ms. Vasudha Aggarwal and Dr. Zaigham Abbas Rizvi for being very supportive and encouraging seniors. I am thankful to my juniors Pieu, Priyanka, Sandeep, Gagan, Rangati, Suchanda and Rahul for respecting and helping me immensely. I am thankful to Kavita and Priyanka for helping me in providing Leishmania and Candida respectively. Geeta is highly acknowledged for the technical help in the lab. My work would not have been completed without the technical help and support from Tarunji, Anwarji and Vijay who were always enthusiastic in helping me.

I am thankful to Nikhat and Munazah for helping their level best and making me optimistic from very first day in JNU till today.

I want to thank

Whatever I have been able to achieve so far is all because of immense love, moral support and blessings of Dadda, Badkeabba, Amma, Abba, Chachi and Chacha. Faizy, Abbas and Ali deserve everything for making me optimistic. This thesis is dedicated to my dearest Baba, my role model since childhood.

It is the mercy of God that has made this work possible ultimately. No words can thank him.

Nilofer Naqvi.

ABBREVIATIONS

µg	microgram
µl	microlitre
AFB	Acid Fast Bacteria
AMP	Anti microbial Peptides
APC	Antigen Presenting Cells
BCG	Bacillus Calmette Guerin
BMMC	Bone Marrow Derived Mast Cells
BSA	Bovine Serum Albumin
CFSE	Carboxyfluorescein N-succinimidyl ester
CFU	Colony Forming Unit
DAPI	(4, 6-diamidino-2-phenylindole)
DMSO	Dimethyl Sulphoxide
ELISA	Enzyme Linked Immunosorbent Assay
ET	Extracellular Traps
FACS	Fluorescence Activated Cell Sorter
FBS	Fetal Bovine Serum
FSC	Forward Scatter
GPI	Glycophosphatidyl Inositol
GPI-GnT	Glycosylphosphatidylinositol-N-acetylglucosaminyltransferase
h	hour
HSC	haematopoietic stem cells
IFN	Interferon
IL	Interleukin
LDH	Lactate Dehydrogenase
LPG	Lipophospphoglycan

MBL	Mannose Binding Lectin
MC	Mast Cells
MCETS	Mast Cell Extracellular Traps
MCP	mast-cell progenitors
MFI	Mean Fuorescence Intensity
mg	milligram
MHC	Major Histocompatibility Complex
mins	minutes
MMC	Mucosal Mast Cell
MOI	Multiplicity of Infection
MPO	Myeloperoxidase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
MΦ	Macrophage
NET	Neutrophil Extracellular Traps
OADC	Oleic acid, Albumin, Dextrose and Catalase
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
PKC	Protein Kinase C
PMC	Peritoneal Mast Cells
PS	Phosphatidyl Serine
RBL	Rat Basophilic Leukaemia
ROS	Reactive Oxygen Species
RPMI	Rosewell Park Memorial Institute
sBCG	Sonicated BCG
SCF	Stem Cell Factor
SEM	Standard Error Mean
SNAP	Soluble *N*-ethylmaleimide-Sensitive Factor Attachment Protein
SNARE	Soluble *N*-ethylmaleimide-Sensitive Factor Accessory

	Protein Receptor
SSC	Side Scatter
TNF	Tumor Necrosis Factor
WHO	World Health Organisation
WT	Wild Type
YEPD	Yeast Extract Peptone Dextrose

CONTENTS

Introduction

Introduction

Mast Cells (MCs) are specialized 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). They are central effectors in anaphylaxis, allergy and asthma (Moon, Befus and Kulka 2014, Moon et al. 2010). All these functions depend on the release of pro-inflammatory mediators following stimulation through cell surface receptors such as the high affinity IgE receptor, FcεRI (Moon et al. 2014). In addition to exocytosis, and participation in inflammation, mast cells also have the ability to phagocytize diverse micromolecular, particulate materials and pathogens. Mast cells are found in large numbers beneath the skin, the mucosa of the genitourinary, gastrointestinal and respiratory tracts. So they are the first immune cells to interact with the invading pathogens or environmental antigens (Marshall 2004).

They possess different receptors which recognize specific components of external environment. To function as immune surveillance cells, MCs have three mechanisms of pathogen recognition: mainly through pathogen associated molecular pattern (PAMP) receptors located on the mast cell surface; complement receptors or immunoglobulin receptors; or recognition of endogenous peptides (Hofmann and Abraham 2009). Depending on the type of receptor interacting on the Mast Cell surface, specific immune responses to the pathogen are stimulated (Qiao et al. 2006). The various responses of Mast Cells include secretion of various mediators, initiation of inflammation, recruitment and activation of other immune cells, direct phagocytosis, production of many antimicrobial peptides, lymph node potentiation through TNFα granules and Mast Cell Extracellular Traps (MCETs).

MCs have important role following infections with *Escherichia coli*, *Klebsiella pneumonia*, and *Bordetella pertussis*. Two other more recent studies have shown that MCs are capable of direct recognition, and uptake of *M. tuberculosis* through lipid raft microdomains, and are activated by these bacteria to release presynthesized and *de novo* synthesized mediators (Munoz et al. 2003, Munoz, Rivas-Santiago and Enciso 2009). Mast Cells have contribution in protective immunity against nonbacterial pathogens as there are various evidences like firstly, parasitic intestinal infections are known to result in MC hyperplasia, MC activation; secondly; parasite-specific IgE contributes to protective immune responses against intestinal parasites

and thirdly, mucosal MCs reportedly contribute to the expulsion of various intestinal parasites (Saha et al. 2004). However, few studies have attempted to provide direct evidence for MC-dependent protective effects in settings of parasite infections of the skin. Thus, the role of MCs as players in antiparasitic immunity remains to be characterized in detail.

Infections with intracellular pathogens like *Mycobacterium tuberculosis* and *Leishmania* species continues to be one of the major global health threats estimating around 2 million lives annually all over the world. *M. tuberculosis* basically attack through respiratory route. From human and animal models of tuberculosis infection, it has been found T lymphocytes and alveolar and interstitial macrophages mostly respond to *M. tubercculosis*. However, other cell types, such as dendritic cells, type II lung epithelial cells, endothelial cells, and fibroblasts also phagocytose *M. tuberculosis*, suggesting that many other cell types may also respond to the immunological control of *M. tuberculosis* in the lung (Munoz et al. 2003). The incidence of candidiasis caused by *Candida albicans* is increasing worldwide. The epidemiology of candidiasis is increasing worldwide because of AIDS epidemic, increased number of patients receiving immunosuppressive therapy for transplantation and the increasing use of antimicrobials in the hospital setups (Giri and Kindo 2012).

Mast cells range from 500 to 4,000 per mm^3 in the lungs (Abraham and Malaviya 1997). Because of their location and capacity to release mediators one early study has reported MCs increment and degranulation in the lungs of infected animals during early phase of infection with *M. tuberculosis* (Ratnam et al. 1977). Large numbers of MCs ranging from 7,000 to 12,000 per mm3 are found in the skin (Abraham and Malaviya 1997), predominantly in the superficial dermis, where *Leishmania* is encountered after the bite of infected sand flies (Maurer et al. 2006). Since Mast Cells are located in those areas where these three intracellular pathogens invade, our objectives were to study the initial interaction of Mast Cells with these pathogens and the consequence of these interactions.

As it is very infectious to work with virulent strains of *Mycobacteria* such as H37Rv so *Mycobacterium bovis* BCG which was developed by Calmette and Guerin in 1921 from virulent strain of *M. bovis* and has lost its virulence will be used for the current study. BCG lung infection in mice is similar to *M. tuberculosis* infection in healthy

human beings. So the mouse BCG infection model is used for studying the interactions between *Mycobacteria* and immune cells in the lung (Saxena *et al*, 2002). While we were studying the initial interaction with *C. albicans*, a report was published detailing MC interactions with *Candida.* It showed that *C. albicans* elicit a temporal response in Mast Cells (Lopes et al. 2015). So we decided to look at role of Glycosylphosphatidylinositol (GPI) anchor protein of *C. albicans* in its interactions with Mast Cells and compared it to Macrophages.

Mast Cells are capable of sensing the two phases of *C. albicans*, and it was suggested that MCs participate as an early inductor of inflammation during the early innate immune response to this fungus (Lopes et al, 2015). The GPI anchor in eukaryotes anchors various proteins to the cell surface. The first step in GPI biosynthesis is the formation of GPI-N-acetyl glucosaminyl phosphatidylinositol (GlcNAcPI) from uridinediphosphate- N-acetylglucosamine(UDP-GlcNAc) and phosphatidylinositol (PI) . It is catalyzed by GPI-N-acetylglucosaminyltransferase (GPI-GnT) complex which consists of six different subunits Gpi1, Gpi2, Gpi3, Gpi15, Gpi19, and Eri1. The role of GPI anchor in immune cell interactions was studied by making use of mutants of *C. albicans* lacking each of these subunits individually and thereby having a defect in GPI biosynthesis.

Well established rodent mast and macrophage cell lines were used for the studies. Interactions with BCG, *Leishmania* and *C. albicans* was studied by monitoring the uptake of CFSE labeled BCG, *Leishmaia* and *C. albicans* and their GPI-GnT mutants by flow cytometry and confocal fluorescence microscopy. The major aim of this study was to explore the direct role for MCs in clearance of these pathogens by both intracellular and extracellular mechanisms. The study highlights the importance of MCs in the disease outcome and vaccine or therapeutic strategy development for these infections.

Review of Literature

1. Mast Cells in general

Mast Cells were first described by Paul Ehrlich in 1878. They are leukocytes that are derived from hematopoetic progenitor cells (Okayama and Kawakami 2006). They circulate in the blood in an immature form and migrate to vascularized tissue where they undergo final maturation and differentiation by stem cell factor which binds to the c-kit receptor and cytokines such as Interleukin-3 (IL3), IL4, IL9 and IL10 secreted by endothelial cells and fibroblasts (Okayama and Kawakami 2006). Stem-cell factor (SCF; also known as KIT ligand) is normally required to ensure mast-cell survival in the tissues, but the phenotype of mature MCs can vary depending on the growth-factor — for example, the presence or absence of additional cytokines with effects on mast-cell proliferation or phenotype, such as IL-3, IL-4, IL-9 and IL-10 (Blechman et al. 1993, Okayama and Kawakami 2006). These cytokines promote differentiation and proliferation of both human and mouse MCs. The SCF receptor (ckit) plays an important role in the hematopoiesis during embryonic development. SCF promotes mast cell adhesion, migration, proliferation, and survival. SCF also promotes the release of histamine and tryptase, which are involved in the allergic response. Mast cell is the only terminally differentiated hematopoietic cell that expresses the c-Kit receptor (Blechman et al. 1993).

Two types of MCs, mucosal and connective tissue MCs, were reported in rodent tissue in the 1960's. These were reported on the basis of histochemical and fixation characteristics whether heparin proteoglycan was present in secretory granule (Metcalfe, Baram and Mekori 1997). Mucosal mast cell (MMC) granules stain blue with copper phthalocyanin dyes, such as Astra blue or Alcian blue, in a staining sequence with safranin, while connective tissue mast cell (MCTC) granules stain red (Feyerabend et al. 2005). In mice, mucosal-type MCs, located in mucosal compartments, express chymase (mouse mast cell protease (mMCP)-1 and mMCP-2). Connective tissue-type MCs, located in the skin and blood vessels, express chymase (for example, mMCP-4, mMCP-5), tryptase (mMCP-6 and mMCP-7) and carboxypeptidase A. IL-3 favors the development of a mucosal mast cell phenotype *in vitro* (da Silva, Jamur and Oliver 2014). In humans MCs are classified into MCTC having both enzyme tryptase and chymase in their granule which resemble rodent connective tissue MCs whereas MCT having only tryptase which resemble rodent mucosal MCs (Metcalfe et al. 1997). Differentiation of MCs from haematopoietic

stem cells (HSCs) has been shown in Figure 1. MCs are generally found "fixed" in tissues and do not circulate through the blood. It is amazing to note that if all human tissue MCs were assembled together in a single organ, it have size equal to normal spleen (Sayed et al. 2008).Thus MCs comprise a substantial population of immune cells.

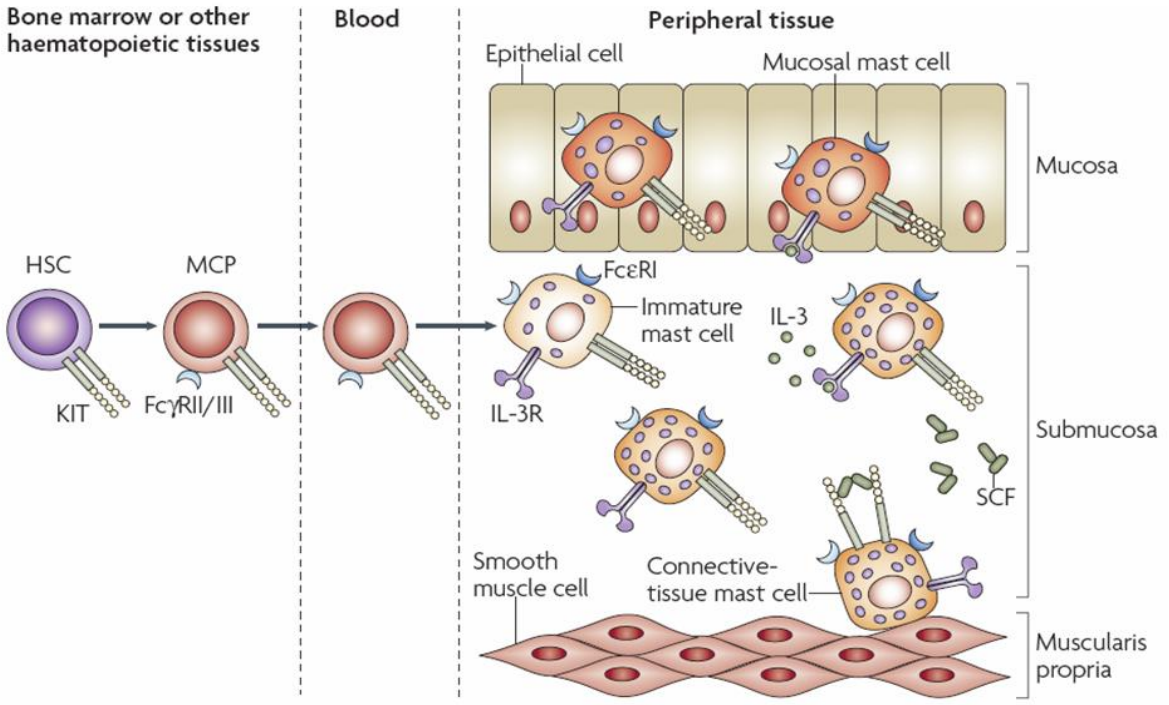

Figure 1: Differentiation of Hematopoietic stem cells (HSCs) to mature Tissue MCs. Tissue mast cells are derived from haematopoietic stem cells (HSCs), which ultimately give rise to mast-cell progenitors (MCPs). MCPs circulate in the blood and enter the tissues, where they undergo differentiation and maturation to become mature mast cells. *Source*: (Galli, Grimbaldeston and Tsai 2008).

1.1 Role of MCs in hypersensitivity

MCs are involved in the pathophysiology of allergic diseases, mainly in IgE-mediated hypersensitivity reactions in airways, skin, and gastrointestinal tract such as asthma, allergic rhinitis, atopic dermatitis, and food allergy (Moon et al. 2010). These responses are due to allergen-specific IgE binding and cross-linking the high-affinity IgE receptor (FCεRI) on MCs, leading to activation and release of inflammatory mediators.

- Effects of MC IgE and / or IgG-mediated activation and degranulation

Upon activation, MCs release many preformed and denovo synthesized mediators. During the early phase of a reaction, MCs can release histamine, tryptase, LTC-4 , prostaglandin D 2 (PGD 2), platelet-activating factor, chemokine ligand 2, IL-13, VEGFA, and TNF, which have immediate effects on epithelial cells, smooth muscle, and endothelial cells and nerves. This mediator release can lead to increased epithelial permeability and mucous production, smooth muscle contraction, vasodilation, and neurogenic signals (Moon et al. 2010). Early release of TNF, LTB 4, IL-8, and chemokine ligand 2 leads to the late phase response by recruiting neutrophils, eosinophils, and basophils (Moon, Befus and Kulka 2014). The late phase response is directed not only by continued MC mediator release, but by activation of newly arrived leukocytes and tissue-resident cells (Moon et al. 2010). These lead to tissue-specific allergic responses and symptoms.

- MCs in asthma

Asthma is one of the most common allergic diseases characterized by chronic airway inflammation and broncho constriction, consisting of edema, increased mucous production, leukocyte infiltration, and smooth muscle contraction (Moon et al. 2010). There are many evidences to believe that MCs have fundamental roles in asthma. It is possible to sensitize to an allergen in a manner that can generate MC-dependent or -independent asthmatic responses using MC-deficient mice such as WBB6F1-$Kit^{W/}Kit^{W-v}$ (W/W^{v}) and reconstituted mice (Lei et al. 2013). There are increased numbers of MCs in airway smooth muscle which is a correlation between MC degranulation and asthma severity (Moon et al. 2010). MCs also contribute to chronic airway thickening, tissue remodeling, and fibrosis, through the release of mediators such as tryptase, transforming growth factor- (TGF), and plasminogen activator inhibitor type 1, which can induce fibroblast proliferation and collagen deposition in the airways (Moon et al. 2010).

- MCs in food allergy

The role of MCs in IgE-mediated food allergy has been extensively researched (Berin and Mayer 2009). Activation of MCs can occur by the transcellular transport of allergen – IgE complexes through epithelial cells, after binding the low-affinity IgE

receptor (CD23) on intestinal epithelial cells (Berin and Mayer 2009). Once activated, MCs release mediators that affect permeability in the intestine by disrupting tight junctions and opening paracellular pathways (Berin and Mayer 2009). This leads to enhanced allergen exposure and further MC activation. Intestinal smooth muscle contraction, increased permeability, and altered water and ion transport by MC mediators are all involved in diarrhoea, which is main symptom of food allergy (Moon et al. 2010).

- MCs in atopic dermatitis and other allergic skin diseases

The role of MCs in allergy in the skin has been studied extensively in rodent models as well as in humans. MC numbers are increased and many are activated in skin following allergen exposure in humans (Kneilling and Rocken 2009). The role of MCs in allergy in the skin has also been studied in humans. Mast cells express IL-17 and IL-22 and during skin disease IL-17 and IL-22 levels increase which is the reason of chronic pathophysiological symptoms of skin disease (Mashiko et al. 2015). MC mediators are responsible for the typical wheal and flare reactions. The symptoms are edema and vasodilation followed by leukocyte recruitment into the affected area.

1.2 Role of MCs in innate immunity

MCs play a very important role in the early immune response to invading pathogens. This newly discovered role for MCs involves the release of various chemo attractants that recruit neutrophils and MCs are known to internalise various pathogens (Arock et al. 1998, Lopes et al. 2015, Shin, Gao and Abraham 2000, Wesolowski, Caldwell and Paumet 2012). MCs have been described in the lowest order of animals (e.g. insects and fishes) (Hakanson et al. 1986). Their presence throughout the evolution suggests a beneficial role. MCs are found in large numbers adjacent to blood or lymphatic vessels but are most prominent immediately beneath the skin and the mucosa of the genitourinary, gastrointestinal and respiratory tracts (Reber et al. 2015). The close association of MCs with both epithelial and endothelial barriers places them amongst the first cells to encounter pathogens along with other innate immune cells including Dendritic Cells (DCs) and Macrophages (MΦs). MCs also sense danger signals from the surrounding tissue, including from epithelial, endothelial, and tissue resident immune cells (St John and Abraham 2013). Depending on the balance of activating stimuli detected by MCs, they release a myriad of inflammatory mediators for a

specific type of pathogen detected (St John and Abraham 2013). Stimuli such as many whole bacteria, parasite products, and even the structure of certain viruses can induce release of mediators by MCs (St John et al. 2011). At a site of infection *in vivo*, host and pathogen-associated products likely have a combinatorial effect to promote extensive degranulation. Release of vesicle encapsulated granules into the surrounding tissue usually begins within seconds of exposure to degranulating stimuli (Kunder et al. 2009). Granules are packed with inflammatory mediators: heparin, proteases, TNF, histamine and others. The dense granule structure help in promoting their travel through lymphatics to draining lymph nodes (DLNs), where they trigger the recruitment of antigen presenting cells (APCs) and T cells (Kunder et al. 2009). Extracellular granules have a contribution in innate immunity by protecting inflammatory mediators from dilution or degradation. It further allows the slow release of associated products (St John et al. 2012). DCs and Mφs can even phagocytose extracellular MC granules (Higginbotham, T. F. Dougherty and Jee. 1956)–this may be a mechanism of communicating to APCs. Thus, MCs act as professional immune sentinels due to various attributes such as increased capacity to detect pathogens, adjustment to promote communication within the tissue and to distant sites, and their prime location as first-responders to pathogens.

1.3 Roles of Mast Cells in adaptive immunity

There are reports that have demonstrated that MCs participate in the sensitization phase of adaptive immune responses via the secretion of mediators, which helps in DC maturation, function, and recruitment to the tissue or their migration to local draining lymph nodes (McLachlan et al. 2008). MCs have important effector function as MCs and T cells of different origin and subsets establish tight cell–cell interactions and modulate their respective effector functions in a bidirectional manner. This has been shown in a variety of models (Gri et al. 2012, Mekori et al. 2016, Valitutti and Espinosa 2010). MCs can present antigens to T cells in a MHC class I- or class II-restricted mechanism (Kambayashi and Laufer 2014, Marshall 2004, Mekori et al. 2016, Stelekati et al. 2009). Antigen internalized into MCs through FcεRI can activate antigen-specific T cell responses *in vitro*; this mechanism is independent of the expression of major histocompatibility complex class II by MCs but requires that such MCs undergo apoptosis and then phagocytosis by antigen-presenting cells (Kambayashi et al. 2008). It has been reported that degranulated, and to a lesser extent

naive or IgE-sensitized, MCs activate both naive and B cell receptor-activated B cells. Increased proliferation, blast formation, and expression of CD19, MHC class II and CD86 in the B cells was shown. MCs can also lead to induction of class-switch recombination in B cells as they stimulated the secretion of IgM and IgG in IgM+ B cells (Palm et al. 2016). The role of MCs in adaptive immunity has been depicted briefly in figure 2.

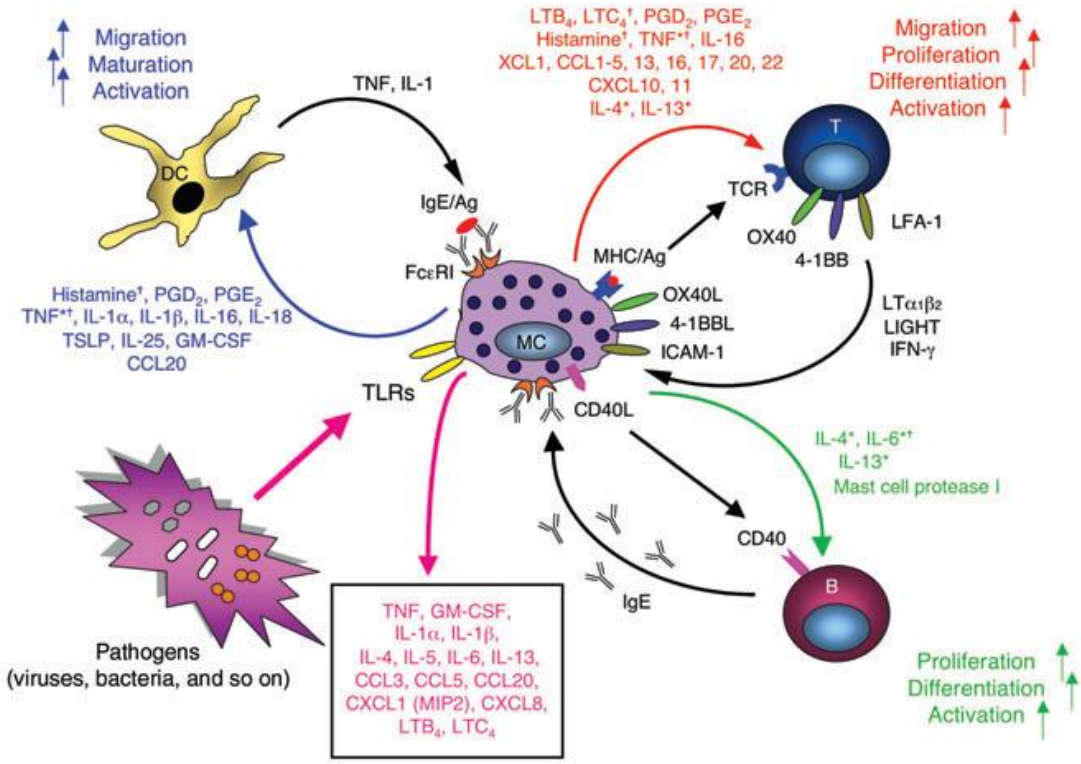

Figure 2: Role of MCs in adaptive immunity. The mast cell surface structures and secreted products that can influence various aspects of the biology of DCs, T cells and/or B cells are presented. *Source*: (Galli, Nakae and Tsai 2005b).

1.4 Mast Cell immunomodulatory functions

MCs are a pivotal source of many mediators that can promote or suppress the development, survival, proliferation, migration, maturation or function of immune cells. Although it is well known that MCs have important roles in allergy and innate immunity, the role of MCs in the inhibition of immune and inflammatory responses has received less importance. Dermal MCs are necessary for the suppression of UVB-induced contact hypersensitivity. Histamine is an important mediator of this MC-dependent suppression (Hart et al. 1998). Anti-inflammatory or immunosuppressive

effects of MC-derived IL-10 have been identified in antigen-specific T-cell responses in Anopheles mosquito bites (Depinay et al. 2006), in contact dermatitis and chronic UVB-irradiated skin pathology by limiting leukocyte infiltration (Grimbaldeston et al. 2007). MCs are essential in $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T-cell-dependent peripheral tolerance to skin allografts (Lu et al. 2006). Immunoregulatory function of MCs is mostly through MC-derived interleukin-10 (IL-10) that has been described briefly in figure 3. IL-10 can promote the development of IL-10-secreting regulatory T cells and inhibit the migration, maturation and activation of DCs. IL-10 can also increase the ability of DCs to inhibit T-cell proliferation and cytokine production through the downregulation of expression of co-stimulatory molecules by the DCs. It can also indirectly decrease the expression of adhesion molecules on vascular endothelial cell by directly inhibiting TNF and IL-6 production by keratinocytes and thus, decrease the recruitment of circulating effector cells. It can directly inhibit the production of prostanoids by neutrophils and pro-inflammatory cytokines by macrophages. IL-10 has been shown to mediate negative immunomodulatory functions *in vivo* (Grimbaldeston et al. 2007).

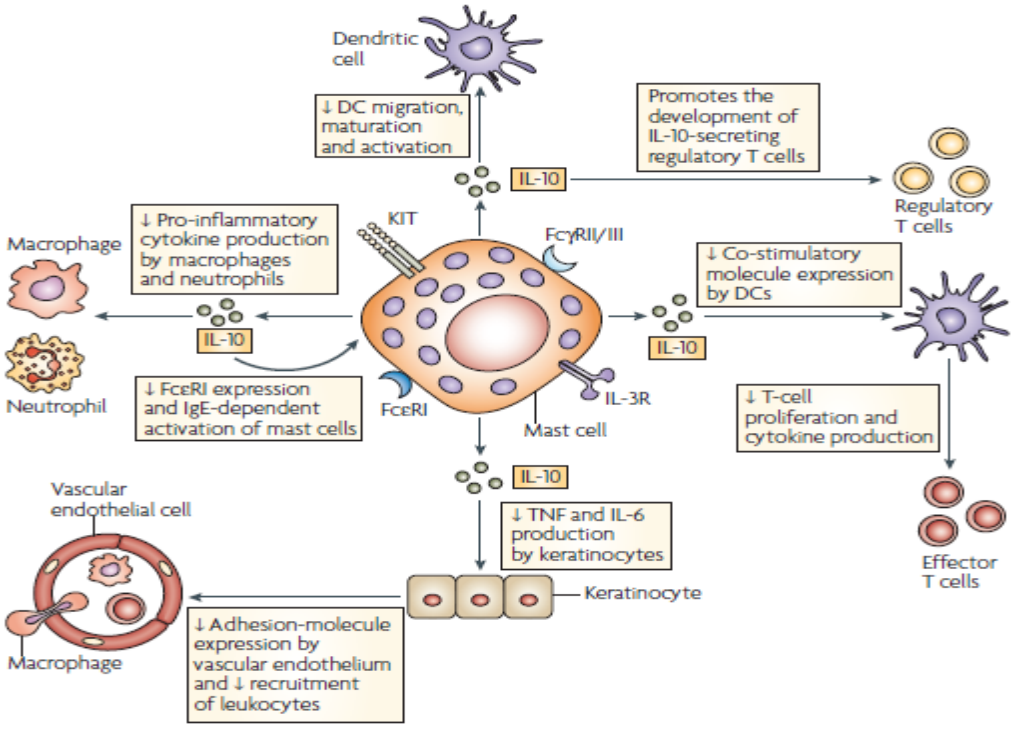

Figure 3: Potential function of mast-cell-derived IL-10. Effects of IL-10 on effector T cells, macrophages, neutrophil, keratinocyte, dendritic cell and regulatory T cells. *Source*: (Galli et al. 2008)**.**

2 Mast Cell Effector Responses

MCs are extremely versatile and act both as effector cells that amplify inflammation, as well as regulatory cells that suppress responses (Galli et al. 2005a). This versatility is due to numerous IgE-independent activation pathways that together interact to modulate the quality and magnitude of the mast cell responses through various receptors present on MCs as shown in figure 4.

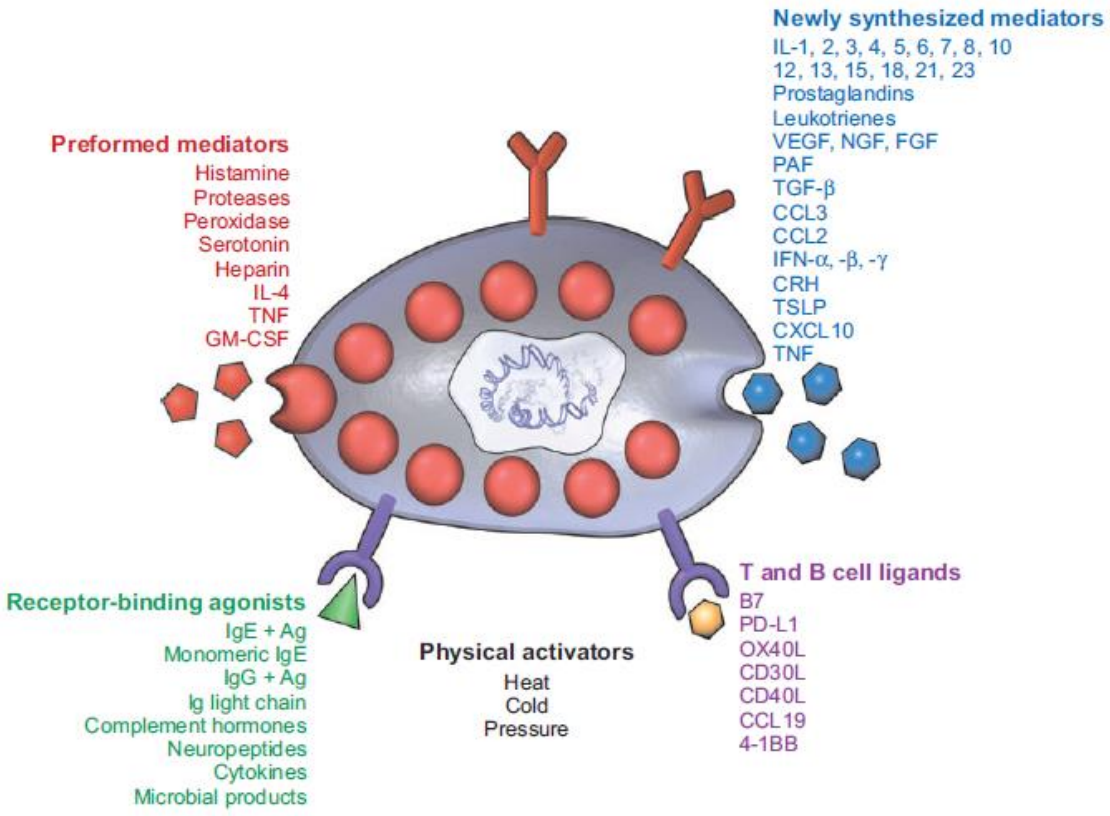

Figure 4: Activators and potential responses of MCs. MCs are versatile as they are activated by a variety of stimuli and produce specific responses ranging from the immediate release of preformed mediators to the *de novo* production of cytokines and chemokines. *Source*: (Sayed et al. 2008).

2.1 Mast Cell Receptors

MCs detect the presence of various pathogens through a combination of various direct and indirect receptors. MCs express TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, CD48, Dectin-1, FcεRI, FcR, C5aR, C3aR, CR2, CR4 and CR5 which recognize various bacteria, fungus, parasites and viruses as shown in figure 5. MCs use Toll-like receptors (TLRs) to respond to products of both Gram-positive and Gram-negative bacteria. MCs contribute to acute inflammatory events *in vivo* through the

engagement of TLR2 and/or TLR4 (Marshall 2004). Rodent and human MCs express TLR9 and produce several cytokines in response to CpG-containing oligodeoxynucleotides (Marshall 2004). TLR3-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 I IFNs. TLR1 and TLR6 which are expressed both in humans and rodents are important because they form functional heterodimers with TLR2 enabling responses to many mast-cell activators, including peptidoglycan and lipopeptides. TLR7 and TLR8 are important for viral infection that are through single-stranded RNA sequences. Functional TLR7 has also been reported to be expressed by human and rodent MCs (Marshall 2004). MCs interact with Gram negative bacteria such as *Escherichia* coli expressing FimH through glycosylphosphatidyl (GPI) – linked protein CD48. Mast cells recognize *Sacharomyces cerevisae* through Dectin -1, a C type lectin through zymosan (Marshall 2004). MCs interact with the complement system through complement receptor 3 (CR3; also known as CD11b–CD18), CR4 (also known as CD11c–CD18) and the receptors for complement product 3a (C3aR) and C5a (C5aR; also known as CD88) (Nilsson et al. 1996). Both C3- and C4- deficient mice are more susceptible to bacterial infection (Marshall 2004).

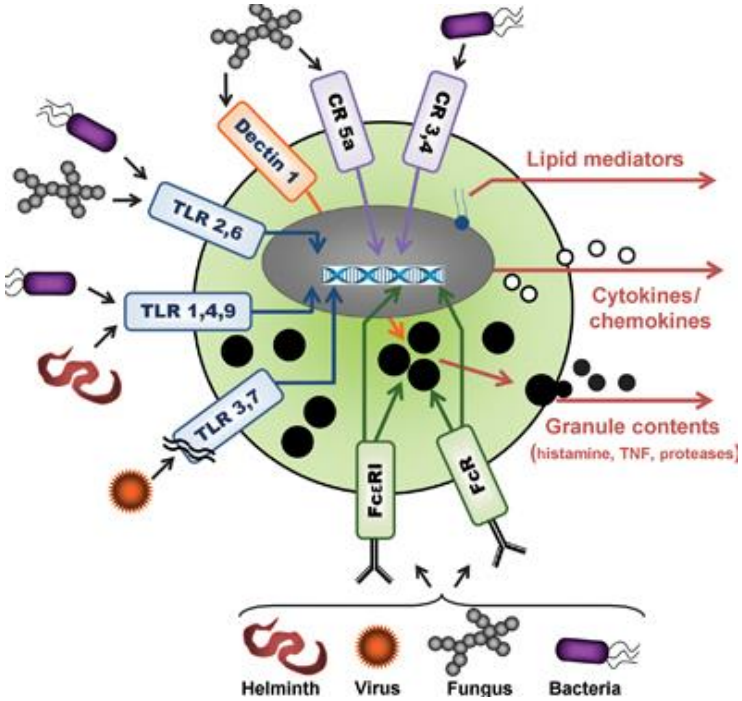

Figure 5: MCs have various receptors for interaction with pathogens. MCs interact with bacteria, virus, fungus and parasites through various receptors present on their surface. *Source*: (Moon et al. 2010).

2.2 Mast Cell Mediators

Mast cells produce three main classes of mediators mainly preformed that are granule-associated mediators; newly generated lipid mediators; and a wide variety of cytokines and chemokines. Tryptase-, chymase- or carboxypeptidase are proteases that are present in mast cell granules. They have various roles in tissue remodelling and cellular recruitment. Vasoactive amines, such as histamine and serotonins, are found in mast-cell granules. MC granules also contain heparin (Marshall 2004, Metcalfe et al. 1997, Moon et al. 2014, Moon et al. 2010). Lipid mediators such as LTC4 and LTB4 have been the most-extensively studied in the context of host defence (Marshall 2004, Metcalfe et al. 1997, Moon et al. 2014, Moon et al. 2010). Cytokines and chemokines that are produced by mast cells include classical pro-inflammatory mediators, such as tumour-necrosis factor (TNF) and IL-1β, as well as cytokines that are associated with anti-inflammatory or immunomodulatory effects, such as IL-10 and transforming growth factor-β. T helper 2 (T_H2)-type cytokines, including IL-4, IL-5 and IL-13, mast cells can also produce T_H1-type cytokines, such as interferon-γ (IFN-γ), IL-12 and IL-18. Mast cells can also be an important source of several chemokines, including those associated with T_H2-type response such as CC-CHEMOKINE ligand 5 (CCL5), and T_H1-type responses, such as CXC-CHEMOKINE ligand 8 (CXCL8; also known as IL-8) and CXCL10 (Marshall 2004, Metcalfe et al. 1997, Moon et al. 2014, Moon et al. 2010).

2.2.1 IgE-Dependent Mast Cell Activation

FcεRI-mediated signaling pathways are among the best characterized in MCs. MCs degranulate and release the contents of intracellular secretory granules in response to the cross-linking of FcεRI by antigens as shown in figure 5. These granules contain a variety of biologically active inflammatory mediators but it is not clear whether these granules are homogenous or there is heterogeneity within the secretory granule population in MCs (Puri and Roche 2008). By using genetically altered mice lacking specific vesicle associated SNARE membrane fusion proteins, Puri et al have found that VAMP-8-deficient MCs exhibited defects in FcεRI-regulated exocytosis, whereas synaptobrevin 2- or VAMP-3-deficient MCs did not. This defect in secretion in VAMP-8-deficient mice was limited to the subpopulation of mast cell secretory granules containing serotonin and cathepsin D whereas regulated exocytosis of

secretory granules containing histamine and TNFα was normal (Puri and Roche 2008).

2.2.2 IgE-Independent Mast Cell Activation

IgE-independent modes of activation include other immunoglobulins such as IgG (Metcalfe et al. 1997), microbial antigens, complement, hormones, peptides, and cytokines (Marshall 2004). Figure 6a andc show how MCs activate in IgE-independent manner.

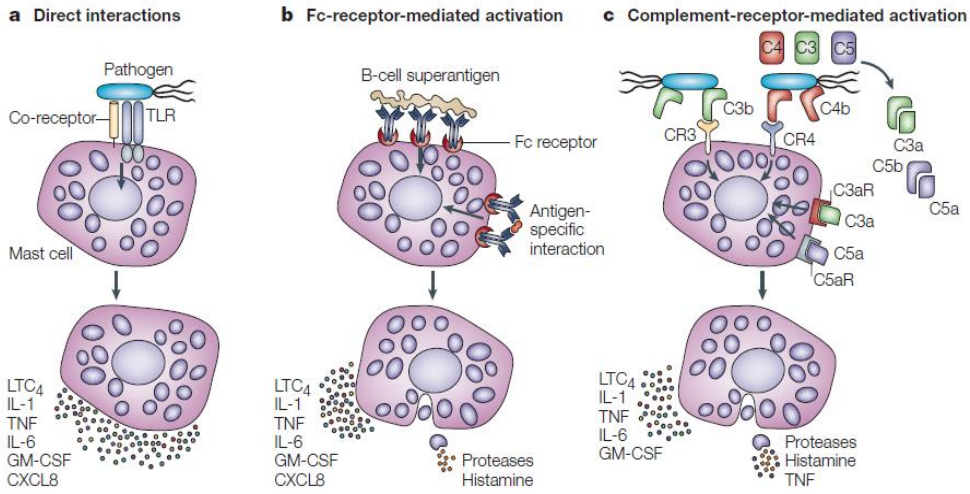

Figure 6: Activation of MCs through IgE independent manner leading to release of mediators. MCs can interact with various pathogens, superantigens and complement protein through various receptors other than FcεRI and leading to release of various mediators. *Source*: (Marshall 2004).

2.3 Exocytosis

Upon activation MCs release a high amount of granule contents. Membrane fusion is necessary for the stimulus-coupled release of granule contents. It requires lipid molecules leave their bilayer orientation to merge two lipid bilayers. MCs are capable of releasing high amounts of their granular content in response to a single stimulatory event. This process is called as degranulation. The regeneration of a granule can take up to 72 h (Lorentz et al. 2012). MCs secretory granules can form channels by fusing with each other. This is called as compound exocytosis. It can either occur in a sequential or in a multivesicular manner. In sequential exocytosis, vesicles initially fuse with the plasma membrane followed by the fusion of underlying next vesicles with the first vesicle. In multivesicular exocytosis, vesicles fuse with each other before interacting with the plasma membrane. Compound exocytosis enables MCs to

discharge their contents very efficiently (Lorentz et al. 2012). In response to some stimuli, MCs also show piecemeal degranulation characterized by gradual loss of granule contents without detectable granule fusion (Lorentz et al. 2012). Thus, piecemeal degranulation allows discharge of discrete packets of granule-associated components without granule exocytosis. Each fusion event mainly requires three basic components: firstly, Rab-GTPases organize the fusion site; secondly, SNARE proteins act during fusion; and thirdly, Nethylmaleimide-sensitive factor (NSF) plus its cofactor - SNAP are required for recycling or activation of the fusion machinery (Ungermann and Langosch 2005).

2.4 Endocytosis / Phagocytosis

Paul Ehrlich named MCs as "Mastzellan" (from the ancient Greek word masto means "I feed"). Although their name "mastzellan" signifies their endocytic role but much of the interest in MCs has centered on their exocytic functions (Abraham and Malaviya 1997). MCs can recognize and attach to a wide variety of opsonized bacteria. *Salmonella typhimurium* coated with the iC3b fragment bind to complement receptor 3 (CR3) on the MC membrane which further leads to phagocytosis and release of various mediators (Sher et al. 1979). As MCs express several IgG receptors such as FcγRI, FcγRII and FcγRIII, opsonized bacteria such as *E. coli* expressing FimH, *E. cloacae* and *K. pneumoniae* bind to these IgG receptors and undergo phagocytosis. These pathogens are killed by the oxidative burst and acidification of phagocytic vacuoles in MCs (Malaviya et al. 1994). *M. tuberculosis* uses a cholesterol-dependent pathway to infect MCs (Munoz et al. 2003, Munoz, Rivas-Santiago and Enciso 2009). Human cord-blood-derived MCs (CBMCs) and MCs from the human leukemic cell line HMC-1 bind and internalize several types of opsonized Gram-negative and Gram-positive bacteria (Arock et al. 1998). This results in a significant loss of bacterial viability. Scanning and transmission electron microscopic pictures showing the interactions of CBMCs with an opsonized clinical strain of *E. coli* are presented in Figure 7.

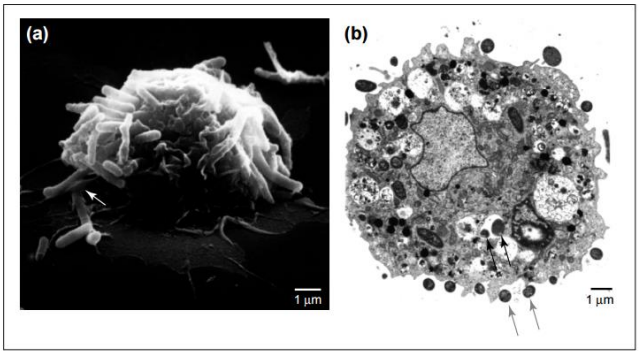

Figure 7: (a) Scanning electron micrographic and (b) transmission electron micrographic examination of human cord-blood-derived MCs (CBMCs), after a three-hour exposure to a noninvasive strain of Escherichia coli in opsonin-dependent conditions. (a) The cell surface of the CBMC is covered with several adherent bacteria. Bacteria appear, in some cases, to be gripped by filapod-like structures (white arrow). (b) Several E. coli are associated with the membrane of the CBMC (gray arrows). In the same CBMC, some of the intracellular bacteria appear to be degraded (black arrows). *Source*: (Arock et al. 1998).

2.5 Etosis

A new mechanism of extracellular killing has been discovered by immune cells in which the cells release DNA and ensnare the pathogens along with histones and mediators which are reported to kill the pathogens which is known as extracellular trap formation known as Etosis. This phenomenon was first discovered in neutrophils and the traps are known as Neutrophil Extracellular Traps (NETs) (Brinkmann et al. 2004). ETosis is triggered by stimuli such as various proinflammatory cytokines (TNF-α, IL-8), platelets, activated endothelial cells (ECs), nitric oxide, monosodium urate crystals, and various autoantibodies (Garcia-Romo et al. 2011, Gupta et al. 2005, Gupta et al. 2010, Kessenbrock et al. 2009, Mitroulis et al. 2011, Neeli, Khan and Radic 2008, Patel et al. 2010). The most frequently used compound to induce ETosis is Phorbol 12-myristate 13-acetate (PMA), a synthetic activator of the PKC family of enzymes. PKC is responsible for activation of NADPH oxidase and ROS production. Many of the inducers of ETs in nature lead to PKC activation (Kaplan and Radic 2012). Histones, antimicrobial peptides (AMPs), and granule proteins are bound to the decondensed chromatin and form web-like structures which mediate entrapment of invading microbes (Brinkmann and Zychlinsky 2012).

Eosinophils, MCs, MΦs, lower vertebrates, and even plants release extracellular traps (Boe et al. 2015, Robb et al. 2014, Tran et al. 2016, Ueki et al. 2013, von Kockritz-Blickwede et al. 2008). The antimicrobial activity of MCs is largely mediated by extracellular mechanisms including degranulation and release of antimicrobial

peptides such as cathelicidins (CRAMP or LL-37), defensins (β-defensins) or proteases (tryptase, chymase). MCs release ETs in a ROS-dependent manner (Figure 8). Mast Cell ETs (MCETs) are composed of DNA and histones, which are the general components of most ETs, as well as mast cell-specific granule proteins like tryptase and CRAMP/LL-37 (von Kockritz-Blickwede et al., 2008). MCs on interaction with microorganisms such as *Streptococcus pyogens, Staphylococcus pneumonia, Pseudomonas aeroginosa, Enterococcus faecalis, Listeria monocytogens* and *Candida albicans* are entrapped in these structures (Campillo-Navarro et al. 2017, Lopes et al. 2015, Scheb-Wetzel et al. 2014, von Kockritz-Blickwede et al. 2008). There is also one report of MCETs formation in psoriasis (Lin et al. 2011). Although, MCETs and NETs share common characteristics, there are several cell type-specific differences. The formation of MCETs can be greatly increased after stimulation of MCs with PMA prior to infection (von Kockritz-Blickwede et al. 2008) similar to NETs (Brinkmann et al. 2004). MCs release less MCETs stimulated with the same stimuli in comparison to neutrophils when studying the respective literature: whereas more than 90% of neutrophils undergo ETosis within 3h upon stimulation with PMA (Fuchs et al. 2007), only 40% of MCs undergo ETosis after 6h of stimulation with the same stimulus and concentration. Another important difference between MCs and neutrophils are their components, which are embedded in the DNA structures: elastase and MPO are essentially involved in the formation of NETs whereas tryptase and LL-37 are involved in the formation of ETs. There is an involvement of the transcriptional hypoxia-inducible factor 1α (HIF-1α) in the modulation of ET release by human and murine MCs (Branitzki-Heinemann et al. 2012) whereas HIF is a well-known factor for its role in the regulation of the inflammatory and innate immune function of neutrophils and MΦs (Goldmann and Medina 2012).

ETs are rather fragile structures, and some effort is required to detect and quantify them. Published methods of NET quantification include microscopy (Brinkmann et al. 2004) and DNA detection either with membrane impermeable DNA dyes (Brinkmann et al. 2004) or by staining the DNA in the supernatant after releasing the NETs with a mild nuclease treatment (Fuchs et al. 2007). Scanning Electron Microscope and Transmission Electron Microscope is also useful in visualizing ETs.

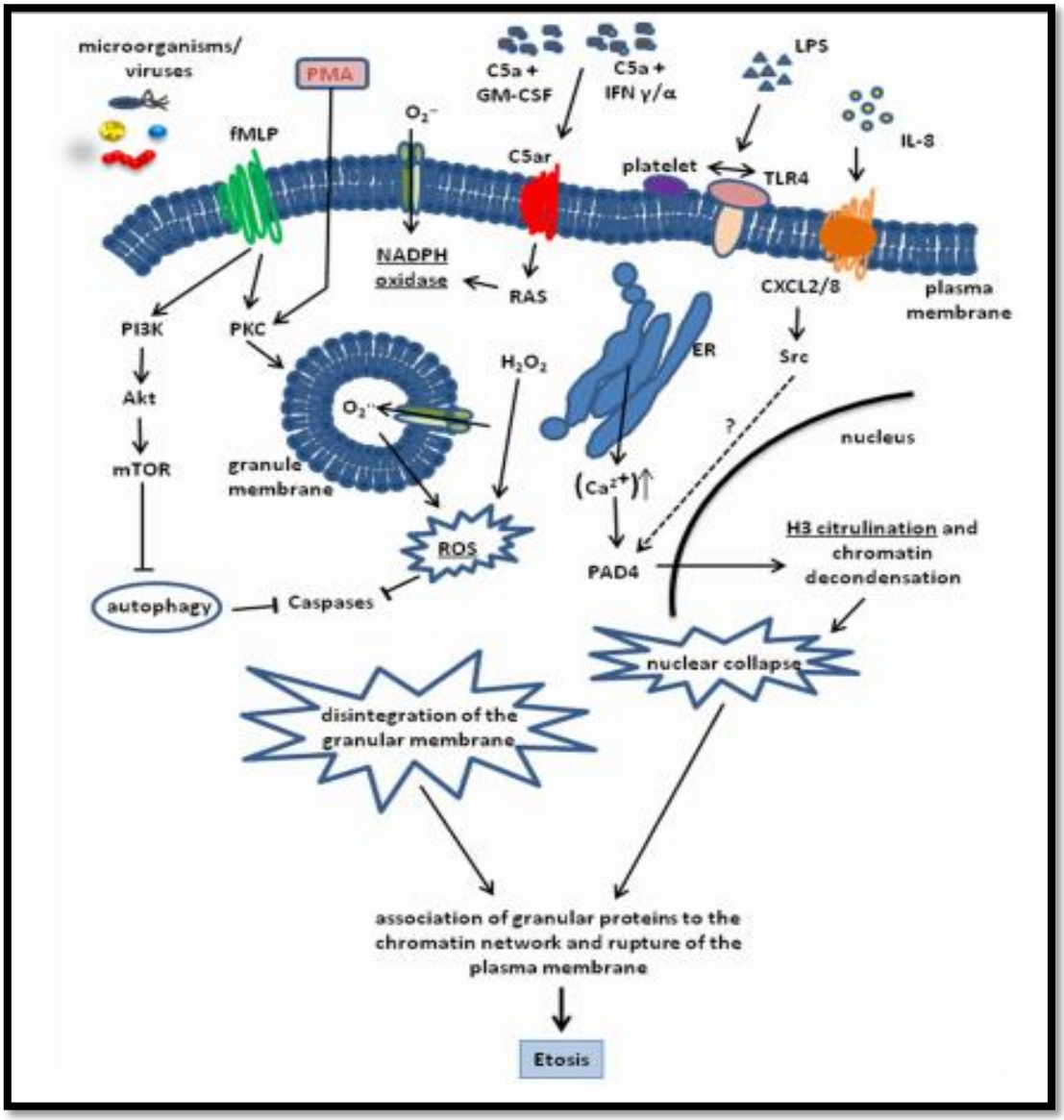

Figure 8: Diagram of cellular processes involved in cellular process of Etosis. MCs are stimulated by contact with bacteria and fungi (yeast and hyphae forms): (a) ultrastructural alterations of nuclear shape with chromatin decondensation, swollen and fragmentation of the nuclear membrane allowing the association of granules and cytoplasmic proteins with the chromatin, and (b) release of extracellular structures consisting of a DNA-backbone, decorated with histones, granular and cytoplasmatic proteins, which ensnare and kill microorganisms. *Source*: (Goldmann and Medina 2012).

3. Response of MCs with Intracellular Pathogens

Intracellular pathogens can be defined as the microbes that cause host damage by intracellular residence, survival and replication. There are two types of intracellular pathogens namely obligate intracellular pathogens which causes host damage and completely depend on a host cell for survival and replication whereas facultative intracellular pathogens do not completely depend on a host cell for survival and replication. They can be cultured in microbiological media in the laboratory. Intracellular pathogenesis can be divided into four phases: (a) entry, (b) survival, (c) replication, and (d) exit from the host cell (Casadevall 2008). Entry can be made by microbe actively or passively. Invasion of *Trypansoma gondi*, *Salmonella* spp., *C. albicans* into the host cells is an example of active entry (Casadevall 2008). Opsonin mediated phagocytosis which delivers the microbe to a phagosome where it is not killed as exemplified by *Crptococcus neoformans* (Casadevall 2008) and Fim H expressing *E. coli* (Malaviya et al. 1999) is an example of passive entry. Entry requires attachment to surface receptors of the host such as *Mycobacterium leprae* which bind to the complement receptor (Casadevall 2008). Several gram-negative bacteria manipulate host cytoskeletal functions and induce their own uptake (Casadevall 2008).

Some microbes survive by subverting intracellular antimicrobial mechanisms such as phagosome-lysosome fusion as exemplified by *M. tuberculosis*, modulating phagosomal pH as exemplified by *C. albicans*, damaging phagosomal membranes as exemplified by *L. monocytogens*, and/or quenching microbicidal oxidative bursts such as *C. albicans* (Casadevall 2008). Some microbes also escape from vesicular compartment to non vesicular compartment of the cytoplasm. Numerous exit strategies also occur in these pathogens such as *L. monocytogens* spread from cell to cell by actin tails, *E. coli* expressing Fim H which gain entry through cellular cavaeloe in MCs also escape through these structures without entering into the endocytic routes (Malaviya et al. 1999).

3.1 Interaction of Mast Cells with intracellular bacteria

MCs are one of the most important cells that strengthens the innate and in few cases adaptive immune system against intracellular bacterial infection. MCs take up *S. typhimurium*, *E. coli* expressing Fim-H, *E. cloacae*, *M. tuberculosis* (Arock et al.

1998, Malaviya et al. 1994, Sher et al. 1979, Munoz et al. 2003) . When MCs recognize *M. tuberculosis* through a GPI anchored protein CD48, release of presynthesized mediators such as histamine and lysosomal enzyme such as β-hexosaminidase and *de novo* synthesized mediators such as TNF-α and IL-6 takes (Munoz et al. 2003, Munoz et al. 2009). A primary bone marrow-derived mast cell–macrophage coculture system significantly inhibited *F. tularensis* LVS live vaccine strain (LVS) uptake and growth within macrophages. Mice deficient in either MCs or IL-4 receptor displayed greater susceptibility to the infection (Ketavarapu et al. 2008). MCs are actively involved in the pathogenesis of *H. pylori* infected gastritis by initiating and promoting the formation of oedema through degranulated and secreted mediators, and releasing multiple chemotactic factors, which induce inflammatory cells to infiltrate to the site of oedema, showing acute inflammatory changes (Nakajima, Bamba and Hattori 2004). The morphological changes, mastocytosis and massive degranulation were observed in *M. leprae* infected mice which is involved in pathophysiology of leprosy (Kumar and Vaidya 1982). MCs on interaction with various intracellular bacteria as already discussed lead to MCET formation and these intracellular bacteria are killed. Thus, MCs phagocytose intracellular bacteria, in few cases such bacteria can subvert the immune response by MCs. MCs can degranulate and increase the pathophysiology of such bacteria and release MCET formation where intracellular bacteria are trapped and killed.

3.2 Interaction of Mast Cells with intracellular Parasites

MCs have long been known to play a role in host defense responses against parasitic infections (Miller and Jarrett 1971). *Toxoplasma gondi* an obligate intracellular parasite inhibits antigen-stimulated degranulation in infected MCs. Antigen-mediated $Ca2^{+}$ release from intracellular stores is significantly reduced because *T. gondi* inhibit tyrosine kinase signaling cascade that results in reduced hydrolysis of phosphatidylinositol4,5-bisphosphate (Smith et al. 2013). MCs can also participate in infection with *Leishmania* parasites. Bone marrow-derived MCs (BMMCs) in contact with live *L. major* or *L. infantum* promastigotes phagocytose *L. major* and release preformed mediators such as β-hexosaminidase and TNF-α (Bidri et al. 1997). In nematode infection, the chymases mMCP-1 and mMCP-9 contribute to their expulsion (Sasaki et al. 2005). Histamine release from MCs via IgE-independent or IgE-mediated immune responses, is known to play a major role in malaria

pathogenesis (Mecheri 2012). It causes release of nitric oxide as well as by enhancing vascular permeability (Van de Voorde and Leusen 1983). Thus, MCs also play an important role in strengthening the immune system against parasitic infection by phagocytosis or degranulation. In few cases, it can also increase pathophysiology of parasitic infection.

3.3 Interaction of Mast Cells with fungus

MCs are prevalent in classical sites of fungal infection. Skin MCs contribute to antifungal immune responses by releasing IL-10 directly at the site of infection. IL-10 plays an important role for controlling infections with several pathogenic fungi (Fierer et al. 1998, Monari et al. 1997). *Aspergillus fumigatus* hyphae can induce MC degranulation in the absence of IgE (Saluja, Metz and Maurer 2012). Fungal zymosan can induce leukotriene production (LTB4 and LTC4) by human MCs through a dectin-1-dependent mechanism. Yeast zymosan can lead to the production of ROS which may have an important role in the defense against fungal infection (Saluja et al. 2012). MCs release mediators during antifungal immune responses in vivo, for example against *P. brasiliensis* in skin lesions with loose granuloma and a Th2 pattern of cytokines (Saluja et al. 2012). *M. sympodialis* extract causes release of cysteinyl leukotrienes and IL-6 from MCs (Saluja et al. 2012). MCs are among the first immune cells to get into contact with *C. albicans* where C-type lectin receptor Dectin-1 is involved in its recognition. Both yeasts and hyphae of C. albicans-induced mast cell degranulation and production of TNF- α, IL-6, IL-10, CCL3 and CCL4, while only yeasts were able to induce IL-1β release. Mast cells also produced ROS after stimulation with both dimorphic phases of *C. albicans* (Nieto-Patlan et al. 2015). MCs reduced fungal viability, internalized yeasts followed by release of cytokines in the supernatant recruiting neutrophils but not monocytes. *C. albicans* could evade ingestion by intracellular growth leading to cellular death. *C. albicans* infected MCs formed extracellular DNA traps, which ensnared but did not kill the fungus (Lopes et al. 2015). Thus, MCs are able to respond to fungus either through degranulation, ROS or MCET formation.

3.4 Interaction of MCs with Viruses

MCs' role in defense in viral infections is not much explored either due to lack of suitable mouse models of viral infection or in MC deficient mouse models so far.

MCs can be activated by viruses via TLR3 (Marshall 2004, Orinska et al. 2005). MCs respond to viral infection by the production of type I IFNs after being exposed to doublestranded RNA and/or virus via TLR3 (Kulka et al. 2004). MCs stimulated via TLR3 are potent regulators of antiviral $CD8^+$ T-cell activities *in vitro* and *in vivo* (Orinska et al. 2005). Mediator release from MCs after viral infection is well explored as described in different reviews (Marshall 2004, Moon et al. 2014). MCs sensitized with dengue virus immune sera and upon contact with Sendai virus leads to degranulation as well as histamine release (Sanchez et al. 1986). The gp120 envelope protein of HIV can induce IL-4 and IL-13 production by MCs (Patella et al. 2000). Antibody-enhanced dengue virus infection among CBMCs and HMC-1 increased expression of the adhesion molecules ICAM-1 and VCAM-1. MCs produce several mediators including IL-1β, IL-6, CCL3, CCL4, and CCL5 after FcγRII mediated dengue virus infection (Brown et al. 2006). MCs further play an important role in the clearance of dengue virus by recruitment of natural killer (NK). MCs co-cultured with respiratory syncytial virus (RSV)-infected A549 airway epithelial cells show degranulation and increased TNF-α secretion (Shirato and Taguchi 2009). Thus, MCs play an important role in limiting viral infections through release of various cytokines and chemokines.

4. Tuberculosis

Tuberculosis (TB) caused by *M. tuberculosis* is a leading cause of morbidity and mortality worldwide, reporting about 9.6 million new cases and 1.5 million deaths annually (Raviglione and Sulis 2016). While anti mycobacterial therapy exists, currently available drugs are only partially effective because of the impermeable nature of the mycobacterial cell wall and development of resistance to M. tuberculosis (Warner and Mizrahi 2006). Moreover, *M. tuberculosis* is able to remain viable within infected hosts for a prolonged time. Infection of a host with *M. tuberculosis* is initiated following the inhalation of droplets (aerosols) containing a small number of bacilli (Kaufmann 2001). Once in the lung, bacilli are internalized through phagocytosis by the resident macrophages of the lung, the alveolar macrophages as shown in Figure 9. Alveolar macrophages activated by the appropriate stimuli can effectively transfer the phagocytosed *M. tuberculosis* to the destructive environment of lysosomes, but some bacilli are able to escape lysosomal delivery and survive within the macrophage (Kaufmann 2001). Infected macrophages can then either

remain in the lung or are disseminated to other organs in the body. Only a minority (10% of infected people [World Health Organization, 2007]) develop tuberculosis, because in most healthy individuals, the immune defense system is sufficient to keep control of *M. tuberculosis* so that the disease is not able to develop. MΦs secrete a variety of cytokines, phagocytose mycobacteria and process/present mycobacterial antigens to T cells when they interact with *M. tuberculosis* through TLR2 (Underhill et al. 1999). Although the primary host cell for *M. tuberculosis* is the macrophage, other cell types, such as DCs, fibroblasts, type II pneumocytes, endothelial cells and MCs, have been reported to phagocyte *M. tuberculosis* (Munoz et al. 2003, Munoz et al. 2009). MCs interact with *M. tuberculosis*, triggering the release of several prestored mediators and cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-6 (Munoz et al. 2003, Munoz et al. 2009). GPI-anchored molecule CD48 is involved in mast cell activation by *Mycobacteria* (Munoz et al. 2003, Munoz et al. 2009). There are also *in vivo* reports suggesting MCs participating in host defense against *M. tuberculosis* infection through the production and secretion of cytokines such as tumor necrosis factor-α, interleukin-1 and chemokines such as keratinocyte-derived chemokine, monocyte chemotactic protein-1 that play a role in the recruitment and activation of inflammatory cells in Balb/c mice infected with *M. tuberculosis* (Carlos et al. 2007).

It is very infectious to work with virulent strains of *Mycobacteria* such as H37Rv so Mycobacterium bovis bacillus Calmette-Guerin (BCG) is used which was developed by Calmette and Guerin in 1921 from virulent strain of M. bovis that has lost its virulence in humans and produce antibodies against tuberculosis by being specially subcultured in an artificial medium containing increasing concentration of bile for 13 years (Calmette 1931). BCG lung infection in mice is a self-limiting infection, similar to M. tuberculosis infection in healthy human beings. For this reason, the mouse BCG infection model has been used extensively for studying the interactions between mycobacteria and immune cells in the lung (Kumari and Saxena, 2011).

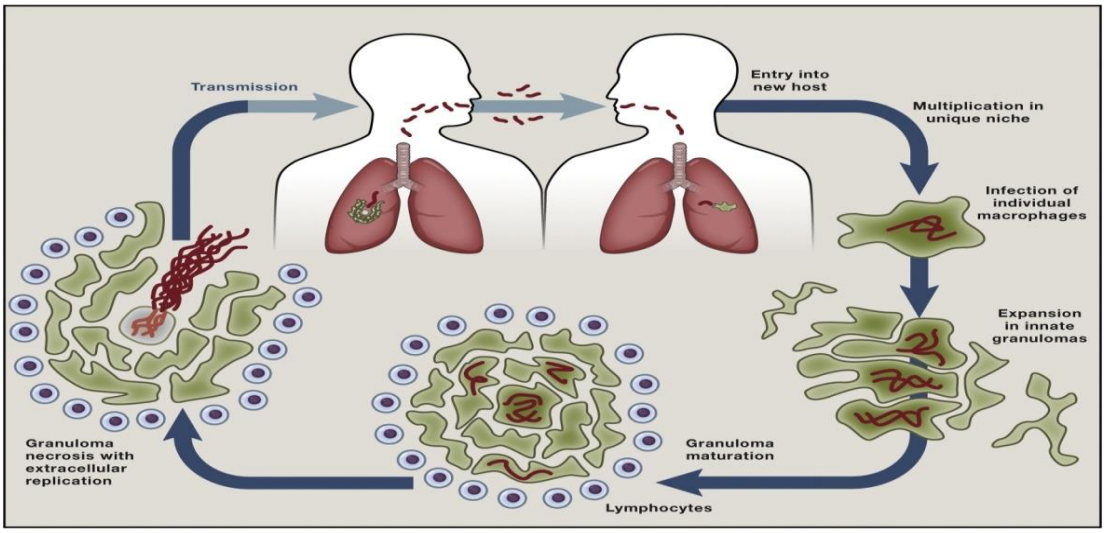

Figure 9: Pathogenic Life Cycle of *M. tuberculosis*. *M. tuberculosis* infection initiates when fine aerosol particles containing the bacteria coughed up by an individual with active disease are transmitted in the lungs of a new host where macrophages are recruited. A new round of macrophage recruitment to the original infected macrophage is initiated, forming the granuloma. The granuloma in its early stages expands infection by allowing bacteria to spread to the newly arriving macrophages. The granuloma can restrict bacterial growth by adaptive immune response. In some case the infected granuloma macrophages can undergo necrosis, forming a necrotic core that makes a good environment for bacterial growth and transmission to the next host. *Source:* (Cambier, Falkow and Ramakrishnan 2014)

5. Leishmania

Few studies have attempted to provide direct evidence for MC-dependent protective effects in settings of parasite infections that originate in the skin. Thus, the role of MCs as players in antiparasitic immunity remains to be characterized in detail. One such important class of parasites is the protozoan parasite of the genus Leishmania, is endemic in tropical and subtropical regions around the world. Parasites of the genus Leishmania cause a spectrum of diseases ranging from self-healing ulcers to disseminated and often fatal infections, depending on the species involved and on the host immune response. Clinical manifestations of leishmaniasis include cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis (MCL), visceral leishmaniasis (VL) and post-kala-azar dermal leishmaniasis (PKDL). Leishmania donovani, the species that causes visceral leishmaniasis (VL), disseminates to spleen, liver and bone marrow (BM), whereas L. major and L. tropica that cause cutaneous leishmaniasis (CL), remain in the cutaneous lesion and the draining lymph node. The majority of VL cases (90%) occur in only six countries: Bangladesh, India, Nepal, Sudan, Ethiopia and Brazil. In the Indian subcontinent, about 200 million people are estimated to be at risk of developing VL; this region harbours an estimated 67% of the

global VL disease burden (Ashford 2000, Chappuis et al. 2007). Currently, about 12 million patients worldwide are believed to suffer from Leishmaniasis and some 500,000 new cases and 59,000 deaths are reported each year (Maurer et al. 2006). So far no vaccine and also very limited therapeutic avenues are available to control Leishmaniasis. Apart from the impact of *Leishmania* on world health, Leishmaniasis represents an elegant infection model that can teach us a lot about host-parasite interactions and immune evasion. The lifecycle of *Leishmania* involves two stages without sexual stage. Leishmaniasis is transmitted through the bite of female sand flies. During their blood meal, infected sand flies inject the infective stage promastigote parasite, into the human host. Injected promastigotes are first phagocytized by macrophages and transform into so-called amastigote parasites. These multiply in the infected cells and also affect different tissues. Depending on the *Leishmania* species, the corresponding clinical manifestation of the disease results. When sand flies take blood meals from an infected host, they take up parasitized macrophages. In the vector fly's midgut, these parasites differentiate into the so-called promastigote form, which multiplies and finally migrates to the fly's proboscis and migrate to the anterior midgut and foregut. Finally to complete the cycle, sand fly injects promastigotes into the skin during a blood meal as shown in figure 10.

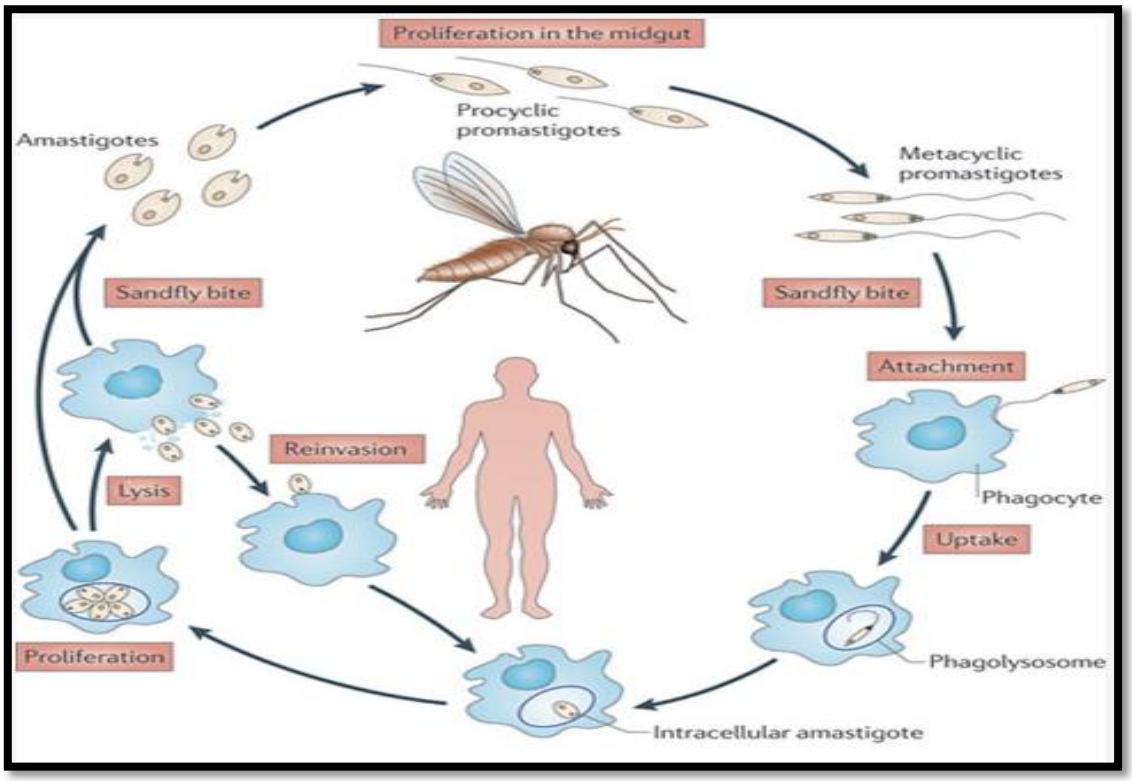

Figure 10: Life cycle of *Leishmania*. *Leishmania* procyclic promastigotes differentiate in sandflies into infective, non-dividing metacyclic promastigotes, which are located ready for transmission. During blood feeding, the sandfly delivers metacyclic promastigotes. The metacyclic promastigotes are then phagocytosed by one of several possible cell types that are found in the local environment. Metacyclic promastigotes transform into aflagellate amastigotes. Amastigotes undergo replication within host cells, which rupture when too many amastigotes are present, allowing reinfection of local phagocytes. The transmission cycle is complete when infected phagocytes are taken up by another sandfly with the blood meal, and amastigotes then convert into promastigotes in the sandfly midgut. *Source*: (Kaye and Scott 2011).

In recent years, a lot of research effort has focused on interaction of the innate immune system with *Leishmania* to enable development of better strategies for vaccine and drug development against Leishmaniasis. These studies of host-pathogen interactions have revealed that driven by co-evolutionary processes, Leishmania parasites have adapted to hide within different cell types to evade the host immune response. Predominantly myeloid cells such as macrophages, dendritic cells, and neutrophils harbour this obligatory intracellular parasite. Macrophages are crucial for parasite survival, as well as for its elimination. After the initial function of neutrophils as host cells, one model based on human neutrophils (and macrophages), called for a 'Trojan horse' function of neutrophils whereby apoptotic neutrophils carrying Leishmania parasites are transferred to macrophages (van Zandbergen et al. 2004). A large numbers of MCs are found in the skin, predominantly in the superficial dermis, where *Leishmania* is encountered after the bite of infected sand flies and their role in *L. major* and *L. donovani* mediated immune responses has been shown. Specifically, *L. major* has been described to activate MCs to induce the release of proinflammatory mediators, and to be phagocytosed by MCs. There is also evidence of their role in establishment of a Th2 response during L. major infection (Bradding and Holgate 1999). Finally, it has been shown that dermal MCs are required for the recruitment of macrophages during cutaneous granuloma formation, a hallmark feature of parasite induced inflammatory responses (Kropf et al. 2003). In contrast, substantial inhibition of IL-10, IL-4, and IL-13 expression and the absence of degranulated MCs within the ears of mice immunized with L. donovani centrin gene deleted live attenuated vaccine candidate and challenged with virulent wild type parasites, suggested a controlled anti-inflammatory response (Dey et al. 2014). As thus observed a distinct dichotomy of opinions exists as to MCs can either help or not in the pathogen replication, therefore we set out to dissect/discover through this study, the very initial events when MCs encounter *Leishmania* parasites in the *in vitro*. It is important to investigate the role of MCs in Leishmaniasis as: firstly, MCs are markedly more numerous at skin regions that are often infected by *Leishmania* sp. Secondly, MCs are critically involved in the induction of innate immune responses against bacteria; and previous studies have suggested that MCs are also involved in the regulation of immunity against various *Leishmania* sp. (Dey et al. 2014, Romao et al. 2009).

6. Interaction of MCs with *Candida albicans*

Candida species are associated with human beings for quite long time as harmless commensals. They are commonly found on the mucosal surfaces of gastrointestinal and genitourinary tracts and skin of humans. In some cases, they become opportunistic pathogens in immunologically weak and immune compromised patients. It can cause a severe, life-threatening bloodstream infection that leads to colonization of Candida in internal organs (disseminated candidemia) which possess serious health problem in these individuals. Mortality rate for these patients is observed between 30% and 50% (Pfaller et al. 1998).

The ability of *C. albicans* to infect such diverse host niches is supported by a wide range of virulence factors and fitness attributes (Mayer, Wilson and Hube 2013). A number of attributes, including the morphological transition between yeast and hyphal forms, the expression of adhesins and invasins on the cell surface, thigmotropism, the formation of biofilms, phenotypic switching and the secretion of hydrolytic enzymes are considered virulence factors. Fitness attributes include rapid adaptation to fluctuations in environmental pH, metabolic flexibility, powerful nutrient acquisition systems and robust stress response machineries as shown in Figure 11.

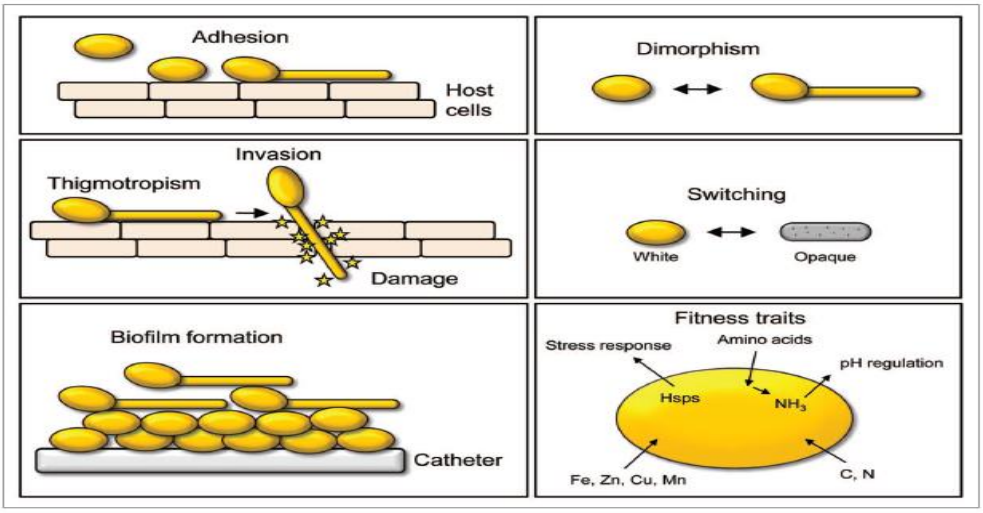

Figure 11: An overview of selected *C. albicans* pathogenicity mechanisms. Yeast cells adhere to host cell surfaces by the expression of adhesins. Contact to host cells triggers the yeast-to-hypha transition by thigmotropism. The expression of invasins mediates uptake of the fungus by the host cell through induced endocytosis. Several fitness traits influence fungal pathogenicity such as robust stress response mediated by heat shock proteins (Hsps); auto-induction of hyphal formation through uptake of amino acids and excretion of ammonia (NH_3). Uptake of different compounds such as carbon (C) and nitrogen (N) *Sources*; and uptake of essential trace metals, e.g., iron (Fe), zinc (Zn), copper (Cu) and manganese (Mn). *Source*: (Mayer et al. 2013).

6.1 Cell wall of *C. albicans*

The cell wall of *C. albicans* contains carbohydrates and cell wall proteins (CWPs) that are not present in the human body so it represents an ideal immunological target to discriminate self from non-self, and consequently the majority of fungal PAMPs (pathogen-associated molecular patterns) that activate and modulate immune responses. The inner layer of the cell wall contains the skeletal polysaccharides chitin and β-1,3-glucan, which confer strength and cell shape as shown in figure 11. The CWPs from the outer layer are attached to this framework predominantly by glycosylphosphatidylinositol (GPI) remnants that are linked to the skeletal polysaccharides through a more flexible β-1,6-glucan as shown in figure12.

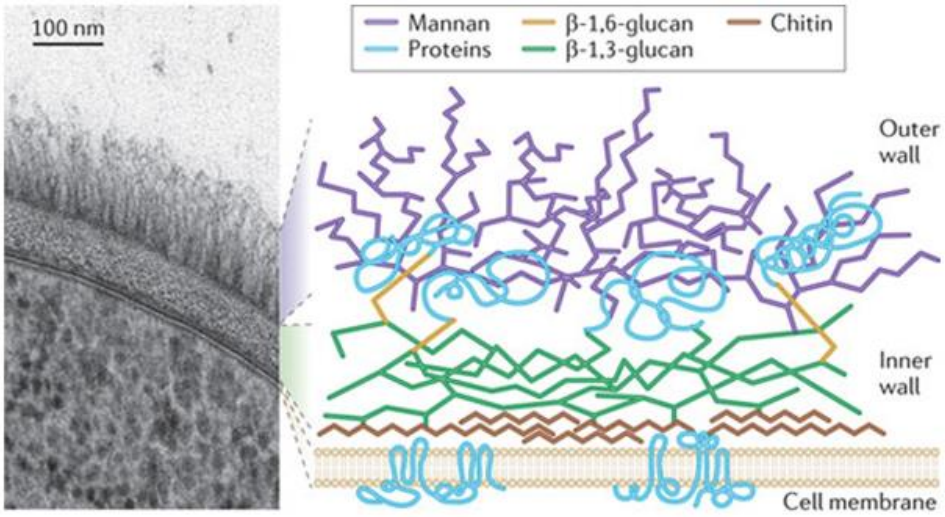

Figure 12: Cell wall of *C. albicans.* Two layers can be easily distinguished in *C. albicans.* The outer layer is enriched with O and N linked polymers (mannans) that are associated with proteins and form glycoproteins. The inner layer contains the skeletal polysaccharides chitin and β-1,3-glucan. The outer cell wall proteins are attached to the inner framework predominantly by GPI. *Source*: (Gow et al. 2011).

6.2 Role of Glycophosphatidyl Inositol biosynthesis in *C. albicans*

The ubiquitous GPI anchor in eukaryotes anchors a variety of proteins to the cell surface. These proteins in the human pathogenic fungus, *C. albicans*, include virulence factors, enzymes involved in host immune response evasion, cell wall biogenesis and maintenance proteins as well as hyphae specific proteins. Inhibiting GPI biosynthesis attenuates its virulence (Yadav et al. 2014). GPI anchoring is a post translational modification which takes place in endoplasmic reticulum by multiple step pathway as shown in figure 13.

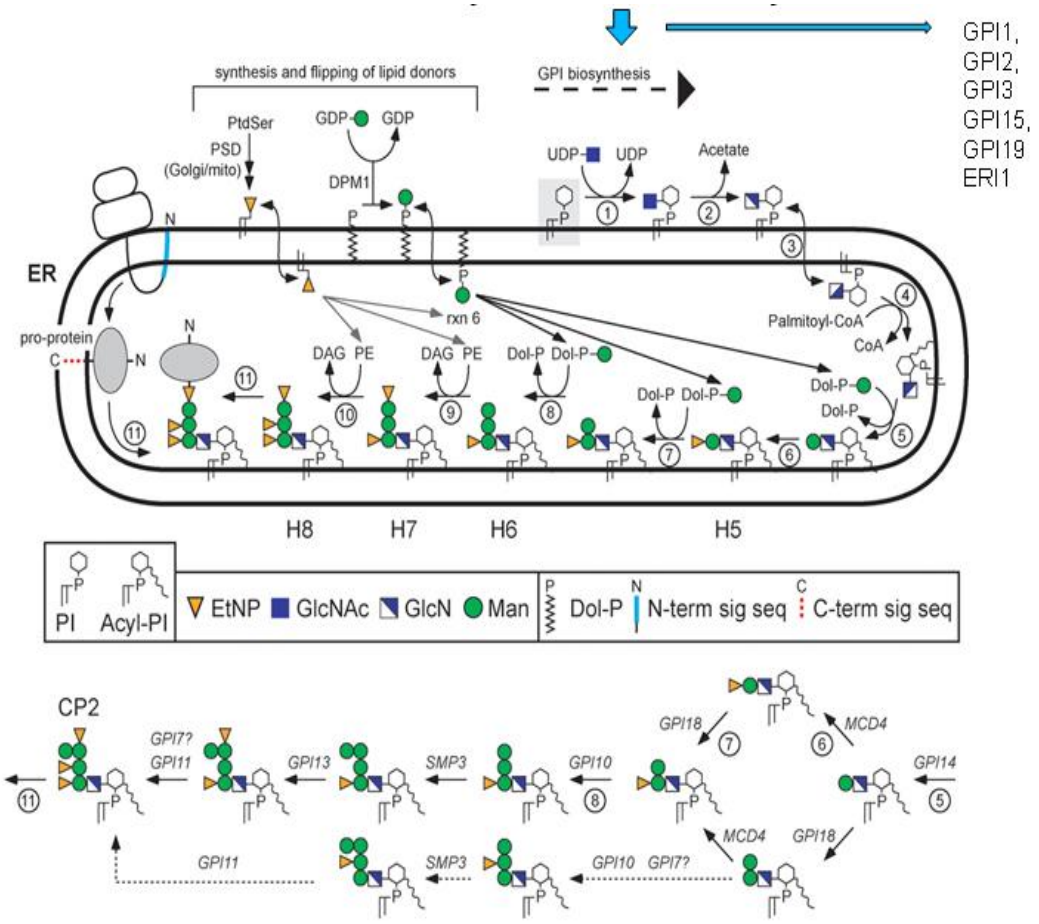

Figure 13: General scheme for GPI biosynthesis in the ER of yeast and mammals. The ER is depicted as a topologically defined compartment. Biosynthesis begins at the top of the figure with a PtdIns acceptor (gray box). In step 1, PtdIns is glycosylated to generate GlcNAc-PI on the cytoplasmic face of the ER. GlcNAc-PI is then de- N -acetylated (step 2) to yield GlcN-PI. GlcN-PI is flipped (step 3) into the lumenal leaflet of the ER, where it is inositol-acylated (step 4), inositol-mannosylated, and modified by Etn-P (steps 5–10). Man is derived from Dol-P-Man (synthesized from Dol-P and GDP-Man in a reaction catalyzed by DPM1 on the cytoplasmic face of the ER), and Etn-P is derived from PtdEtn [synthesized typically by decarboxylation of PtdSer via the action of PSD (PtdSer decarboxylase); synthesis from CDP-Etn via the Kennedy pathway is also possible]. Both Dol-P-Man and PtdEtn must be flipped into the lumenal leaflet of the ER to participate in GPI biosynthesis. The Etn-P-capped GPIs that are synthesized by this pathway [H7 and H8 in mammals; CP2 in yeast] (panel B) are attached (step 11) to ER-translocated proteins displaying a C-terminal GPI signal sequence. Step 11 is catalyzed by GPI transamidase. Man 4 - versions of the lipids H6, H7, and H8 may also be synthesized. Adapted: (Orlean and Menon 2007).

The first step is the formation of GPI-N-acetyl glucosaminyl phosphatidylinositol (GlcNAcPI) from uridinediphosphate- N-acetylglucosamine (UDP-GlcNAc) and phosphatidylinositol (PI) as shown in figure 11. It is catalyzed by GPI-N-acetylglucosaminyltransferase complex which consists of six different subunits Gpi1, Gpi2, Gpi3, Gpi15, Gpi19, and Eri1. Gpi2 and Gpi19 mutually coregulates with ergosterol biosynthesis. Gpi2 regulates hyphal morhphogenesis via Ras signaling and governs pathogenecity. Both Gpi2 and Gpi19 subunits negatively regulate each other

(Yadav et al. 2014). By observing phenotypic effects it was found that the single allele disruption of GPI15 (*GPI15* heterozygote) makes the strain azole sensitive and hypofilamentous compared to wild type. Deletion of one allele of GPI19 and GPI2 in the GPI15 heterozygote makes it further sensitive drugs and hypofilamentous while overexpression reverts the phenotypic effects. Gpi15 governs both ergosterol biosynthesis and Ras signaling via Gpi19 and Gpi2 and that Gpi15 can act as a good antifungal target (Kalpana Pawar 2015). This has been described in figure 12.

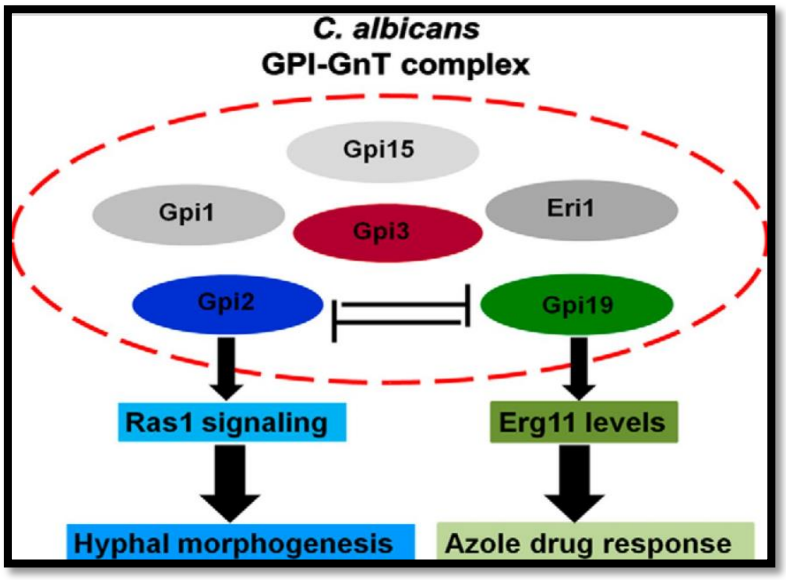

Figure 14: Sub units of Glycosylphosphatidylinositol-Nacetylglucosaminyltransferase (GPI-GnT) complex. GPI-GnT complex includes different subunits. GPI2 and GPI19 subunits of GPI-GnT complex are negatively co-regulated. Thus, whereas GPI19 seems to directly affect ERG11 levels, and thereby alter azole response of *C. albicans*, GPI2 primarily affects Ras signaling and thus affects hyphal morphogenesis in *C. albicans*. The effect of GPI2 disruption on ERG11 appears to be via its interaction with GPI19, whereas the effect of GPI19 on Ras1 activity is dependent on its interaction with GPI2. *Source*: (Yadav et al. 2014).

MCs are among the first immune cells to get into contact with *C. albicans*. *C. albicans* induced mast cell degranulation *in vitro* and *in vivo*. Cell wall fraction but not culture supernatant and cell membrane fraction prepared from hyphally grown *C. albicans* induced β-hexosaminidase release in RBL-2H3 cells. Cell wall mannan and soluble β-glucan fractions also induced β-hexosaminidase release. Histological examination of mouse forestomach showed that *C. albicans* gut colonization induces mast cell degranulation. However, intragastric administration of cell wall fraction failed to induce mast cell degranulation. It was proposed that cell wall polysaccharides are responsible for mast cell degranulation in the *C. albicans*-colonized gut (Sakurai, 2012). Both yeasts and hyphae of *C. albicans* induced mast

cell degranulation and production of TNF- α, IL-6, IL-10, CCL3 and CCL4, while only yeasts were able to induce IL-1β. MCs also produced ROS after stimulation with both dimorphic phases of *C. albicans*. When MCs were activated with yeasts and hyphae, they showed decreased expression of IκBβ and increased presence of phosphorylated Syk. Blockade of the receptor Dectin-1, but not Toll-like receptor 2, decreased TNF- αproduction by mast cell in response to *C. albicans*. These results indicate that MCs are capable of sensing the two phases of *C. albicans*, and suggested that MCs participate as an early inductor of inflammation during the early innate immune response to this fungus. *Candida albicans* is the most common human fungal pathogen. Mast cell responses included the release of initial, intermediate and late phase components determined by the secretion of granular proteins and cytokines. Initially MCs reduced fungal viability, internalized yeasts followed by secreting factors in the supernatant recruiting neutrophils but not monocytes. *C. albicans* could evade ingestion by intracellular growth leading to cellular death. Furthermore, secreted factors in the supernatants of infected cells recruited neutrophils, but not monocytes. Late stages were marked by the release of cytokines that are known to be anti-inflammatory suggesting a modulation of initial responses. *C. albicans*-infected MCs formed extracellular DNA traps, which ensnared but did not kill the fungus. They can evade MCETs. These results suggest that *C. albicans* elicit a temporal response in MCs (Lopes et al, 2015).

7. Limitations in vaccines or therapy available for above three pathogens

Many researchers are focussing in developing vaccines and discovering new therapeutic approaches to overcome the diseases related to these pathogens

7.1 Treatment of *M. uberculosis*

Antibiotics such as isoniazid, rifampin, pyrazinamide, and ethambutol are used for treatment of Tuberculosis (Centres for Disease and Prevention 2009). But resistance is developing due to these antibiotics. BCG is the only vaccine available for tuberculosis. The efficacy of these vaccines varies. There is difference in opinion related to the variation. Differences in the strain of BCG, the age at vaccination, or methodological differences are important factors for this variation. The most widely accepted hypothesis for this variation is geographical location where exposure to nontuberculous *Mycobacteria* is common. High efficacy is seen when BCG is used to

vaccinate newborns. The efficiency wanes over a period of 10 to 15 years (Ottenhoff and Kaufmann 2012, Pitt et al. 2013).

7.2 Treatment with Leishmaniasis

Current treatment is based on chemotherapy with drugs such as sodium stibogluconate and meglumine antimoniate, Pentamidine and amphotericin B. There is a problem of toxicity, resistance and side effects which can be life threatening by usage of these drugs (Croft, Sundar and Fairlamb 2006). It has been a goal to develop of a successful vaccine to prevent Leishmaniasis but nothing promising has been achieved so far.

Sub unit vaccines or heat-killed parasites have been disappointing when tested in field studies. The first generation vaccines based on killed parasites is replaced by leishmanization. Leishmanization is also used in which minimal live inoculums of *Leishmania* is administered and the individual becomes refractory to reinfection (Khamesipour et al. 2005). This method is also not promising due to a range of reasons including quality control, parasite persistence, emergence of HIV and ethical reasons. The first generation of vaccine is replaced by second generation of vaccine. The second generation vaccines include genetically modified parasites such as *Leishmania* having deletion in centrin1 (Selvapandiyan et al. 2004), defined subunit vaccines or recombinant bacteria and viruses expressing leishmanial antigens. *Leishmania* vaccine development has proven to be a challenging task because of complexity in pathogenesis of *Leishmania*

7.3 Treatment with *Candidiasis*

Various drugs such as Amphotericin B, Flucanazole, Diflucan are used to treat Candidiasis (Shokohi et al. 2016). As drugs have side effects new approaches are being developed to cure candidiasis. Cell wall proteins are an ideal vaccine targets for inducing a protective immune response in the host. Since recombinant proteins have been generally produced in *E. coli*, the expressed proteins aren't glycosylated as they would be in eukaryotic cells because of which they differ in natural structure. But there is a challenge to the development of effective vaccines to elicit immune responses in immunocompromised individuals who are most at risk for invasive fungal infections. The vaccine usually elicits a weak immune responses or generate

side effects in immunocompromised patients. Under these circumstances passive immunotherapy is useful which involves opsonisation, complement fixation, immune response and protective antibodies (Moriyama et al. 2014).

8. Usage of MCs for therapeutic approaches for these three pathogens

Most inflammatory signals are not very long lived so immune system has adaptated in such a way to prolong the survival of extracellular signals and deliver to specific targets. The delivery of TNF to draining lymph nodes during inflammation by MC-derived particles is an example of such an adaptation (Kunder et al. 2009). MC particles are specialized for delivery of very small quantities of cargo from one specific site to another specific site. Heparin, the predominant component of MC particles, being negatively charged minimizes interaction with adjacent cells and other structures in the extracellular environment, and hence favours their movement into the lymphatic drainage. Synthetic MC granule can function as adjuvants during vaccination to promote and polarize immunity in lymph nodes (St John et al. 2012). Because MCs can help to initiate adaptive immune responses by inducing or enhancing the migration of antigen-presenting cells to draining lymph nodes and through lymphocyte activation, one group has hypothesized that the administration of small-molecule mast cell activators (such as compound 48/80) with vaccine antigens might enhance the development of a protective antigen-specific immune response (McLachlan et al. 2008). Scientists have found that subcutaneous or nasal administration of such activators enhances the trafficking of dendritic cells and lymphocytes to draining lymph nodes and increases antigen-specific serum IgG responses. Moreover, nasal administration of compound 48/80 with B5R poxvirus protein protects immunized mice against infection with vaccinia virus *in vivo* but B5R poxvirus protein alone does not (McLachlan et al. 2008).

Thus, a lot more needs to be explored in MCs so make it as an attractive tool for therapeutic approaches.

Materials and Methods

Animals

Inbred C57BL/6 female mice were obtained from National Institute of Nutrition, Hyderabad, India. They were used for *M. bovis* BCG study. Inbred BALB/c female mice (8 to12 weeks old) were used to isolate Peritoneal Mast Cells (PMCs). Animals were obtained from Jamia Hamdard University, New Delhi, India and were maintained in the animal house facility at Jawaharlal Nehru University (JNU), New Delhi, India under pathogen free conditions in positive pressure air-conditioned units (25°C, 50% relative humidity) at a 12 h light and dark cycle. Both water and food were provided *ad libitum.* Institutional Animal Ethics Committee (IAEC), JNU (registration no: 19/GO/ReBi/S/99/CPCSEA) approved all experimental protocols (IAEC Code:13/2013 and 27/2014) requiring the use of animals. All experiments were performed under relevant guidelines and regulations.

Intra-tracheal instillation of BCG

Female C57BL/6 mice were anaesthetized and exposed to BCG (1 Million cfu) or PBS vehicle by intra-tracheal instillation (Saxena et al. 2002). Briefly, mice were anaesthetized and then suspended on a 60 degree incline board by the help of a rubber band through their incisors. The mouse tongue was gently drawn out and while exerting gentle pressure on its thoracic cavity, BCG was administered intratracheally with the help of a gavage needle. Pressure from thoracic cavity was then released to allow the mice to iinhale with great force, thus depositing the treatment directly into the lungs of the mouse. Each BCG instillation experiment was accompanied by one set in which only PBS was intra-tracheally instilled.

Production of mouse polyclonal antibodies to BCG

Mouse polyclonal antibodies to BCG were raised in three female C57BL/6 mice that were injected intraperitoneally (i.p.) with 100 µg protein from sBCG in PBS. This injection was followed by weekly booster doses of 50 µg each for 3 weeks. Four days after the final booster dose, whole blood was collected aseptically by retro-orbital bleeding in an microfuge tube. The blood was then kept at room temperature for 2 h, the clot was detached from walls of the tube after 3-4 h. Then the tubes were kept overnight at 4°C. The next day serum was obtained by centrifugation at 1800 g, 4°C for 10 mins. Indirect ELISA was done to quantitate the antibody titre. The

complement proteins in the serum were inactivated by incubating serum at 56°C for 30 mins. Then the decomplemented serum was aliquoted and stored at -20°C for further use.

BCG ELISA

The microwells of 96 well ELISA plate were coated with 10 µg protein from sonicated BCG in 100 µl of coating buffer (0.1 M Sodium Carbonate-Bicarbonate buffer). Unbound antigens were washed away with wash buffer (PBS + 0.05% Tween-20). The plates were then blocked with 100 µl of blocking buffer (3% BSA in PBS) at room temperature for 2 h. The wells were washed five times with wash buffer followed by addition of 50 µl of serial dilutions of polyclonal antibodies or pre-immune serum prepared in assay diluent (3% BSA in PBS). The incubation was continued at room temperature for 1 h. The wells were washed five times with 100 µl of wash buffer and incubated with 100 µl of a 1:20000 dilution of secondary anti-mouse IgG-horseradish peroxidise conjugated antibody for 1 h. After the wells were washed five times with wash buffer, 100 µl of 1X 3,3',5,5'-Tetramethylbenzidine (TMB) was added and the plates were incubated in dark until the blue colour developed. The enzyme reaction was stopped by adding 1M H_2SO_4 and the absorbance at 450 nm was determined by using ELISA plate reader (Bio-Rad microplate reader, Model no. 680). Each serum was tested in duplicate, and the mean absorbance for control (no antigen) well was subtracted from that for antigen-coated wells before analysis

Isolation and co-culture of PMCs

PMCs were isolated from peritoneal lavage of female BALB/c mice by $26^{1/2}$ gauge needle having 3 ml volume of syringe. After ACK lysis cells were incubated with anti-mouse CD16/32 Fc block for 20 min on ice prior to staining. Cells were then incubated with APC anti mouse CD117 (Biolegend, San Diego, CA, USA) and PE anti mouse CD45R (Biolegend, San Diego, CA, USA) for 30 min on ice. Cells were washed twice with staining buffer (PBS containing 2%FBS). Cells were filtered through 70 µ filter and were sorted through BD FACS Melody. The isolated PMCs were co-cultured in RPMI media supplemented with 20ng/ml IL-3 (Peprotech, Rocky Hill, NJ, USA) and Stem Cell Factor (Peprotech, Rocky Hill, NJ, USA) (Dahdah et al. 2014) .

Materials

The reagents Trypsin, Bovine Serum Albumin (BSA), Lucifer Yellow (LY), Cytochalsin D (CTD), Ionomycin, Dimethyl Sulfoxide (DMSO), Tween-20, RPMI-1640 Medium, phenol red free RPMI medium, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, Minimum Essential Medium Eagle, sheep anti-mouse IgG HRP conjugated antibody were all purchased from Sigma Aldrich (India). Iscove's Modified Dulbecco's Medium and Fetal Calf Serum were purchased from Invitrogen (India). Tween-80 was purchased from Sigma, MO, USA. Middlebrook 7H11 agar was purchased from Difco Laboratories. Anti-mouse CD16/32, Allophcocyanin (APC) anti-mouse CD117 were purchased from BD Pharmingen (USA). 3,3',5,5'Tetramethylbenzidine (TMB) was purchased from eBiosciences San Diego, CA, (USA). Bradford dye was purchased from Bio-Rad (USA). Costar (NY, USA) was the source of all plastic disposable culture ware. All the syringes and needles were procured from Becton Dickinson (Singapore). All other chemicals were purchased locally and were of analytical grade.

Maintenance of cell line

The Rat Basophilic Leukaemia (RBL-2H3) mast cell line was a kind gift from Dr. Paul Roche, NIH, Bethesda, MD, USA. They were maintained in RBL complete medium containing equal parts of Minimum Essential Medium Eagle with Earle's salts (Gibco, Life technologies, Grand Island, NY, USA) and Iscove's Modified Dulbecco's Medium (Gibco, Life technologies, Grand Island, NY, USA) and supplemented with 25mM HEPES (Sigma, MO, USA) (N-[2- 100 hydroxyethyl]piperazine-N0-[2-ethanesulfonic acid]), 50 µg/ml gentamicin sulfate, and 20% heat- inactivated Fetal Bovine Serum (FBS) (Gibco, Life technologies, Grand Island, NY, USA) in a humidified atmosphere containing 5% CO_2 at 37°C. Cell lines were maintained as adherent cultures and subcultured by trypsinization. Murine alveolar macrophage (MH-S) cell line was a kind gift from Prof. R. K. Saxena, South Asian University, New Delhi, India. They were grown in RPMI-1640 medium supplemented with 10% FBS, 50 µg/ml gentamicin sulfate, in a humidified atmosphere containing 5% CO_2 at 37°C. Exponentially growing cells were used in all experiments. Both the cell lines were maintained as adherent cultures and subcultured by trypsinization. Viability of cells were counted using trypan blue exclusion method

using formula, Percent (%) Viability = (Live cells / Total number of cells) × 100. For taking DIC images, cells were grown overnight on cover slips at 37°C followed by washing with PBS and fixing with PFA and then visualizing under Nikon Ti-E microscope.

Culture of BCG

A seed culture of *Mycobacterium bovis* Bacillus-Calmette-Guerin (BCG) [*M. bovis* Pasteur, TMCC no. 1011] was kindly provided by Prof. R. K. Saxena, South Asian University, New Delhi. BCG culture media was prepared using a published recipe comprising of 4.0 g L-asparagine, 2.0 g citric acid, 0.50 g potassium dihydrogen phosphate, 0.50 g magnesium sulphate, 0.05 g ferric ammonium citrate 60 ml glycerol and 0.05% Tween-80 whenever required. (Oleic acid, Albumin, Dextrose and Catalase) OADC supplement was added to Middlebrook 7H11 agar to prepare OADC plate. All inoculations and manipulations of BCG were carried out in a Biosafe Laminar Flow Chamber. Cultures were initiated from frozen stocks. For initial seeding, a loopful of cells were scraped from the surface of frozen stock and inoculated in a culture flask containing 20 ml of Sauton's medium and incubated at 37°C shaker for three weeks. Cells were then sub cultured in increasing volumes of Sauton's medium (with 0.05% Tween 80) and harvested in the late log phase. Growth was monitored by measuring absorbance of the bacterial suspension at 600 nm. For rapid estimation of bacterial concentration in a given suspension, absorbance of 0.4 at 600 nm corresponded with a concentration of 1.1×10^8 CFU/ml. When culture reached a cell density of 1.1×10^8 cells/ml bacteria were harvested by centrifuge at 1800 g for 20 min and the pellet was washed twice with PBS at 4°C. Resulting bacterial cells were either inoculated into fresh medium or aliquots were frozen at -70°C in medium with 20% glycerol. Bacterial viability and CFU count was determined by plating on 7H11 agar OADC plates. Prior to every use in *in vitro* assays, BCG was sonicated for 2 mins in Branson sonicator.

Maintenance of promastigotes of *Leishmania*

Leishmania donovani 1S (a cloned line from strain 1S, WHO designation: MHOM/SD/62/1S), and *Leishmania tropica* (Wright) Luhe (ATCC® 50129™) were used in all experiments. The promastigotes were cultured and harvested as described previously (Selvapandiyan et al. 2004). Briefly, the parasites were grown *in vitro* in

T25 cm^2 culture flasks at 26 °C in medium 199 (Sigma, MO, USA) (pH 6.8) having 8µM 6-Biotin (Sigma, MO, USA), 25mM Hepes, 0.1mM adenine (Sigma, MO, USA) in 25mM Hepes), 8µM hemin (Sigma, MO, USA) [4mM stock made in 50% triethanolamine (Sigma, MO, USA)], 100 U/ml each of penicillin G (Gibco, Life technologies, Grand Island, NY, USA) and streptomycin (Gibco, Life technologies, Grand Island, NY, USA) and 10% (v/v) heat-inactivated fetal bovine serum.

Maintenance of *C. albicans* WT and GPI-GnT mutants

C. albicans WT and GPI-GnT mutants were a kind gift from Prof. Sneha Sudha Komath, School of Life Sciences, JNU, New Delhi (Kalpana Pawar 2015, Victoria et al. 2012, Yadav et al. 2014). They were grown in YEPD medium at 30°C in incubator shaker at 220 rpm for overnight.

CFSE labelling of pathogens

BCG, *Leishmania* and *C. albicans* were labelled with Carboxyfluorescein N-succinimidyl ester (CFSE). Its acetate groups are cleaved by intracellular esterases inside the cells, and the molecules are converted to fluorescent esters. CFSE is retained within the cell and covalently couples to intracellular molecules via its succinimidyl group. Due to this covalent coupling reaction, fluorescent CFSE can be retained within the cell for an extremely long period. Once the dye has been incorporated within the cell, it is not transferred to adjacent cells due to its stable linkages. 1×10^8 BCG were washed with PBS twice at 4000 g for 10 mins followed by incubating 10µM Carboxyfluorescein N-succinimidyl ester (CFSE) (Sigma, MO, USA) in 500µl PBS at 37°C for 1h. The bacterial pellet obtained by centrifugation at 4000 *g* for 10 mins is washed thrice with PBS (Beatty and Russell 2000). 1 million parasites in late log phase were labelled with 5µM CFSE for 10 min in water bath at 37°C. After two washes with Phosphate Buffer Saline (PBS) at 2000g for 5 min at 4°C parasites were syringe separated with 26 ½ gauge needle. 100 million *C. albicans* were labelled with 10µM CFSE for 30 mins at room temperature followed by two washes with Phosphate Buffer Saline (PBS) at 2000g for 5 min at 4°C. Flow cytometric analysis by a BD FACS Calibur flow cytometer at FL1 channel using Cell Quest software indicated that by using this protocol, more than 95% of the pathogens were labelled with CFSE and labelling was stable upto hyphae formation. For all

FACS experiments, relative fluorescence intensity of 10,000 cells was recorded as single parameter histograms (logscale 1024 channels, 4 decades).

Uptake of CFSE labelled Pathogens and pathogen derived antigens

0.1 million RBL-2H3 cells cultured in 48 well plate were incubated with CFSE labelled BCG, CFSE labelled *Leishmania* and CFSE labelled WT or mutant *C. albicans* at different MOI (1:100 or 1:10), (1:1 or 1:10) and (1:1 or 1:5) respectively for indicated time period at 37°C in CO_2 incubator. The cells were then harvested at mentioned time, washed and fixed in 2% PFA. Uptake of labelled BCG, *Leishmania* and *C. albicans* by RBL cells was analyzed by flow cytometry. 8×10^8 BCG were washed with PBS thrice followed by incubating with 1 mg/ml of sulfo-NHS-XX-biotin (Sigma, MO, USA) for 1 h. Cells were washed with PBS thrice. BCG was then sonicated for 10 mins on ice. 0.2 million RBL-2H3 was incubated with biotinylated sonicated BCG (sBCG) for various time points at 37°C. The cells were then washed with PBS thrice and were then fixed with 2% PFA at room temp for 10 mins followed by permeabiling with 0.01 % Saponin (Sigma, MO, USA) and 1% NP40 (Sigma, MO, USA) for 10 mins. The cells were then treated with Streptavidin FITC (eBiosciences, San Diego, CA) for 20 mins. The cells were washed with PBS twice and then run through flowcytometer.

Standardization of inhibitors for studying effect on uptake

Cytochalasin D Treatment

Different doses of Cytochalasin D (0.5µg/ml, 1.5µg/ml, 2.5µg/ml, 5µg/ml and10µg/ml) and different incubation time (3h, 12h and 24h) were used to check its toxicity on adherent RBL-2H3 cells by calculating viability using trypan blue exclusion method. Figure represent cell viability (percent of total) in RBL-2H3 after Cytochalasin D treatment at different concentrations and indicated time points. It is clear treatment with Cytochalasin D for more than 3 h was toxic for RBL-2H3. So a dose of 2.5µg Cytochalasin D for 3 h was chosen to study the effect of cytoskeleton disruption of BCG as this dose gave a viability 95 ± 3.5%. For studying the effect of Cytochalasin D (Cyt D) on BCG uptake by RBL-2H3 cells, these cells were cultured in a 48-well culture plate. Cyt D (2.5 µg/ml) (Sigma, MO, USA) was added 1 h before the addition of CFSE labeled BCG and incubation continued for an additional 2 h

(Kumari and Saxena 2011). For *C. albicans* study cells were pretreated with Cytochalasin D for 3 h followed by washing with PBS and further incubating with CFSE labeled *C. albicans.*

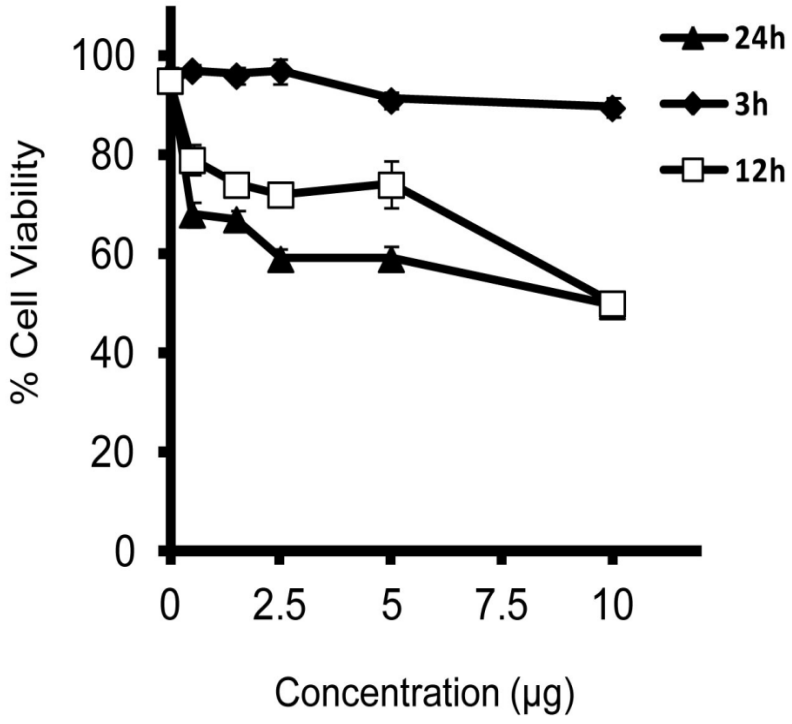

Figure 15: Treatment of RBL-2H3 with Cytochalasin D. 0.1×10^6/ml cells were treated with different concentrations of Cytochalasin D for indicated time points at 37°C. Recoveries of viable cell numbers were assessed by using trypan blue exclusion method using a hemocytometer. Panel A and B represent cell viability (percent of total) in RBL-2H3. Each data point represents the mean ± SEM of results obtained from three independent assays.

Treatment with MβCD

Different doses of MβCD (2.5mM, 5mM, 7.5mM and 10mM) and different incubation time (20 min, 30 min and 60 min) were used to check its toxicity on adherent RBL-2H3 cells by calculating viability using trypan blue exclusion method. Figure represent cell viability (percent of total) in RBL-2H3 after MβCD treatment at different concentrations and indicated time points. It is clear treatment with MβCD for more than 30 min was toxic for RBL-2H3. So a dose of 2.5 mM MβCD for 30 min was chosen to study the effect of cholestrol removal on uptake of BCG as this dose gave a viability 92 ± 1.5%. MβCD treatment was done for 30 mins followed by

washing and adding CFSE labelled BCG for 1 h. The cells were then harvested and were run through flowcytometer.

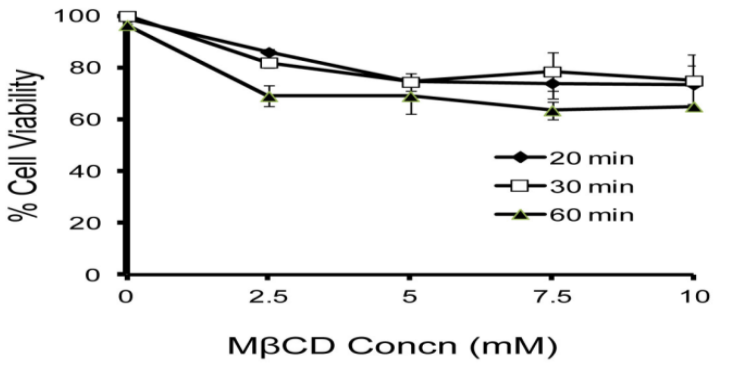

Figure 16: Treatment of RBL-2H3 with MβCD. 0.1 × 10^6/ml cells were treated with different concentrations of MβCD for indicated time points at 37°C. % Cell viability was assessed by using trypan blue exclusion method using a hemocytometer. Panel A and B represent cell viability (percent of total) in RBL-2H3. Each data point represents the mean ± SEM of results obtained from three independent assays.

Treatment with Nystatin

Different doses of Nystatin (5µg/ml, 10µg/ml and 15µg/ml) and different incubation time (5 min, 15 min and 30 min) were used to check its toxicity on adherent RBL-2H3 cells by calculating viability using trypan blue exclusion method. Figure represent cell viability (percent of total) in RBL-2H3 after Nystatin treatment at different concentrations and indicated time points. It is clear treatment with Nystatin for more than 5µg/ml was toxic for RBL-2H3. So a dose of for 30 min was chosen to study the effect by disassembling caveolae on uptake of BCG as this dose gave a viability 91 ± 2.6%.

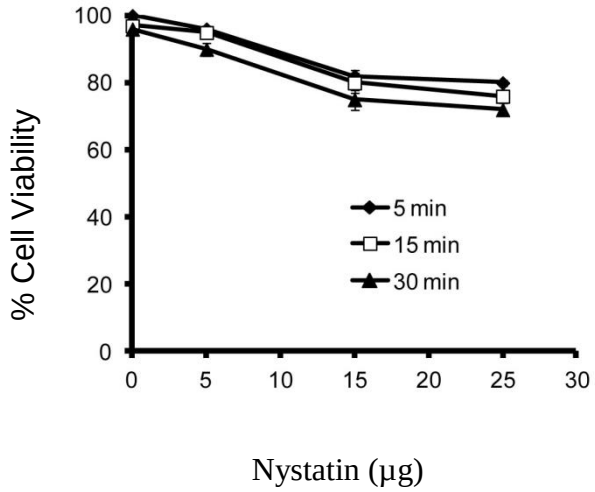

Figure 17: Treatment of RBL-2H3 with Nystatin. 0.1×10^6/ml cells were treated with different concentrations of Nystatin for indicated time points at 37°C. % Cell viability was assessed by using trypan blue exclusion method using a hemocytometer. Panel A and B represent cell viability (percent of total) in RBL-2H3. Each data point represents the mean ± SEM of results obtained from three independent assays

Treatment with Filipin

Cells were cultured on glass cover slips overnight. Cells were washed with PBS and fixed with 2% paraformaldehyde for 30 min at room temperature. It was then quenched with ammonium chloride followed by washing with PBS. Cells were stained with 2.5 µg/ml Filipin (Sigma, MO, USA) for 2 h at RT followed by with PBS. These cells were examined using a confocal laser scanning microscope (Olympus FluoView FV1000). Similarly, cells were also pretreated with 2.5 µg/ml Filipin (Sigma, MO, USA) for 30 mins and uptake was carried on for one hour. Cells were harvested by trypsinization, washed with PBS, and fixed in 2% PFA for flow cytometric analysis.

Treatment with Wortmanin

RBL Mast Cells were incubated with 100mM Wortmanin for different time periods to check its toxicity. Viability was calculated using trypan blue exclusion method. Figure represent cell viability (percent of total) in RBL-2H3 after Wortmanin treatment at 100mM and indicated time points. It is clear treatment with Wortmanin at 100mM for more than 30 min was toxic for RBL-2H3. So a dose of 100mM for 30

min was chosen to study the role of phosphoinositide 3-kinase on uptake of BCG as this dose gave a viability 98 ± 1.2%. Cells were pretreated with 100 nM Wortmanin (Sigma, MO, USA) for 30 mins and uptake was carried on for one hour. Cells were harvested by trypsinization, washed with PBS, and fixed in 2% PFA for flow cytometric analysis.

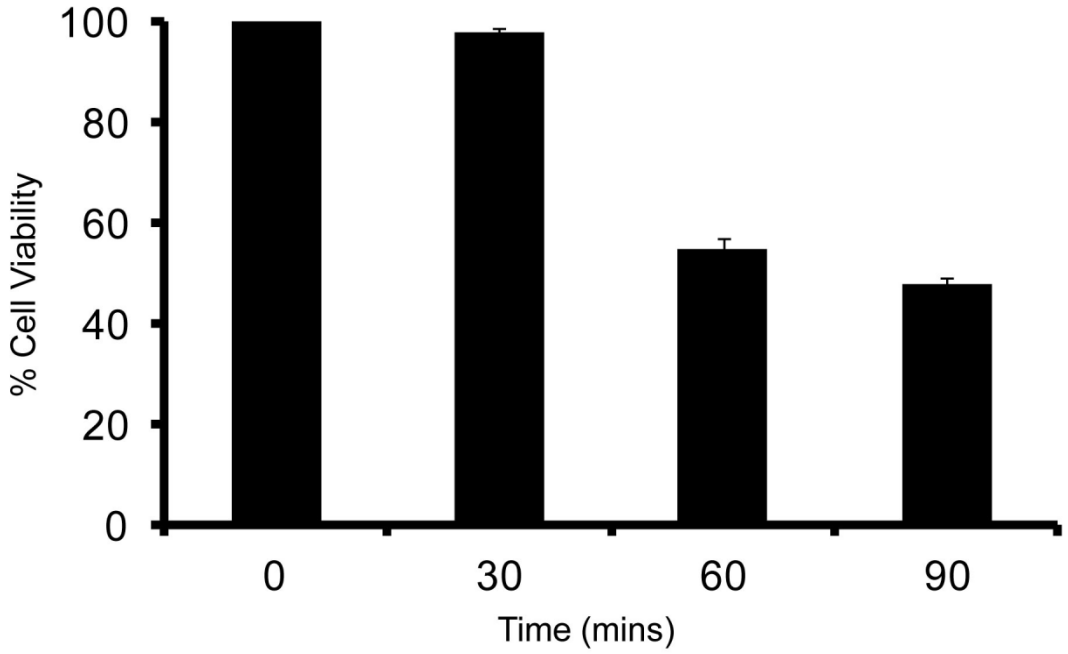

Figure 18: Treatment of RBL-2H3 with Wortmanin. 0.1 × 10^6/ml cells were treated with 100 mM Wortmanin for indicated time points at 37°C. % Cell viability was assessed by using trypan blue exclusion method using a hemocytometer. Panel A and B represent cell viability (percent of total) in RBL-2H3. Each data point represents the mean ± SEM of results obtained from three independent assays.

Opsonisation of BCG uptake

1×10^8 BCG were incubated with 10% anti-BCG serum suspended in RPMI media (Gibco, Life technologies, Grand Island, NY, USA) for 30 mins on rotisserie at 5 g followed by three times washing with PBS (Sendide et al. 2005). They were then labeled with CFSE. Overnight cultured RBL-2H3 were incubated with opsonized CFSE labeled BCG for 12 h followed by harvesting by trypsinization, washing with PBS, and fixing in 2% PFA for flow cytometric analysis.

Co-culture of MCs and pathogens *in vitro*

In some co-culture experiments, 0.3 million MCs were seeded in the lower chamber of a transwell polystyrene plate (polycarbonate membrane with 0.4-µm pore size, 6.5-mm diameter; (Corning Costar, Cambridge, MA) and promastigotes were added to the top chamber of the transwell plate in 24 well plate. In some assays, 0.1 million MCs were pre-treated with cytochalasin D (Sigma-Aldrich) at a final concentration of 10µg/ml to inhibit phagocytosis and promastigotes were co-cultured. To disrupt MCETs, MCs were pre-treated with 40 U/ml DNaseI (Sigma-Aldrich)to degrade DNA and then promastigotes were co-cultured (von Kockritz-Blickwede et al. 2008). To inhibit ROS 25 U/ml PEG catalase (Sigma Aldrich, India) was used. Viability of MCs as well as promastigotes was assessed through trypan blue staining.

Experimental Treatments for uptake by BCG derived antigen

Ionomycin treatment of RBL-2H3 in suspension

Activation of RBL-2H3 cells in suspension was accomplished by harvesting $1.5x10^6$ cells each in respective microcentrifuge tubes and doing the experiment in duplicates and were washed thrice with phenol red free RPMI medium and resuspending in 250 µl of phenol red free RPMI medium. Pre-warmed 250 µl of 10 µM Ionomycin was added and incubation was continued at 37°C for 10 mins. Mock degranulation studies were conducted in parallel by using medium alone. Control and Ionomycin treated RBL cells were washed thrice with PBS at 1200 g for 5 mins and media was added to cells. Uptake was studied by flow cytometry as described before. In order to confirm the activation of RBL-2H3 by Ionomycin treatment, release of β-hexosaminidase into the supernatant was assayed as discussed earlier.

Cyt D treatment of RBL-2H3

RBL-2H3 cells were treated with different doses of Cytochalasin D at 37°C. Cytochalasin D was not found toxic at all the concentrations tested Figure. So RBL-2H3 cells were pre-treated with 10µg of Cytochalasin D before studying sBCG uptake. It was able to inhibit sBCG uptake.RBL-2H3 cells were treated with different concentrations of Cyt D at 37°C for 30 mins. Sonicated BCG was added and incubation continued for additional 30 mins followed by washing of cells and flowcytometry as described earlier (Hohn et al. 2009).

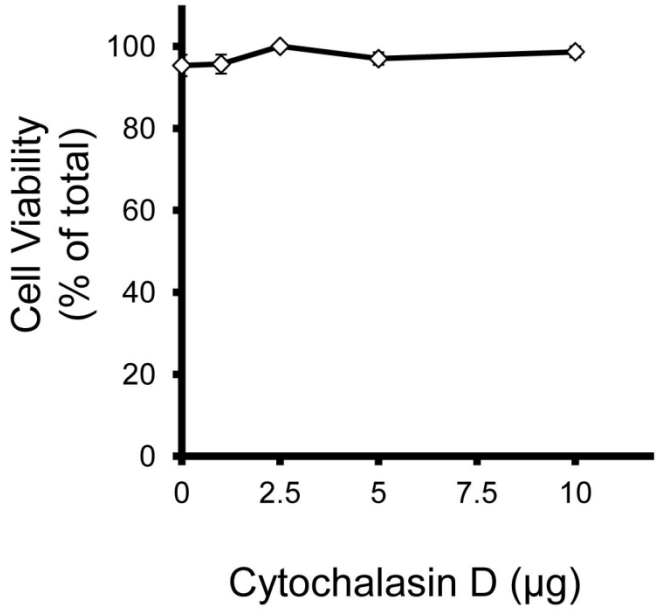

Figure 19: Effect of Cytochalasin D treatment on viability of RBL-2H3 cells in suspension. Different doses of Cytochalasin D (1 µg, 2.5µg, 5µg, 10µg) were added to 1 x10^6 RBL-2H3 suspended in 100µl of respective medium and incubated for 1 h at 37°C. Recoveries of viable cell numbers were assessed by trypan blue exclusion method using a haemocytometer. The data shown are mean ± SEM of three independent assays.

Cell Viability assay using Lactate Dehydrogenase (LDH) kit

Cell viability assay was done using the LDH cytotoxicity kit (Cayman, USA) as per the manufacture's protocol with minor changes. Briefly, two sets of replicates for each condition were used: one high control and one for the actual assay. Replicates of 100 ml of media without cells will be used to serve as the blank for that condition. Control and treated cells were centrifuged for 5 min at 1500rpm. A multichannel was used to transfer 100ul of supernatants for the top of all the wells for the experimental culture plate to the assay plate. 100ul of the mixed detection kit reagent was then added to each of the assay wells on top of the supernatants in rapid succession. After this plates were read using a standard plate reader (Bio-Rad, CA, USA) with a reference wavelength of 490 nM.

MTT assay

To determine cell viability the colorimetric MTT metabolic activity assay was used. MCs in 100µl medium were cultured in a 96-well plate at 37 °C in 5% CO_2, and cocultured with promastigotes for 24 h. Cells treated with medium only served as a negative control group. After removing the supernatant of each well and transferring to another 96-well plate 20 µl of MTT solution (Sigma, MO, USA) (5 mg/ml in PBS) were then introduced. After incubation for another 4 h, the resultant formazan crystals were dissolved in dimethyl sulfoxide (100 µl) and the absorbance intensity measured by a microplate reader (Spectramax M2, USA) at 595nm. The relative cell viability (%) was expressed as a percentage relative to the untreated control cells.

Determination of ROS

MCs were cultured at a concentration of 0.3 million cells/ml in 24 well cell culture plates overnight. Adhered MCs were cocultured with promastigotes directly or in transwell washed with complete medium to remove promastigotes, debris and dead cells. These cells were washed, and resuspended in pre-warmed PBS supplemented with 2% FBS and incubated with 5 µM CMH_2DCFDA stain (Molecular Probes; Eugene, OR, USA) in the dark for 30 minutes at 37°C in 5% CO_2 in air. The oxidative conversion of CMH_2DCFDA to its fluorescent product by ROS was measured immediately by BD FACS Calibur flow cytometer in FL1 channel using Cell Quest software.

Assessment of apoptosis

MCs were cultured at a concentration of 0.1 million cells/ml in 48 well cell culture plates. After overnight culture, cells were washed with complete medium to remove debris and dead cells. Cells were co-cultured with promastigotes of *Leishmania* for 24 h and promastigotes were removed. Promastigotes were washed and double stained with 7-amino actinomycin- D (7AAD) (Biolegend, San Diego, CA, USA) and annexin V FITC conjugate (Biolegend, San Diego, CA, USA) to assess the apoptotic and necrotic cells using flowcytometry.

Quantification of DNA released by MCs

DNA release by MCs was quantified using a Sytox Green-based assay. MCs (5×10^4 cells/well) were seeded in a 96 well plate wrapped in aluminium foil in the presence of 2.5μM Sytox Green (Molecular Probes; Eugene, OR, USA) and infected with promastigotes of *L. donovani* and *L. tropica* - MOI 1:1, 1:10- or left untreated. The same amount of *L. donovani* and *L. tropica* served as viability control whereas triton-lysed MCs served as a reference for 100% DNA release. The fluorescence intensity was measured by a fluoremeter when excited by 450-490 nm source.

Confocal Microscopy

For visualization of uptake of CFSE labeled BCG, *Leishmania* by RBL-2H3, these cells were cultured on glass cover slips overnight. Cells were then washed and incubated with CFSE labeled BCG and *Leishmania* at MOI (1:100) and (1:10) for 24 h at 37°C. Then they were washed, fixed with 4% PFA followed by washing twice with quencher (Ammonium Chloride). The PFA fixed cells were then incubated with 3% normal goat serum (Sigma, MO, USA) for 1 h to prevent nonspecific protein binding. Anti-AD1 antibody (BD Biosciences, San Diego, CA) diluted in the same buffer were added to the cells, and incubation was conducted for 2 h. After washing, they were incubated for 30 min in the presence of secondary goat Abs conjugated to Alexa Fluor 546 (red). For uptake of sBCG by RBL-2H3 cells were cultured on coverslips and were processed in a similar way as discussed above. Then mounted in Vecta shield containing 4, 6-diamidino-2-phenylindole (DAPI) (Vector, Vector Lab. Inc.) to stain nucleus. For visualization of Extracellular Traps, Rabbit Anti-Histone H2A (acetyl K5) antibody (Abcam, Cambridge, MA) and Mouse anti-Mast Cell Tryptase antibody (Abcam, Cambridge, MA) diluted in the same buffer were added to the cells, and incubation was conducted for 2 h. After washing, this was followed by 30-min incubation in the presence of secondary goat Abs conjugated to Alexa Fluor 546 (red). Coverslips were mounted in Vecta shield containing DAPI (Vector, Vector Lab. Inc.) to stain nucleus. The images were visualized under Nikon Real Time Laser Scanning Confocal Microscope Model A1R with motorized inverted microscope having Live Cell and Spectral Imaging – Model Ti-E at 60X.

Live Cell Imaging

RBL and MH-S cells were seeded in a 3.5 cm culture dish overnight at 37°C. Next day *C. albicans* were added and observed by a live cell imager Andor Spinning Disk Confocal Microscope (Nikon Eclipse TiE, Software – Andor iQ 2.7) in 5% CO_2 chamber at 37°C. The movies were captured immediately after addition of *C. albicans* and were continued for 3h. All the images were analysed by NIS element AR ver4.

Transmission Electron Microscopy

15 million RBL cells were co- cultured with BCG for 3h. The cells were washed with PBS and then fixed with 2% glutaraldehyde for 1 h. The samples were processed for Transmission Electron Microscope (JEOL 2100F) in Advanced Instrument Facility (AIRF, Jawaharlal Nehru University).

Scanning Electron Microscopy

2.5×10^6 MCs were seeded in 6 well plate for overnight. *L. donovani* and *L. tropica* at their late log stage from culture were syringe separated and were added at MOI 1:10. After 24 h, parasites were removed and the cells were harvested followed by fixing with 2% glutaraldehyde for 1h and were processed for Scanning Electron Microscope Zeiss EV040 in Advanced Instrumentation Research Facility.

Histology staining (Hematoxylin and Eosin, Toluidine Blue and Acid Fast Bacterial staining) of Lungs

Intra-tracheal BCG infection was established in mice with 1×10^8 cfu of BCG. The infection was allowed for four weeks, thereafter mice were euthanized and lungs were isolated. These organs were washed in PBS and then fixed in 10% methyl formaline solution. After fixation, organs were fine – sectioned and stained with Hematoxylin and Eosin (HE) solution. Eosin is pink and stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining (Cardiff, Miller and Munn 2014). Acid Fast Bacterial (AFB) staining and Toluidine were also processed similarly followed by staining with Acid Fast Bacterial solution and Toluidine Blue. Mast cells were first recognized by virtue of metachromasia in 1877 by Paul Ehrlich. The compound responsible for metachromasia was identified as heparin, a heteroglycan rich in half-sulfate esters.

Toluidine Blue is a small weakly hydrophilic cationic dye. Attached to DNA or RNA, in chromatin or Nissl substance, this dye has a blue color. Attached to glycosaminoglycans, in mast cell granules or cartilage matrix, the dye displays a purple metachromatic color (Sridharan and Shankar 2012) The images were captured for at least 5 different fields in 3 mice.

Stimulation of RBL-2H3 mast cell exocytosis

Exocytosis in the RBL-2H3 mast cells was triggered using ionomycin and *M. bovis* BCG. RBL-2H3 cells (0.3×10^6) were washed with RPMI (without phenol red) and degranulation was induced by different concentrations (5 µM, 10 µM and 15 µM) of ionomycin in a final volume of 1.5 ml. Plates were incubated at 37°C, aliquots of the medium were withdrawn at various times and β-hexosaminidase activity released into the medium was measured. Mock degranulation studies were carried out in parallel by using medium alone (Vaidyanathan, Puri and Roche 2001). At the end of the assay, cells were lysed with 1 ml of 0.2% Triton X-100 in RPMI to measure residual cell-associated β-hexosaminidase. To determine β-hexosaminidase activity, aliquots (200 µl) of the supernatants and cell lysates were incubated with 50 µl of the substrate solution (1.3 mg/ml of *p*-nitrophenyl- *N*-acetyl-β-D-glucosaminide in 0.1 M citrate buffer (pH 4.5) for 90 mins at 37 °C. The reaction was terminated by the addition of 100 µl of 0.2 M NaOH/0.2 M glycine. Absorbance was read at 405 nm in an enzyme-linked immunosorbent assay reader, and the amount of exocytosis was expressed as the percentage of total β-hexosaminidase activity released in the supernatant. Release of histamine was measured by a commercial ELISA kit. The amount of histamine released was expressed as a percentage of the total amount of histamine secreted into the medium as compared with the total amount of histamine secreted into the medium plus that remained in cell lysates.

RBL and MH-S mediated *C. albican* killing

To determine the extent of killing and/or phagocytosis, 0.1 million RBL-2H3 and MH-S cells were seeded in 48 well cell culture plate and kept for adherence followed by addition of *C. albicans* at MOI 1:5 for 18h followed by lysing the mammalian cells using 0.2% Triton-X-100 (Sigma, MO, USA) and plating on the Yeast Extract Peptone Dextrose (YPD) plates and kept at 30°C for 24h. The number of *C. albicans*

was determined as CFU/ml. The formula used is cfu/ml = (no. of colonies x dilution factor) / volume of culture plate

Statistics

Statistical analysis was performed using Graph Pad Prism 5 software (San Diego, USA), MS office and Sigma Plot Software. The significance of any difference was calculated by using Mann-Whitney U Test, Student's t-test and ANOVA. Data are presented as mean ± SEM. *$p<0.05$, **$p<0.005$ and ***$p<0.0005$ represent statistically significant difference between control and treated cells.

Chapter-1

Initial Interaction of Mast Cells With Intracellular Pathogens

As it has already been reviewed that MCs are found in large numbers in skin, and mucosal lining, they may represent one of the first immune cells encountered by invading pathogens (Abraham and Malaviya 1997). They have been suggested to contribute to protective immunity against bacterial, nonbacterial (Arock et al. 1998, Lopes et al. 2015, Shin, Gao and Abraham 2000, Wesolowski, Caldwell and Paumet 2012) and even parasitic pathogens (Bidri et al. 1997, Saha et al. 2004), it was important to study the initial interaction of Mast cells with bacteria, parasite and fungus.

BCG infects lungs where large numbers of MCs also reside. Intracellular pathogens such as *M. bovis* BCG, *Leishmania tropica*, *Leishmania donovani* and *Candida albicans* were studied extensively for deciphering the role of Mast Cells in initial interaction with these pathogens. Since a large numbers of MCs are found in the skin, predominantly in the superficial dermis (Maurer et al. 2006), where *Leishmania* is encountered after the bite of infected sand flies, interaction of Mast Cells was studied with *L. tropica* and *L. donovani.* Another intracellular pathogen that we were interested was *C. albicans* which is a commensal, colonizing predominantly in skin and mucosal surfaces. Mast cells are present in tissues prone to fungal colonization. So they are among the first immune cells to get into contact with *C. albicans*. The GPI in eukaryotes anchors a variety of proteins to the cell surface which include virulence factors, enzymes involved in host immune response evasion, cell wall biogenesis, and maintenance proteins as well as hyphaspecific proteins. The pathway affects the functioning and viability of the organism. The pathway is of 10–12 steps that generates the precursor GPI anchor core in the endoplasmic reticulum. The enzymatic subunit for each step has been identified, and its role in the catalytic step has been specified based on yeast and mammalian studies. The role of the accessory subunits in these steps is unclear. The accessory proteins provide a regulatory control on the pathway (Victoria et al. 2012, Yadav et al. 2014). So we also looked at the role of accessory proteins of Glycosylphosphatidylinositol-N-acetylglucosaminyltransferase (GPI-GnT) complex in interaction with MCs and MΦs thereby affecting virulence.

Mast Cell recruitment to lungs infected with *M. bovis* BCG *in vivo*

It was necessary to visualize what happens to lungs after BCG infection. 1×10^8 BCG were infected in lungs of C57BL/6 female mice by intratracheal instillation. Infection

was established for 1 month. Mice were sacrificed and lungs were fixed with methyl formaline. Thin sections were prepared and were stained with haematoxylin and eosin stain.. Figure 20 panel A shows low power photomicrograph of lung from uninfected animal showing normal pulmonary tissue whereas panel B shows high power photomicrograph of lung from uninfected animal showing normal alveolar spaces and thin alveolar septae. Low power photomicrograph of lung from infected animal showed pulmonary tissue with foci of inflammatory cells shown by black arrow (Figure 20C) whereas high power photomicrograph of lung from infected animal showing heavy inflammatory cell infiltration shown by black arrow in the peribronchial tissues (Figure 20D).

Similarly, by Acid Fast Bacterial staining low power photomicrograph of lung from uninfected animal showing normal pulmonary tissue whereas high power photomicrograph of lung from infected animal showing epithelioid cells and lymphocytes in the granuloma marked with arrow (Figure 21). Thus, these results suggest that BCG infection has led to inflammation and granuloma formation.

Further to investigate whether there is recruitment of MCs upon infection with BCG, Toluidine staining was done. High power photomicrograph of lung from uninfected animal stained with Toluidine Blue for Mast Cells showing two mast cells in the peribronchial area. High power photomicrograph of lung from infected animal stained with Toluidine Blue for Mast cells marked with arrow (Figure 3A). The peribronchial area shows approximately 48 MCs in the upper left corner marked with arrow (Figure 3B). Thus, these results clearly show that more MC numbers are increased in lungs upon infection with *M. bovis* BCG.

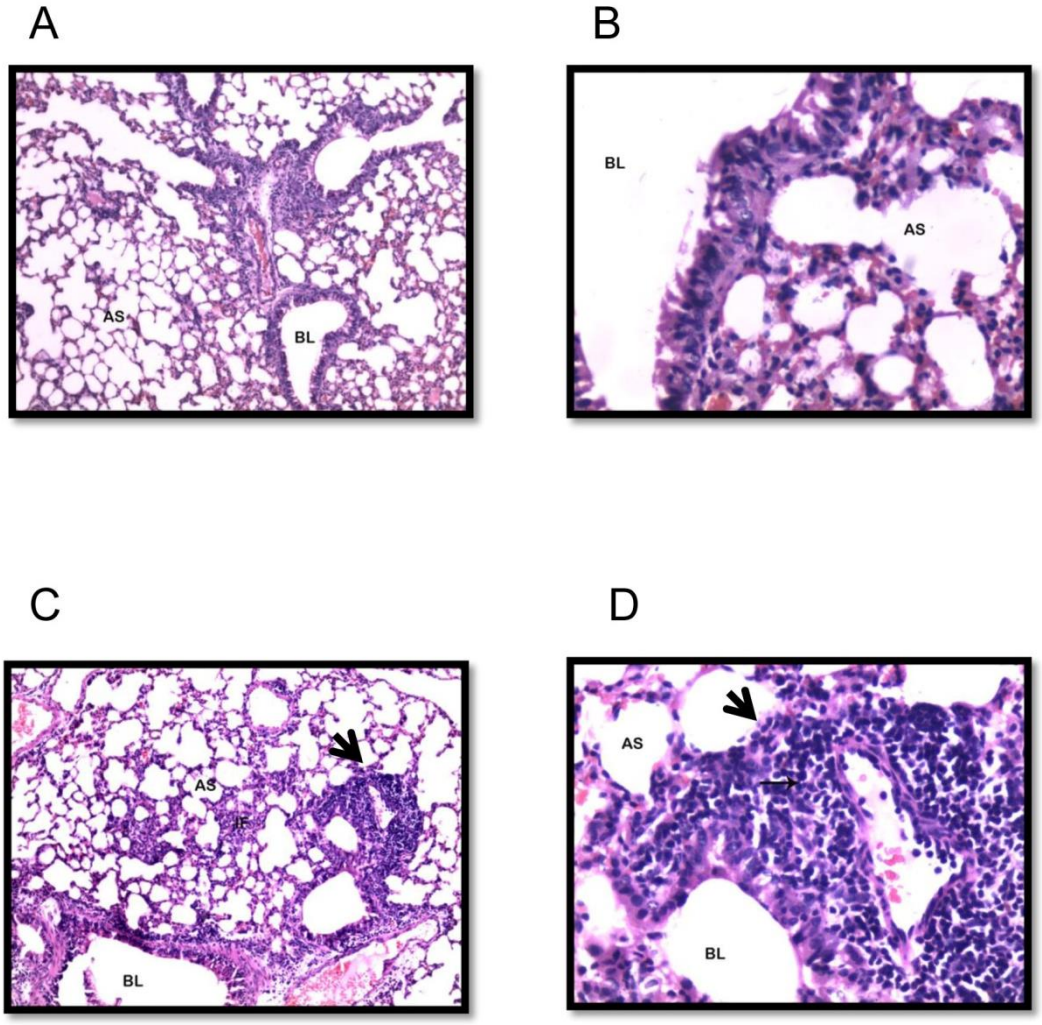

Figure 20: Morphology of lungs after BCG infection by Hematoxylin and Eosin Staining. Intratracheal infection was established in mice. Lungs were isolated from mice and were fixed with methyl formaline. These organs were fine sectioned and stained with Hematoxylin and Eosin. Panel A and B show H and E stain for lungs of uninfected mice at 10X and 40X. Panel C and D show H ans E stain for lungs of infected mice at 10X and 40X. . BL = Bronchial Lumen, AS = Alveolar Space. Arrow indicate inflammatory cells. This is a representative image out of five images captured from at least three three mice.

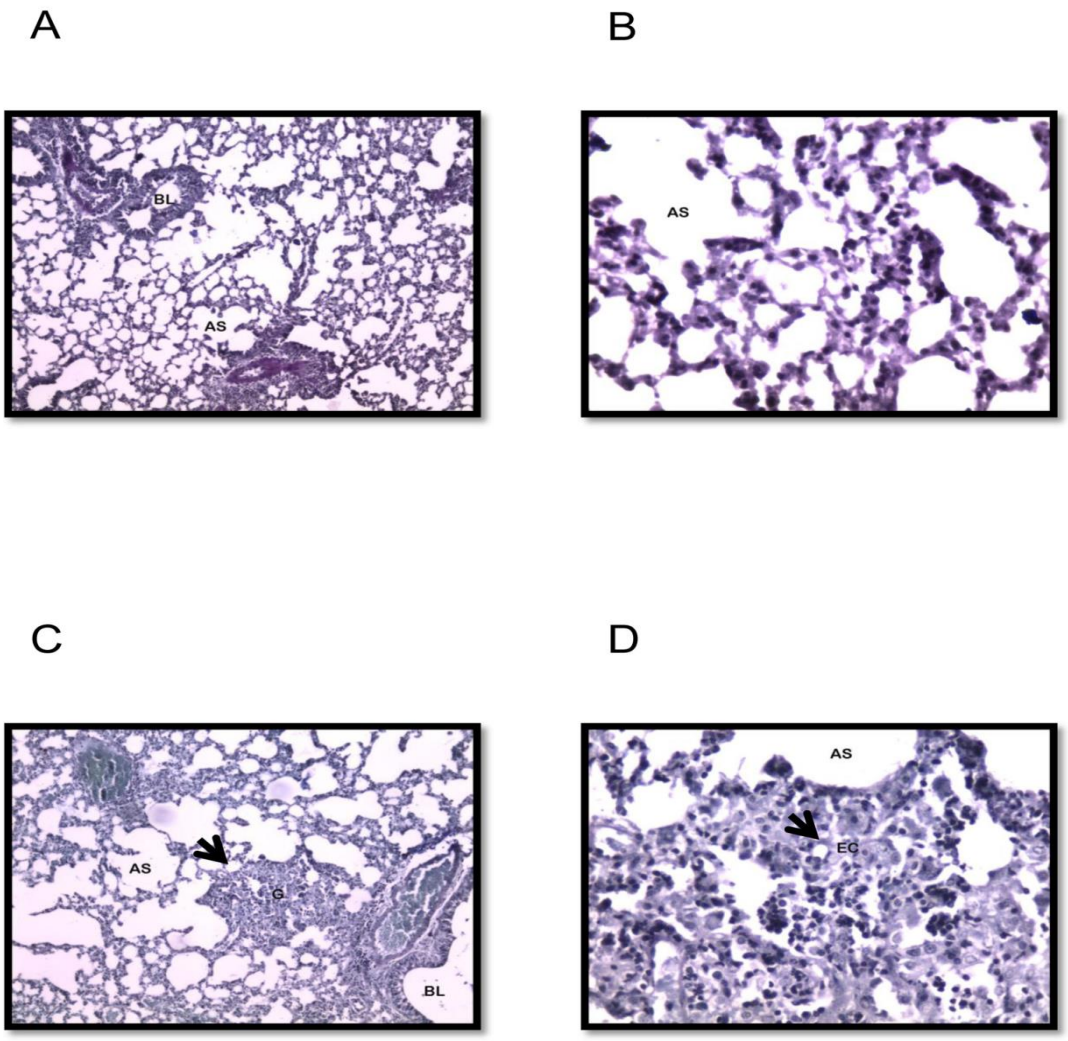

Figure 21: Morphology of lungs after BCG infection by Acid Fast Bacterial Staining. Intratracheal infection was established in mice. Lungs were isolated from mice and were fixed with methyl formaline. These organs were fine sectioned and stained with Acid Fast Bacterial stain. Panel A and B show Acid Fast Bacterial staining for lungs of uninfected mice at 10X and 40X. Panel C and D show Acid Fast Bacterial Staining for lungs of infected mice at 10X and 40X. . BL = Bronchial Lumen, AS = Alveolar Space, G = Granuloma and EC = Epithelioid cells. Arrow in panel C indicate granuloma and in panel D indicate epitheloid cells and lymphocytes in the granuloma. This is a representative image out of five images captured from at least three mice.

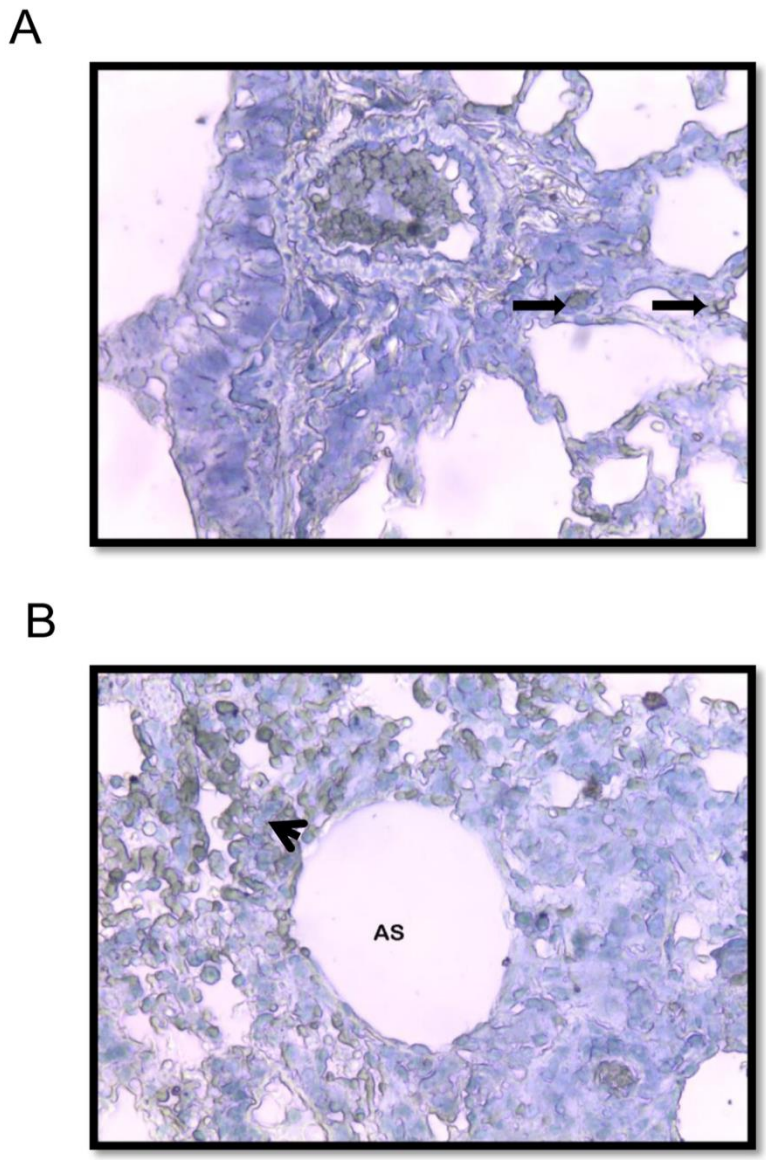

Figure 22: Visualization of MCs in lungs by Toluidine Blue Staining. Intratracheal infection was established in mice. Lungs were isolated from mice and were fixed with methyl formaline. These organs were fine sectioned and stained with Toluidine Blue stain. Panel A shows Toluidine Blue staining of lungs from uninfected mice showing two mast cells in the peribroncheal area at 40X magnification. Panel B shows Toluidine Blue staining of lungs from infected mice showing several mast cells in the upper left corner of the peribroncheal area. Arrow indicate MCs. This is a representative image out of five images captured from at least three independent mice.

Phagocytosis of live *M. bovis* BCG by RBL-2H3 MCs

As it is seen that *M. bovis* BCG infection in mice is able to recruit many MCs it was necessary to investigate how these *M. bovis* BCG is able to interact with MCs. So RBL-2H3 was used as a model system to study the interaction of *M. bovis* BCG with MCs. RBL-2H3 is a rodent mast cell line that has been extensively used to study MC biology in general and MC-pathogen interactions in particular (de Bernard et al. 2005, Mollerherm, von Kockritz-Blickwede and Branitzki-Heinemann 2016, Munoz et al. 2003, Sakurai, Yamaguchi and Sonoyama 2012, Wesolowski et al. 2012).

Labeling of BCG with CFSE stain

Before performing interaction studies it was necessary to label BCG with a fluorescent dye CFSE which is able to passively diffuse into cells for easy visualization by microscopy or assay by flowcytometry. To accomplish this BCG were stained as described in Materials and Methods. The labelled BCG were then analysed by flow cytometry. Figure 23A represents flow histogram of unstained BCG whereas panel B represents 91.21±2.5% staining of CFSE labelled BCG whereas an overlay of unstained and stained BCG is illustrated by figure 23C. To check stability of this dye after 24h labelled BCG suspended in media were incubated at 37°C for 24h followed by washing and analyzing through flowcytometer. Figure 24A shows 92.96±3.2% staining of CFSE labelled BCG at 0 h immediately after labeling whereas 5B represents 91.29±2.8% staining of CFSE labelled BCG after 24 h of incubation in RBL complete mediumin CO_2 incubator. Hence, the labelling was stable. Further, staining with CFSE was confirmed by confocal fluorescent microscopy and observed in green channel (Figure 25). CFSE labelled green coloured bacilli were seen.

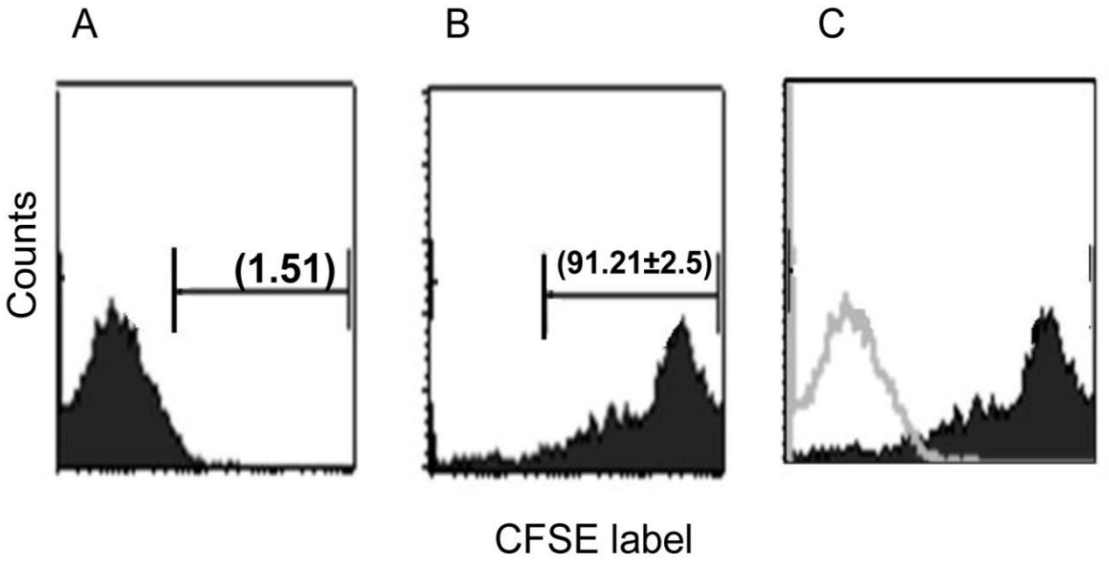

Figure 23: Staining of BCG. BCG (1×10^8 CFU) were resuspended in 500µl of phosphate-buffered saline (PBS) containing 10µM CFSE dye and incubated in the dark at 37°C for 1 h on rotisserie at 5 rpm. The bacterial pellet obtained by centrifugation at 6000 *g* for 15 min was washed thrice with PBS. Flow histograms of unstained BCG is represented by panel A whereas of stained BCG is represented by panel B. Values in parenthesis represent percent positive cells stained with CFSE stain. The overlay histogram of unstained and stained BCG is represented in panel C**.** Grey line histogram represents unstained and filled grey histogram represents stained BCG. Each percent value represents mean ± SEM of values obtained from three independent assays.

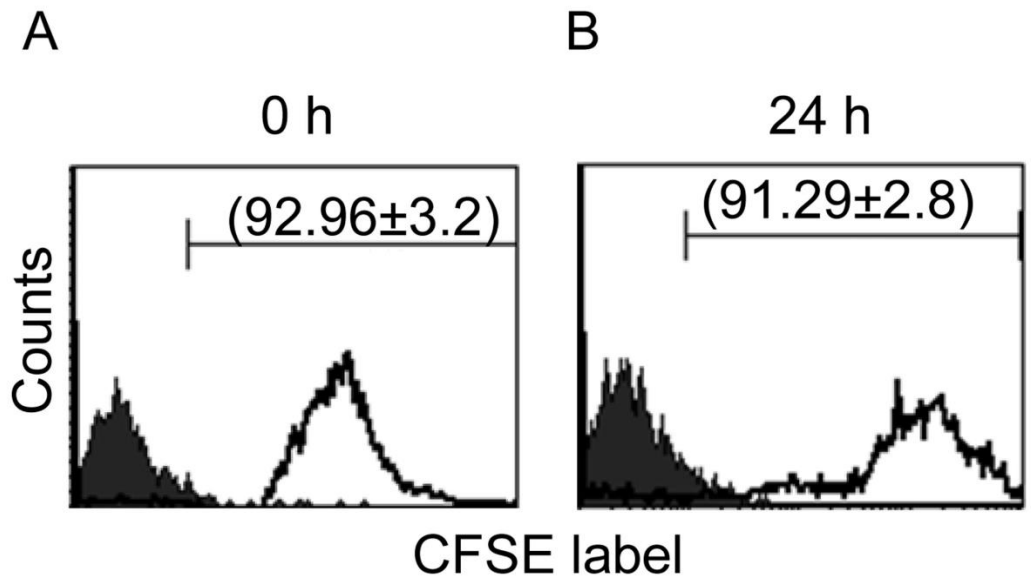

Figure 24: Stability of CFSE labelling of BCG. BCG (1×10^8 CFU) were resuspended in 500µl of phosphate-buffered saline (PBS) containing 10µM CFSE dye and incubated in the dark at 37°C for 1 h on rotisserie at 5 rpm. The bacterial pellet obtained by centrifugation at 6000 *g* for 15 min was washed thrice with PBS. Values in parenthesis represent percent positive cells stained with CFSE stain. Grey filled histogram represents unstained whereas black line histogram represents CFSE stained BCG. The overlay histograms of unstained and stained BCG at 0 h and 24 h are represented respectively. Each point represents mean ± SEM of values obtained from three independent assays.

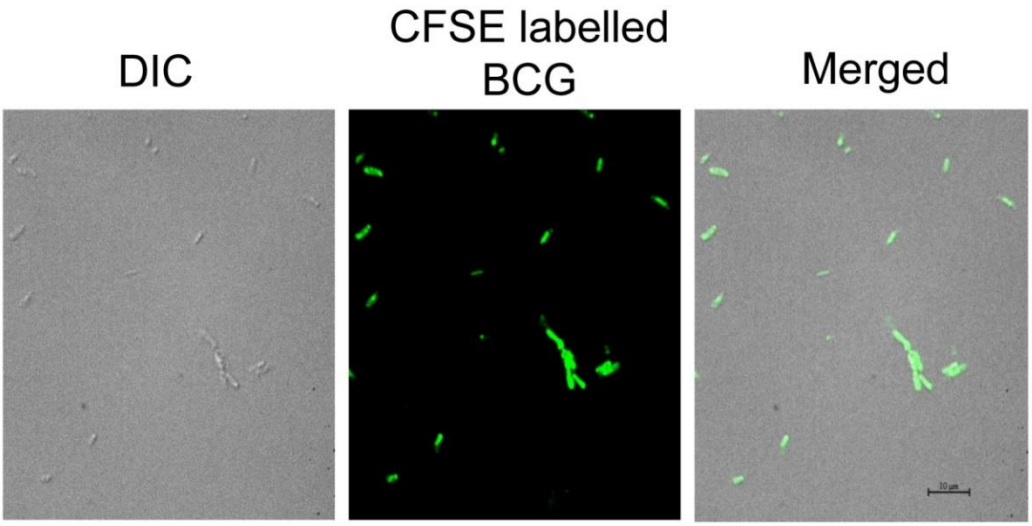

Figure 25: Staining of BCG. BCG (1×10^8 CFU) were resuspended in 500µl of phosphate-buffered saline (PBS) containing 10µM CFSE dye and incubated in the dark at 37°C for 1 h on rotisserie at 5 rpm. The bacterial pellet obtained by centrifugation at 6000 *g* for 15 min was washed thrice with PBS. CFSE labelled BCG were put on coverslip and were examined by Confocal fluorescent Microscope at 100X magnification and scale bar is 10µm.

Phagocytosis of CFSE labeled BCG by RBL-2H3 at different doses and time

CFSE labeled BCG were incubated at different MOI (1:10, 1:100) with RBL-2H3 cells for indicated time points. RBL-2H3 were then washed and BCG uptake was analyzed by flow cytometry. The uptake of BCG by RBL-2H3 occurs in a dose dependent manner on the basis percentages of cells having phagocytosed BCG. At low MOI 1:10, very low level of uptake less than 20% of RBL were able to take up BCG. On per cell basis also the uptake is low because of low MFI. At high MOI significantly high uptake around 40% RBL MCs take up at 6 h and almost 90% MCs at 24 h (Figure 26A). Per cell also MFI is increasing with time (Figure 26B). Each MC is able to take up higher number of BCG. As flowcytometric analysis cannot differentiate between adhered versus internalized pathogens, confocal fluorescence microscopy was performed. Also to confirm their internal location the late endosomes were marked by staining for LAMP-3 which is a lysosomal membrane marker.

Visualization of internalized CFSE labelled BCG by RBL-2H3 through confocal microscopy.

To visualize whether CFSE labelled BCG was just adhering to the cell surface or was engulfed by the cell confocal microscopy was employed. Cells were cultured on coverslips incubated with CFSE labelled BCG (MOI 1:100) at 37°C for 24 h followed by washing and fixing and were examined using a confocal laser scanning microscope. It is found that this CFSE labelled BCG reaches to late endosomes which appear red due to antibody staining of Lysosome Associate Membrane Protein (LAMP). Arrow indicates green coloured BCG inside red coloured late endosomes. Not only is BCG internalized it is able to reach late endosome lysosomal compartments (Figure 27).

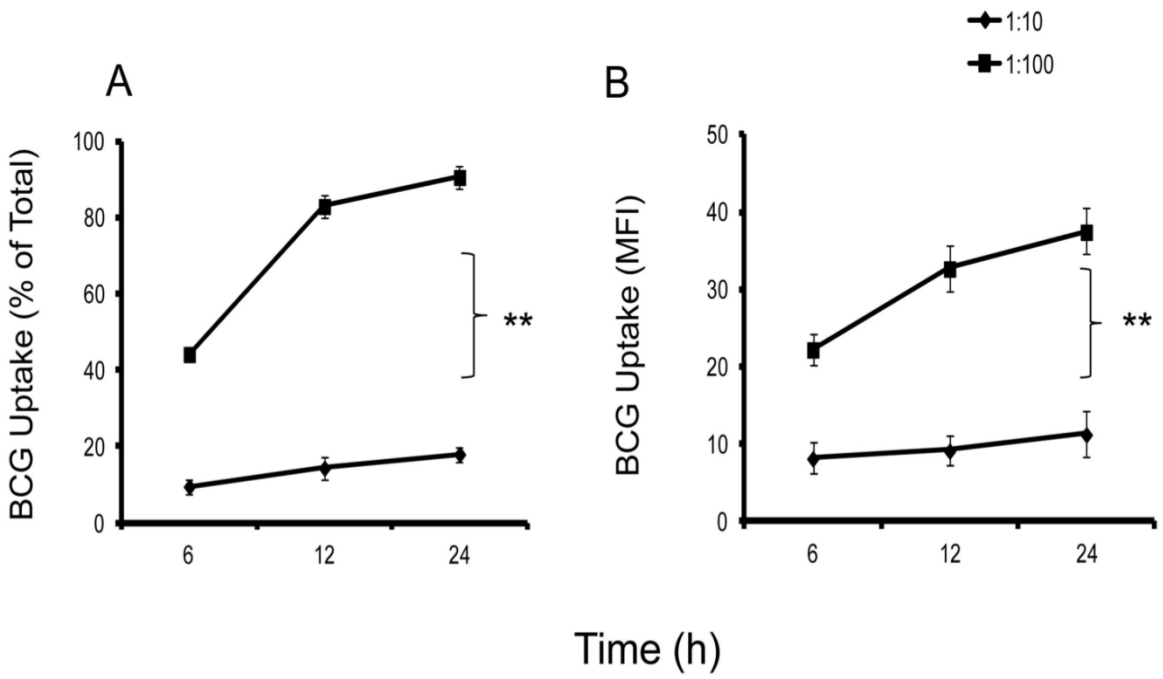

Figure 26: Uptake of BCG by RBL Mast Cells. RBL-2H3 cells were cultured on coverslips. 0.1 million cells were cultured in a 48-well culture plate for overnight. CFSE labelled BCG at MOI (1:10) and (1:100) were incubated for indicated time. Cells were harvested by trypsinization, washed with PBS, and fixed in 2% PFA for flow cytometric analysis. Graph represents MCs positive for BCG at MOI (1:10) and (1:100). Each point represents mean ± SEM of values obtained from three independent assays **P<0.005 is given by ANOVA.

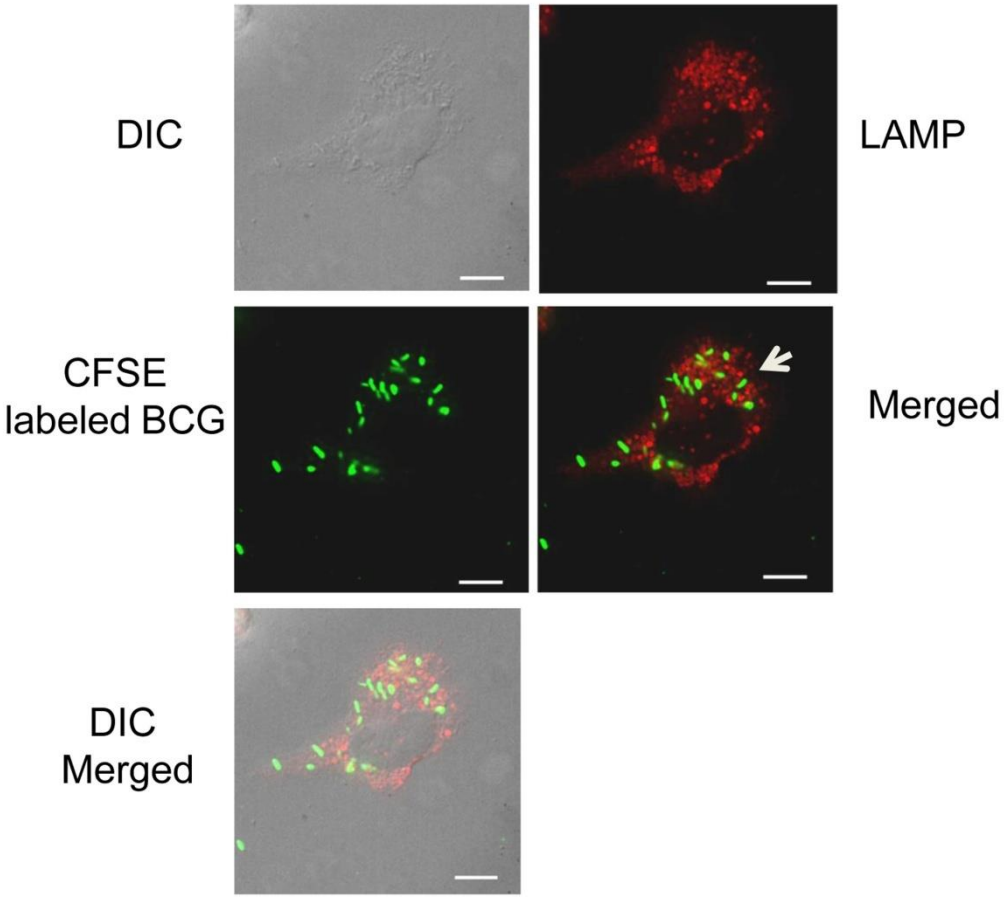

Figure 27: Uptake of live BCG by RBL MCs. RBL-2H3 cells were cultured on coverslips. After 12h, cells were co-cultured with CFSE labelled BCG for 24h followed by fixing with PFA and staining with LAMP-3 and were mounted and examined using a confocal laser scanning microscope. (magnification 100X and scale bar represents 10µm). This is a representative image out of five images captured from at least three independent experiments.

Mechanism involved in phagocytosis of BCG by RBL-2H3

Phagocytosis which is classically defined as a mechanism for internalising and destroying particles greater than 0.5μm size is a receptor-mediated, actin-driven process. The best-studied phagocytic receptors are the opsono-receptors, FcγR and CR3. Phagocytic uptake involves actin dynamics including polymerisation, bundling, contraction, severing and depolymerisation of actin filaments (Hatsuzawa 2008). So, we decided to understand the mechanism involved in phagocytosis of BCG by Mast Cells.

Involvement of cytoskeleton in phagocytosis by RBL-2H3

As phagocytosis is mediated by cytoskeleton, Cytochalasin D which is a cell permeable mycotoxin blocks the formation of microfilaments and microtubules (Hohn et al. 2009) was used to understand the role of cytoskeleton in phagocytosis by Mast Cells. A treatment with 2.5 μg/ml dose for 3h brought about morphological changes in RBL-2H3 cells Figure 28 indicates the disruption of cytoskeleton. The cells became spherical in shape. RBL-2H3 were cultured in a 48-well culture plate. Pre-treatment of RBL-2H3 cells with Cytochalasin D inhibited the BCG uptake by RBL-2H3. In Control cells 37.87±3% were able to phagoctose BCG whereas in cells treated with Cytochalasin D 7.49±1.49% were able to phagocytose BCG. This reduction in phagocytosis is statistically significant ($p<0.0005$, Student's t-test).

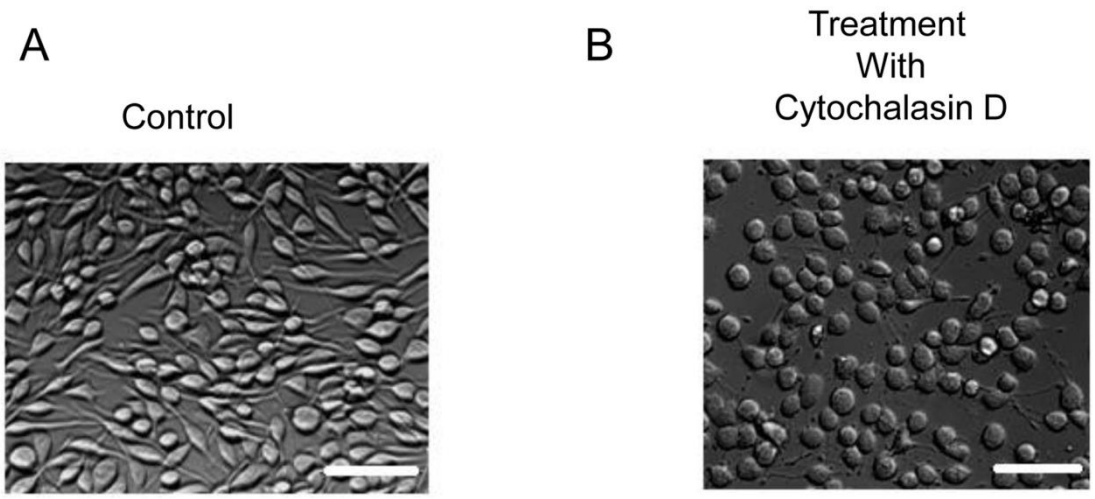

Figure 28: DIC images showing changes in morphology of RBL-2H3 on CTD treatment. (A) Control and (B) Treated DIC image of RBL after 2.5 µg CTD treatment for 3h. Images were taken at 40X magnification using Nikon Ti-E microscope. Scale bar represents 50µm.

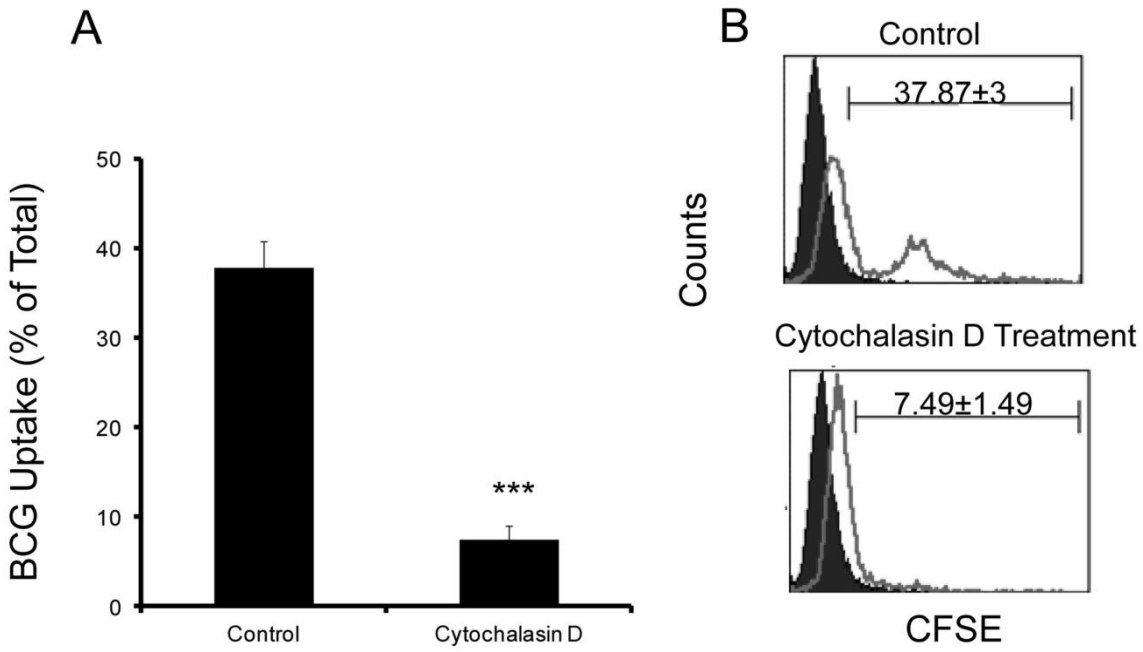

Figure 29: Effect of Cytochalasin D on uptake of CFSE labelled BCG by RBL-2H3. RBL-2H3 cells were cultured in a 48-well culture plate. Cytochalasin D (2.5µg/ml) was added to the cultures for 30 min before the addition of CFSE stained BCG (MOI, 100:1), and incubation continued for an additional 3 h. After 3 h, cells were harvested by trypsinization, washed with PBS, and fixed in 2% paraformaldehyde for flow cytometric analysis. ***$p<0.0005$ represent statistically significant difference between control and CTD treated cells. Panel B shows representative overlay histograms of unstained and BCG uptake by RBL-2H3 in control and Cytochalasin D treatments respectively. For FACS data filled histogram represents unstained RBL MCs and line histogram represents BCG uptake by RBL MCs. Each value represents mean ± SEM of values obtained from three independent assays.

Involvement of Rafts in phagocytosis of BCG by RBL-2H3

The dynamic clustering of sphingolipids and cholesterol form membrane microdomains called rafts that move within the fluid bilayer (Manes, del Real and Martinez 2003). Bacteria such as *Mycobacterium* spp., Fim H expressing *Eschericia coli*, *Salmonella typhimurium* and *Brucella* spp. exploit host rafts to generate phagosomes that allow them to enter and survive in professional and non professional phagocytic cell (Baorto et al. 1997, Garner, Hayward and Koronakis 2002, Gatfield and Pieters 2000, Watarai et al. 2002).

To explore the mechanism of phagocytosis and involvement of lipid rafts in the uptake of BCG by RBL-2H3 MβCD, Filipin and Nyastatin were used. MβCD removes cholesterol and Filipin sequesters cholesterol whereas Nystatin is a sterol-binding agent that disassembles caveolae and cholesterol in the membrane. It does not affect the clathrin-dependent internalization (Arthur, Heinecke and Seyfried 2011, Chen et al. 2011, Subtil et al. 1999, Zhu et al. 2011).

To check whether MβCD was able to remove cholesterol MβCD treated cells were stained with Filipin. Filipin is a naturally fluorescent polyene antibiotic that binds to cholesterol but not to esterified sterols (Maxfield and Wustner 2012). Thus, it is useful for detecting free cholesterol in biological membranes. In control RBL-2H3 Filipin was able to stain cholesterol of RBL-2H3 completely whereas in 2.5 mM MβCD treatment Filipin was able to stain cholesterol of RB-2H3 partially. Thus, it was concluded that 2.5 mM MβCD for 30 min was able to remove cholesterol partially (Figure 30). RBL-2H3 cells were cultured in a 48-well culture plate. Pre-treatment of RBL-2H3 cells with MβCD, Filipin and Nystatin resulted in 9.19±2.32%, 8.45±1.92% and 11.8±1.02% uptake of BCG compared to 21.6±0.98% BCG uptake by control cells. This represents almost 40% to 60% inhibition. Results of treatment with inhibitors of raft showed statistically significant decrease in uptake compared to control cells (Figure 31). This shows that uptake is partially via lipid rafts.

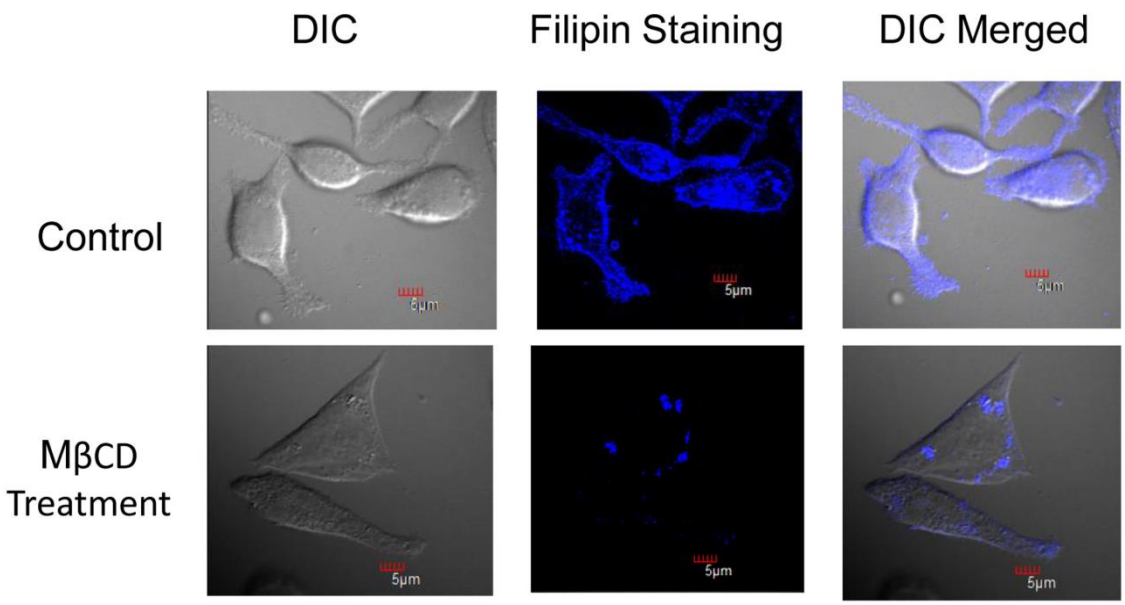

Figure 30: Confirmation of removal of cholesterol depletion by MβCD through Filipin staining. Cells were cultured on glass cover slips overnight. Cells were treated with 2.5 mM MβCD for 30 mins and were washed with PBS and fixed with 2% paraformaldehyde for 30 min at room temperature. It was then quenched with ammonium chloride followed by washing with PBS. Cells were stained with 2.5 µg/ml Filipin for 2 h at RT followed by washing with PBS. These cells were examined using a confocal laser scanning microscope (Olympus FluoView FV1000). Images were captured at 100X magnification and scale bar is 5 µm. This is a representative image out of five images captured from at least three independent experiments.

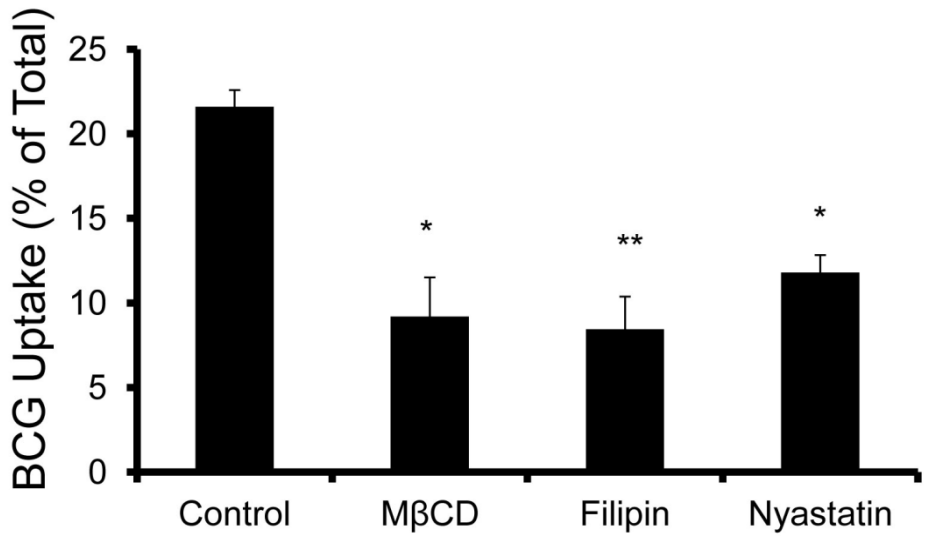

Figure 31: Involvement of rafts in BCG uptake. 0.1 million RBL MCs were seeded on 48 well cell culture plate and kept for overnight for adherence in CO_2 incubator at 37°C. Cells were pretreated with 2.5 mM MβCD for 30 mins, 5 µg/ml Nyastatin, 2.5 µg/ml Filipin for 30 mins and uptake was carried on for one hour. The samples were harvested and processed for flowcytometry. Each point represents mean ± SEM of values obtained from three independent assays. *P < 0.05 and **P <0.005 show statistically significant difference between control and treated cells is given by Student's t test.

Phosphoinositide 3-kinase is not involved in phagocytosis of BCG by RBL-2H3

Phosphoinositide 3-kinase (PI 3-kinase) has been shown to play an important role in vesicular membrane traffic. Wortmanin which is a PI 3-kinase inhibitor inhibits Fc receptor mediated phagocytosis but has little effect on receptor mediated endocytosis (Araki, Johnson and Swanson 1996). We investigated whether PI 3-kinase has any role in uptake by RBL Mast Cells. There was no change in uptake of BCG by RBL-2H3 on treatment with Wortmanin in comparison to control. Hence PI 3-kinase has no role in uptake of BCG by Mast Cells (Figure 32).

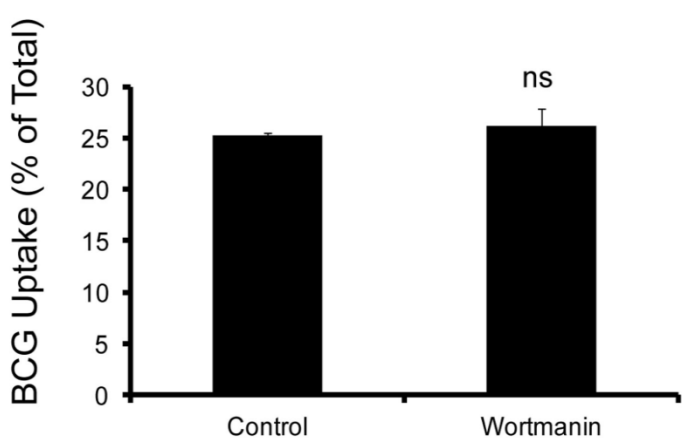

Figure 32: Involvement of Phosphoinositide 3-kinase in uptake of BCG. 0.1 million RBL MCs were seeded on 48 well cell culture plate and kept for overnight for adherence in CO_2 incubator at 37°C. Cells were pretreated with 100 mM Wortmanin and uptake was carried on for one hour. The samples were harvested and processed for flowcytometry. Each point represents mean ± SEM of values obtained from three independent assays.

Augmentation of BCG uptake by MCs by opsonisation of BCG

Opsonisation is known to enhance phagocytosis of pathogens by professional phagocytes like macrophages up to many folds. Our studies so far have indicated that RBL-2H3 cells are also able to phagocytose BCG. To further examine if opsonisation may have an effect on uptake of BCG by RBL-2H3 cells, it was decided to raise polyclonal antibodies to BCG in rodents and use them in opsonisation study. Antibodies to BCG by immunizing C57BL/6 mice were raised as decribed in Materials and Methods. The immunization schedule is given in Figure 33 A. Later on an indirect ELISA was done to quantitate antibody titer which came around 1:32,000 (Figure 33 B). BCG was opsonised with 10% anti-BCG serum and then was labelled with CFSE as before use in studies of phagocytosis. The labeling of opsonised BCG with CFSE dye was checked by flow cytometry as represented by flow histogram in Figure 34 depicting that even after opsonisation the labelling was above 95%.

These opsonised and CFSE labeled BCG were incubated with RBL-2H3 cells at low dose (MOI, 1:10) for 12 h. Figure 35 shows flow histogram of opsonised BCG uptake by RBL-2H3 cells. Under unopsonised condition 27.50±2.4% BCG were phagocytosed by RBL-2H3 whereas under opsonised condition 97.1±3.28% BCG were phagocytosed by RBL-2H3. Even the mean fluorescence intensity (MFI) has also increased. Under unopsonised condition MFI is 22.21 ±2.24 whereas opsonised condition MFI is 49.92±1.36.

Almost all MCs are now able to take up BCG even at a low dose. This is a very significant increase in uptake of BCF by RBL-MCs on opsonisation.

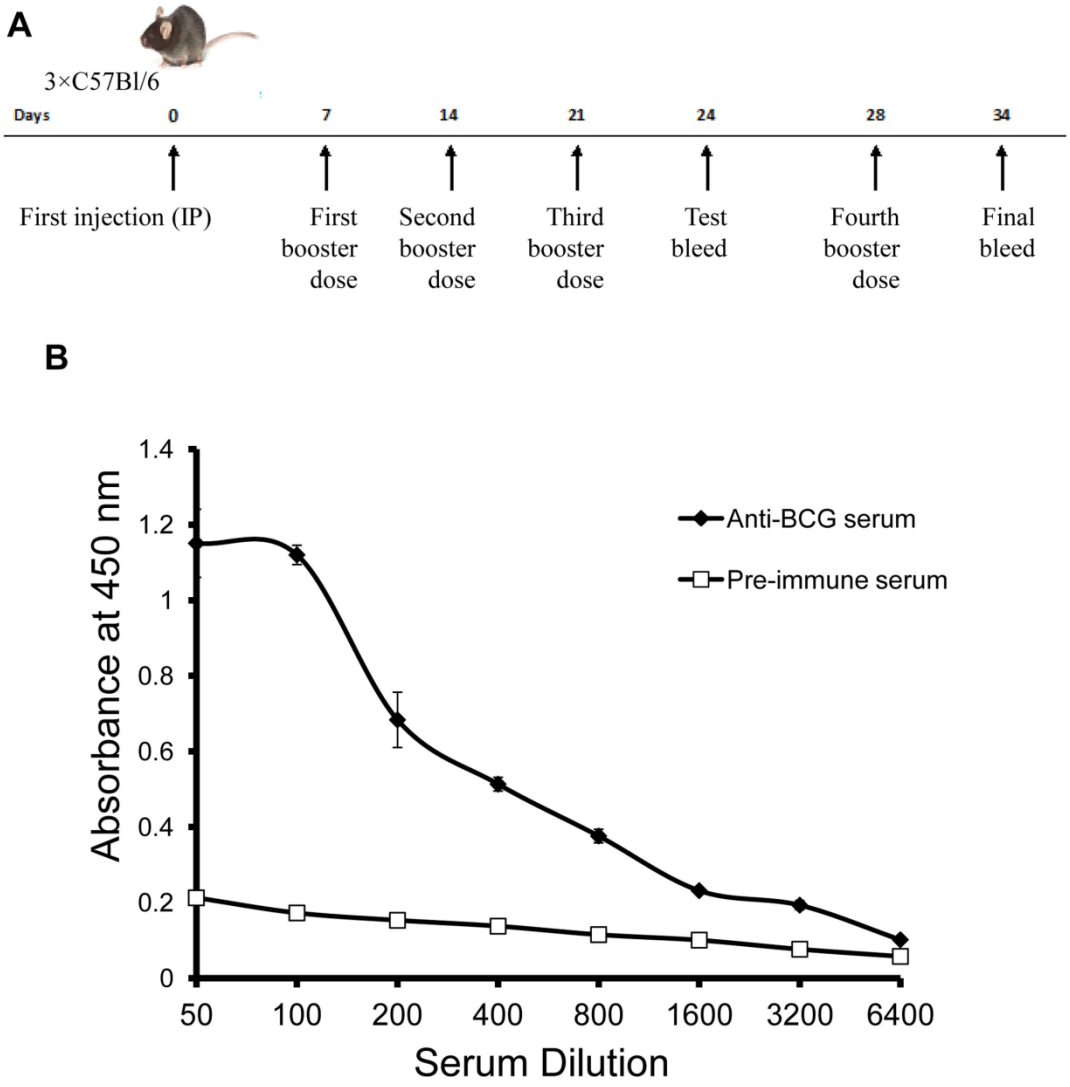

Figure 33: Generation of anti-BCG antibodies in mouse. 100µg antigen was injected intraperitoneally followed by 50µg booster doses for four weeks as shows in panel A timeline. The mice were then finally bled and indirect ELISA was performed to quantitate antibody titer. (B)10µg/well of sonicated BCG was coated on a 96 well ELISA plate. After washing, dilutions of post immune sera or pre immune sera were added to each wells at different dilutions followed by washing and adding 20000 times diluted secondary antibody. The wells were washed and 1X TMB was added which was incubated for 20 minutes followed by stopping the reaction by 1M H_2SO_4. The absorbance was read at 450nm.The data shown in panel B are mean ± SEM of three independent assays**.**

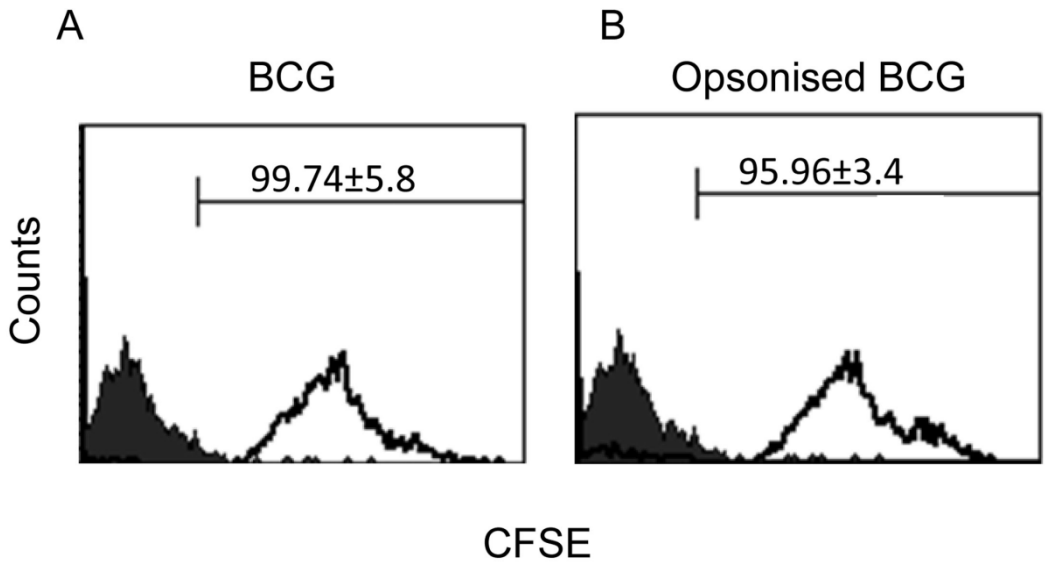

Figure 34: Staining of opsonized BCG with CFSE stain. 1×10^8 BCG were incubated with 10% anti-BCG serum suspended in RPMI media for 30 mins on rotisserie at 5 rpm followed by three times washing with PBS. They were labeled with CFSE stain as mentioned in materials and methods. The overlay histograms of unstained and stained BCG at in unopsonised and opsonised condition are represented by Panel A and B respectively. Each point represents mean ± SEM of values obtained from three independent assays. For FACS data filled histogram represents unstained BCG and line histogram represents CFSE stained BCG. Each value represents mean ± SEM of values obtained from three independent assays.

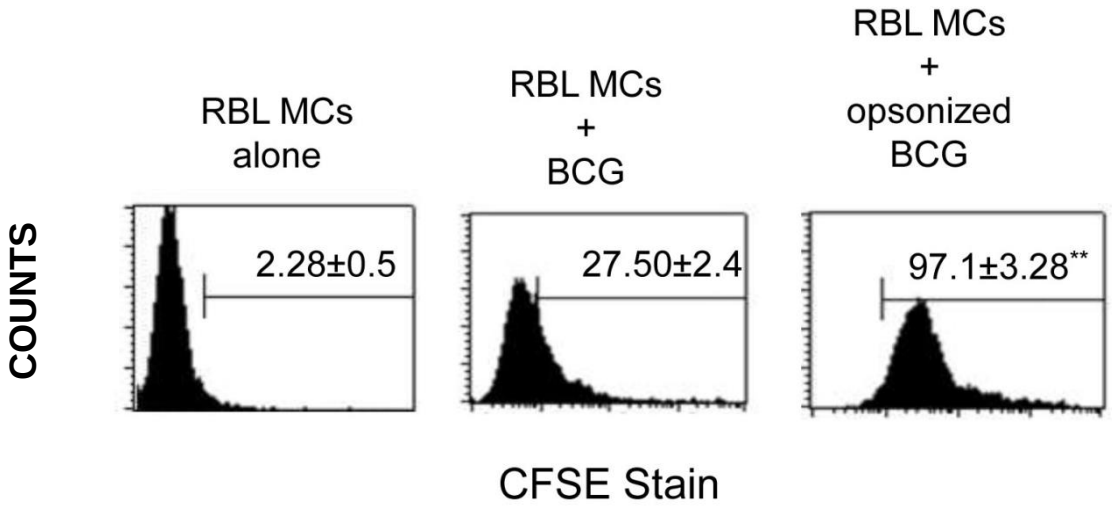

Figure 35: Augmentation of phagocytosis by opsonisation. $0.1x10^6$ µm cells were seeded in 48 well cell culture plate for overnight. 1×10^8 BCG were incubated with 10% anti BCG serum suspended in RPMI media for 30 mins on rotisserie at 5 rpm followed by three times washing with PBS. They were labeled with CFSE as mentioned in materials and methods. These opsonized BCG labeled with CFSE were incubated at MOI 1:10. After 12h cells were trypsinized, fixed and analyzed through flow cytometer. Panels A represents flow histogram of BCG uptake by RBL-2H3 at different conditions. ** $p<0.005$ represent statistically significant difference between BCG uptake under opsonised and unopsonised condition by RBL-2H3 cells. Each point represents mean ± SEM of values obtained from three independent assays.

Uptake of BCG derived antigen by Mast Cells

Another mode of uptake is macropinocytosis, a form of bulk uptake of fluid and solid cargo into cytoplasmic vacuoles, called macropinosomes which has been studied mostly in relation to antigen presentation (Swanson 2008). Major APCs namely Macrophages and DCs are highly active in macropinocytosis, internalizing up to 200% of their surface area every hour and therefore sampling the environment for their role as APCs of the immune system. Also immature dendritic cells are able to macropinocytose large quantities of exogenous solute as part of their sentinel function (Norbury 2006). So it was decided to explore whether Mast Cells are also capable of take up *M. bovis* derived antigen by macropinocytosis.

M. bovis BCG were biotinylated followed by sonication. The sonicated biotinylated BCG were incubated with RBL Mast Cells. The uptake of sBCG by RBL-2H3 cells occur in a dose dependent manner on the basis of percentages of cells having taken up sBCG. At lower MOI there is lesser uptake whereas at higher MOI it is picked up (Figure 36). It also illustrates that at higher MOI and after incubating for more time uptake is pretty much saturable. Further, the uptake was also confirmed through microscopy. RBL Mast Cells were cultured on coverslips for adherence overnight. Biotinylated sonicated BCG were incubated with RBL Mast Cells for 2 h followed by fixing and staining with Streptavidin FITC. Green coloured sBCG is seen in the cytoplasmic region surrounding the DAPI stained blue coloured nucleus (Figure 37).

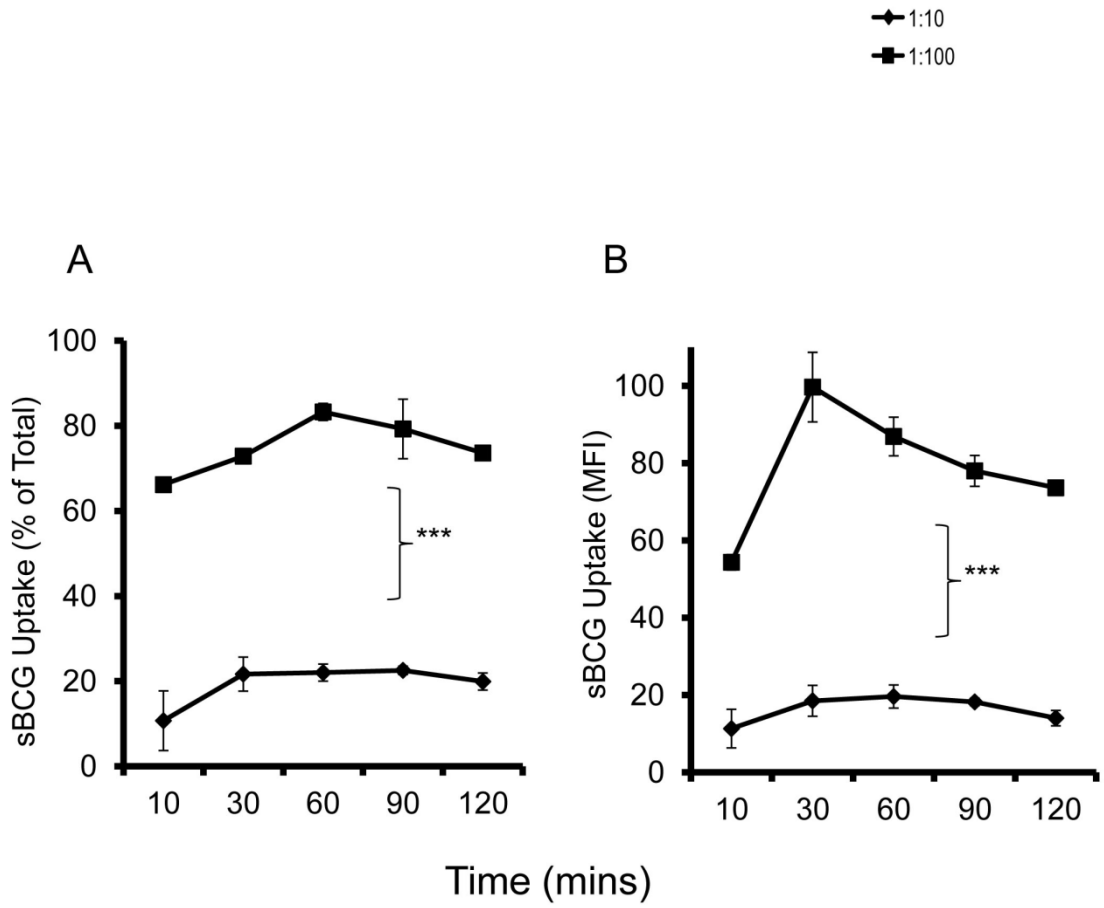

Figure 36: Uptake of BCG derived antigen by RBL Mast Cells. 0.1 million cells were cocultured with biotinylated sonicated BCG for various time points followed by fixing PFA, permeabilizing and staining with Streptavidin FITC and running through flowcytometer. Panel D represent sonicated BCG percent positive cells at MOI (1:10) and (1:100). Each point represents mean ± SEM of values obtained from three independent assays. **P<0.005 is given by ANOVA.

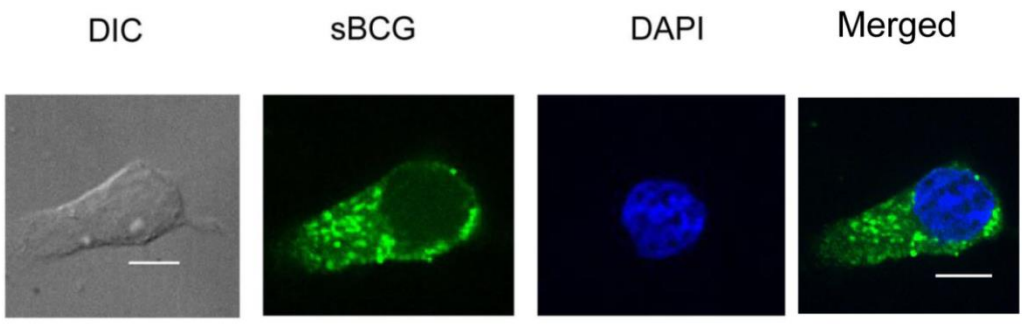

Figure 37: Uptake of BCG derived antigen by RBL Mast Cells. RBL-2H3 cells were cultured on coverslips. RBL-2H3 cells were cultured on coverslips. After 12h, cells were cocultured with biotinylated sonicated BCG for 1h followed by fixing PFA permeabilizing and staining with Streptavidin FITC and were mounted with DAPI and examined using a confocal laser scanning microscope. (magnification 100X). Scale bar represents 10μm. This is a representative image out of five images captured from at least three independent experiments.

Mechanism of macropinocytosis in RBL-2H3 and MH-S cells

Macropinocytosis has various characteristic features such as it is inhibited at low temperatures, it is an actin-driven endocytic process which is not directly co-ordinated by the presence of cargo but can be induced upon activation of growth factor signalling pathways (Lim and Gleeson 2011). So it is important to determine the mechanism employed for macropinocytosis by Mast Cells and to examine the functional regulation of macropinocytosis in Mast Cells by micro environmental factors.

Temperature dependence of BCG derived antigen uptake by RBL Mast Cells

RBL-2H3 cells were incubated at different temperature with different doses. At 4°C there was significantly lesser uptake of BCG derived antigen compared to uptake at 37°C in RBL-2H3. Almost 75% inhibition has taken place at 4°C compared to control at 37°C (Figure 38). Thus, uptake of BCG derived antigen in the cells was optimal at 37°C.

Involvement of cytoskeleton in BCG derived antigen uptake by RBL Mast Cells

Cytochalasin D, an agent that blocks the actomyosin system in the cellular membrane was used (Kumari and Saxena 2011) to test the involvement of cytoskeleton in uptake of BCG derived antigen by RBL-2H3 cells. Almost 50% inhibition has taken place compared to control (Figure 39). Results of Cytochalasin D treatment experiments show that macropinocytosis is partially dependent on cytoskeleton in Mast Cells.

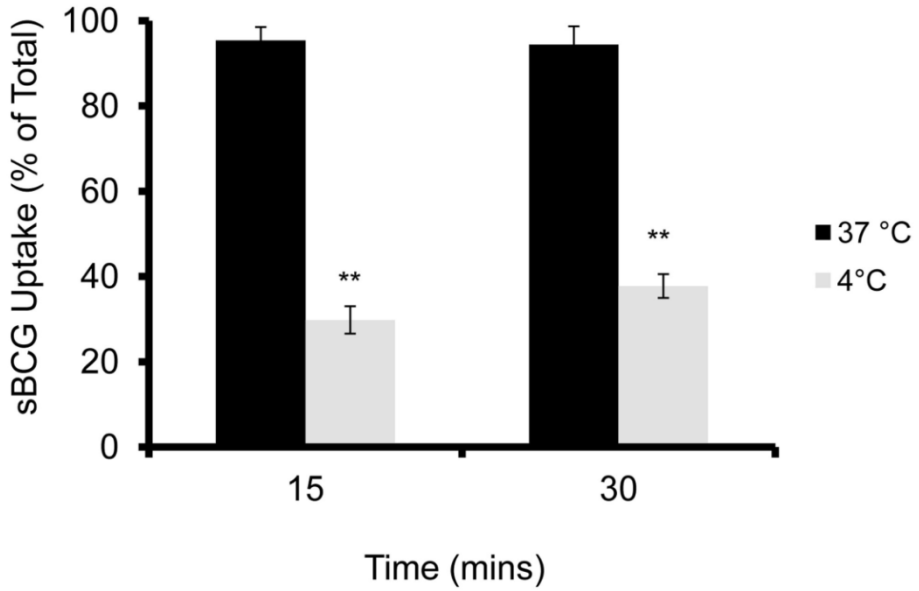

Figure 38: Effect of temperature on uptake of BCG derived antigen. 0.2 million cells were co-cultured with biotinylated sonicated BCG for various time points at 37°C and 4°C at MOI (1:100) followed by fixing with PFA permeabilizing and staining with Streptavidin FITC and running through flowcytometer. Each point represents mean ± SEM of values obtained from three independent assays.**P<0.005 between control and treated cells is given by Student's t test.

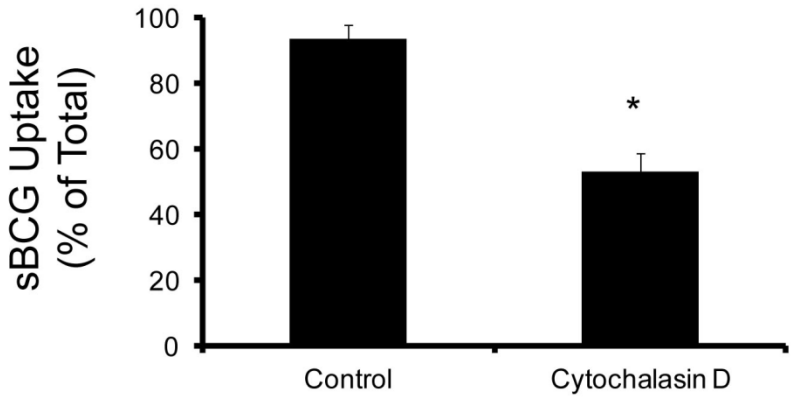

Figure 39: Effect of Cytoskeleton on BCG derived antigen. 0.2 million cells were pretreated with Cyt D cocultured with biotinylated sonicated BCG for 1h at MOI (1:100) followed by fixing PFA permeabilizing and staining with Streptavidin FITC and running through flowcytometer. Each point represents mean ± SEM of values obtained from three independent assays.*$P<0.05$ between control and treated cells is given by Student's t test.

sBCG uptake by RBL-2H3 cells activated by Ionomycin

It is well known that activation of receptor tyrosine kinases, like the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) lead to a more global increase in actin polymerization at the cell surface, resulting in an elevation in actin-mediated ruffling and ultimately increasing macropinosome formation (Swanson and Watts 1995). Calcium ionophores like Ionomycin are lipophilic ion carriers that allow calcium randomly across membranes lead to the release of calcium from intracellular stores. So ionomycin induced activation of RBL-2H3 MCs was used to explore macropinocytosis under activated conditions in Mast Cells. Since the assay was performed in suspension and RBL-2H3 is an adherent cell line, standardization was performed to activate RBL-2H3 cells with Ionomycin in suspension. RBL-2H3 cells were activated by Ionomycin at 5µM concentration for 10 mins. This trigger showed 35±2.5% secretion of β-Hexosaminidase enzyme (Figure 40 A). Control and Ionomycin treated RBL cells were washed thrice with PBS at 1200 g for 5 mins and sonicated BCG at MOI (1:1) was added to cells. In resting MCs 17.54±2.2% were sBCG positive whereas in activated MCs 74.56±3.4% were sBCG positive. There is a fourfold increase in uptake of sBCG by activated RBL-2H3 in comparison to control (Figure 40 B). Activation of Mast Cells seems to upregulate their capacity for macropinocytosis to a great extent.

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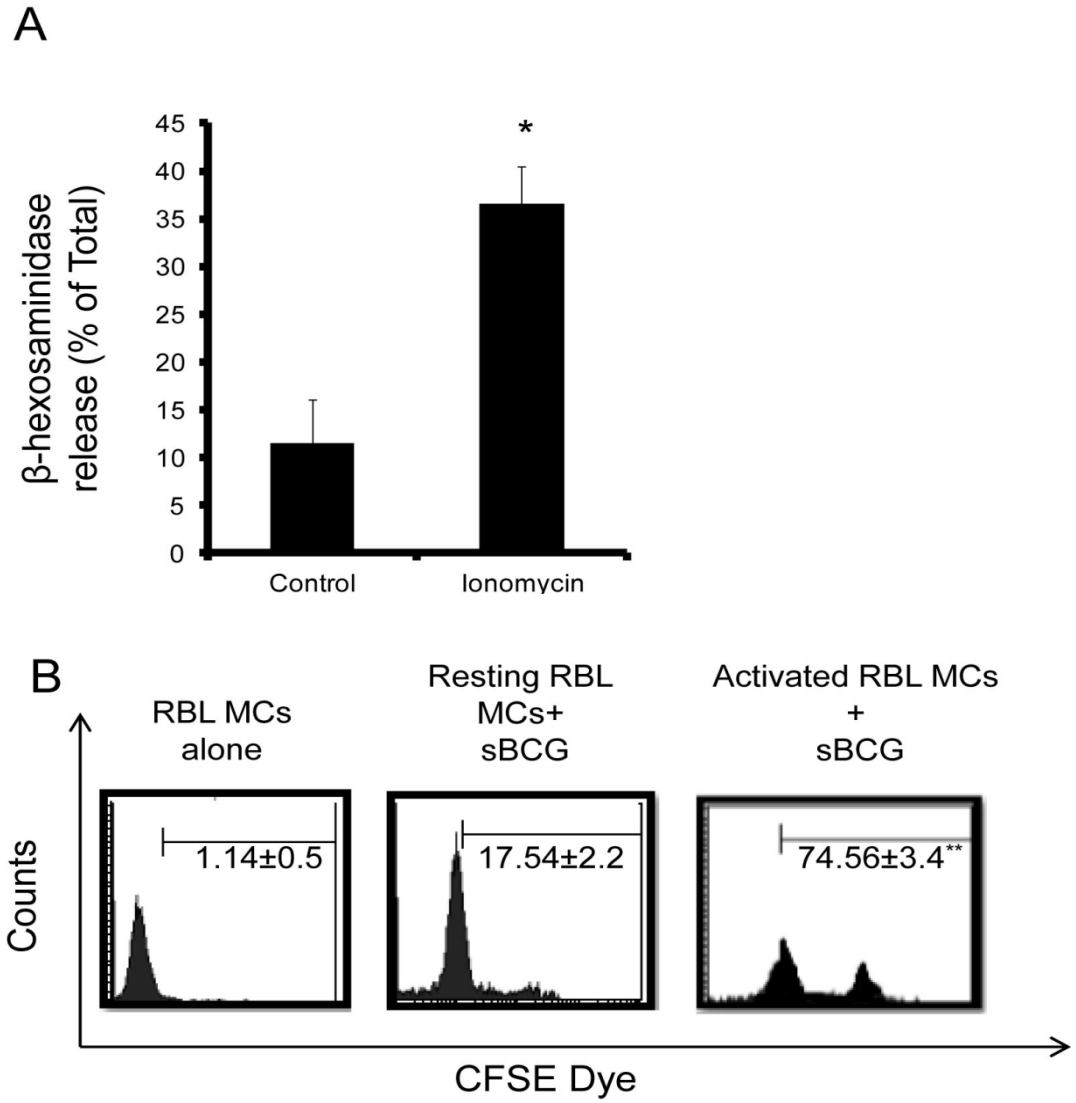

Figure 40: Enhancement of uptake of BCG derived antigen by RBL Mast Cells. Activation of RBL-2H3 cells in suspension was accomplished by harvesting $1.5x10^6$ cells each in microcentrifuge and doing the experiment in duplicates, washing it thrice with phenol red free RPMI medium and resuspending in 250µl of phenol red free RPMI medium. Pre-warmed 250 µl of 10 µM Ionomycin was added and incubation was continued at 37°C for 10 mins. Mock degranulation studies were conducted in parallel by using medium alone as represented in panel A. Sonicated BCG uptake was studied as discussed before. Panel B represents flow histogram of sonicated BCG uptake by RBL-2H3 at different conditions. Each point represents mean ± SEM of values obtained from three independent assays. *$P < 0.05$ and**$P < 0.005$ between resting and activated MCs is given by Student's t test.

Uptake of *L. tropica* by RBL Mast Cells

In order to study initial interaction of *L. tropica* with Mast Cells, promastigotes of *L. tropica* were CFSE labelled. 100×10^6 promastigotes of *L. tropica* and *L. donovani* were labelled with 10 µM CFSE for 30 minutes. Staining efficiency was checked through flowcytometer. It was found that 98±2.6% *L. tropica* were CFSE labelled and after 24 h the labelling was 91.8±2.3% (Figure 41). Thus, the labelling was stable. When these CFSE labelled promastigotes were co-cultured with MCs, 7.2%±2.2% and 11.7%±2.7% MCs were found to be positive for CFSE labelled *L. tropica* at 18h and 24 h respectively (Table1). Confocal microscopy also further confirmed *L. tropica* being taken up by MCs after 24 h of co-culture (Figure 42).

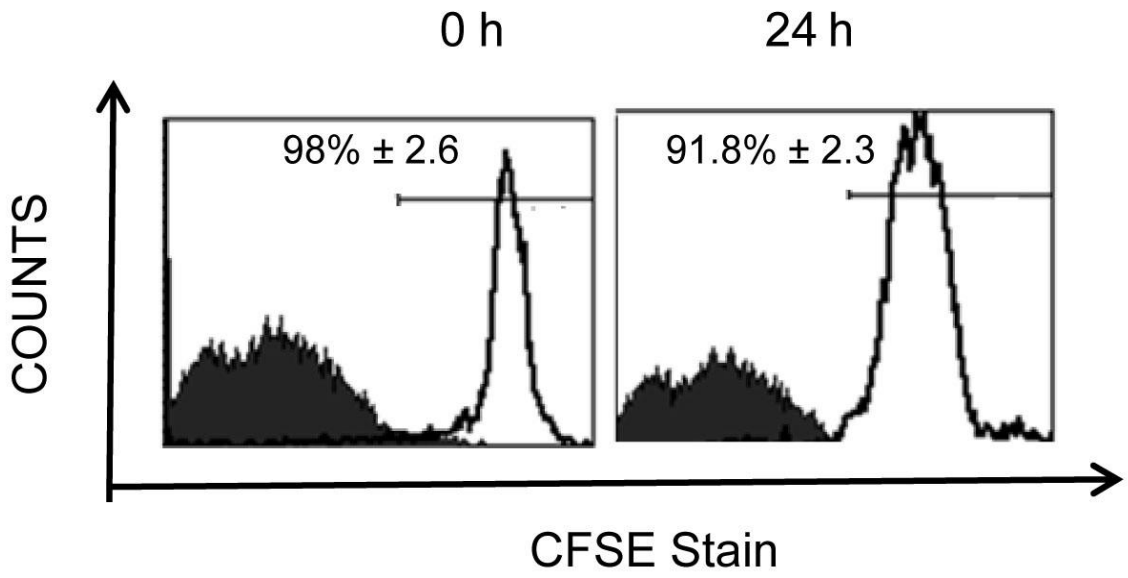

Figure 41: Staining *L. tropica* with CFSE. Promastigotes of *L. tropica* were labelled with CFSE and their stability was checked after 24 h. Closed grey histogram represents unstained *Leishmania* whereas line histogram represents stained *Leishmania.* Each point represents mean ± SEM of values obtained from three independent assays.

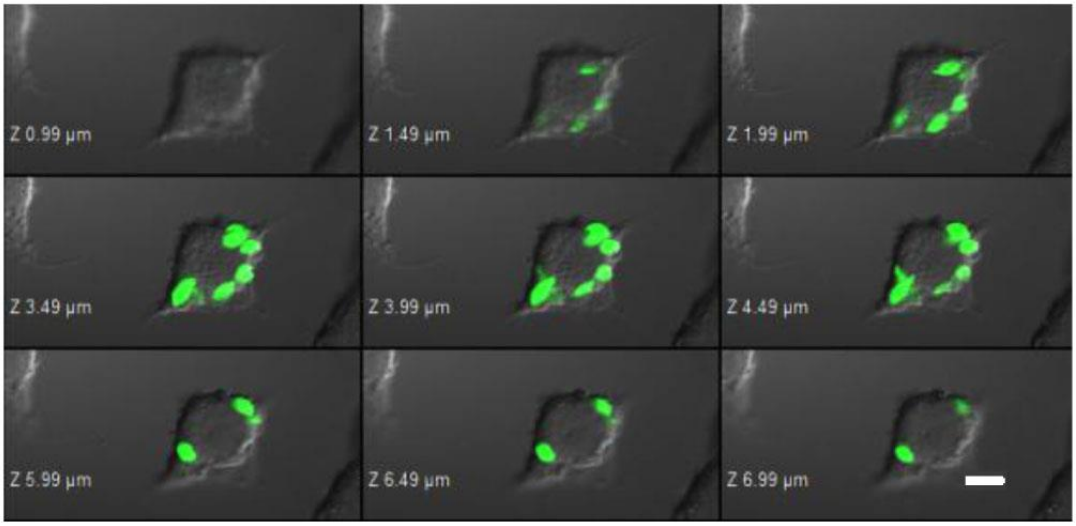

Figure 42: Uptake of *L. tropica* by RBL Mast Cells. CFSE labelled promastigotes were syringe separated and incubated with overnight cultured MCs at an MOI 1:10 in 48 well cell culture plate and were processed as discussed in materials and methods. RBL-2H3 were cultured on coverslips overnight. Cells were incubated with CFSE labeled *L. tropica* for 24 h and were processed as discussed in materials and methods. Cells were examined using a confocal laser scanning microscope *L. tropica* (magnification 100X). Scale bar represents 5µm. This is a representative image out of five images captured from at least three independent experiments.

Table 1: Uptake of *L. tropica* with RBL Mast Cells

MCs cocultured with the *Leishmania*	**Time (h)**	***Leishmania* Uptake in MCs (% Gated)**	***Leishmania* Uptake in (MFI)**
Mast Cells alone	18	1.16±1.2	4.9±2.1
	24	1.8±0.8	4.8±1.6
Mast Cells + *L. tropica*	18	7.2±2.2*	18.5±2.3*
	24	11.7±2.7*	23.8±3.4*

*P < 0.05between MC alone and MCs + *L. tropica* is given by Mann Whitney u test .

Role of Cytoskeleton on uptake of *L. tropica*

Further to confirm that the reduction in live cell recoveries of promastigotes is because of phagocytosis by MCs, we pre-treated MCs with Cytochalasin D, a known inhibitor of actin polymerization, and therefore phagocytosis (Casella, Flanagan and Lin 1981), and co-cultured with *L. tropica* and calculated viability. The viability of promastigotes of *L. tropica* at 24 h of co-culture with cytochalasin D treated MCs was found to be 65.3%±4%. Hence the viability still shows a statistically significant decrease from cell viability of promastigotes cultured alone, but a statistically significant increase from viability obtained on co-culture of promastigotes with untreated MCs (Figure 43). Further, since phagocytosis is only partially responsible for reduction in recoveries, other non-phagocytic pathways may also be involved in clearance of *Leishmania* by MCs.

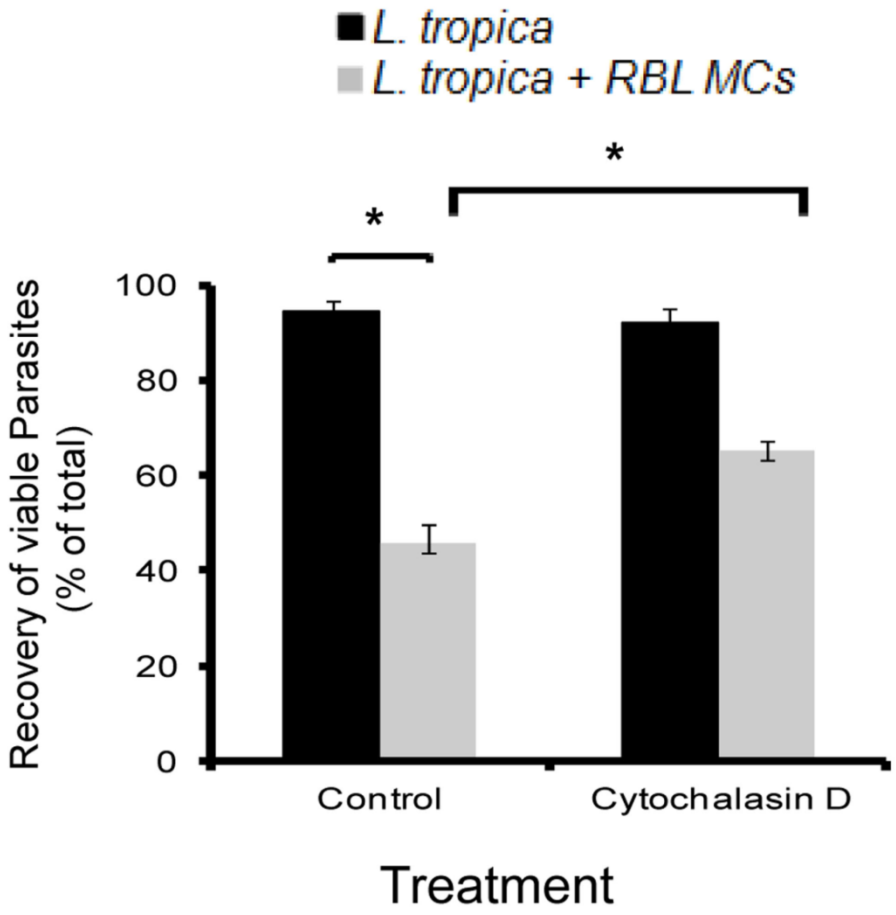

Figure 43: Cell viability of *L. tropica* on co-culture with MCs treated with Cytochalasin D. 0.1 million RBL Mast Cells were seeded in 48 well plate. Cells with pretreated with Cytochalasin D for three hours followed removing the medium and addition of fresh medium and adding *L. tropica* at MOI 1:10. % cell viability was assessed through trypan blue staining. Each point represents mean ± SEM of values obtained from three independent assays. *$p<0.05$ by Mann Whitney u test

Uptake of *L. donovani* by RBL Mast Cells

It was found that 91.8±2.5% *L. donovani* were CFSE labelled and after 24 h the labeling was 84.8±6.4% (Figure 44). Thus, the labeling was stable. But no uptake of CFSE labelled *L. donovani* by MCs was seen at these time points (Table 2). Confocal microscopy also further confirmed *L. donovani* not being taken up by MCs after 24 h of co-culture (Figure 45). Cell recovery of *L. donovani* are not affected by Cytochalasin D treatment on co-culture with RBL Mast Cells (Figure 46).

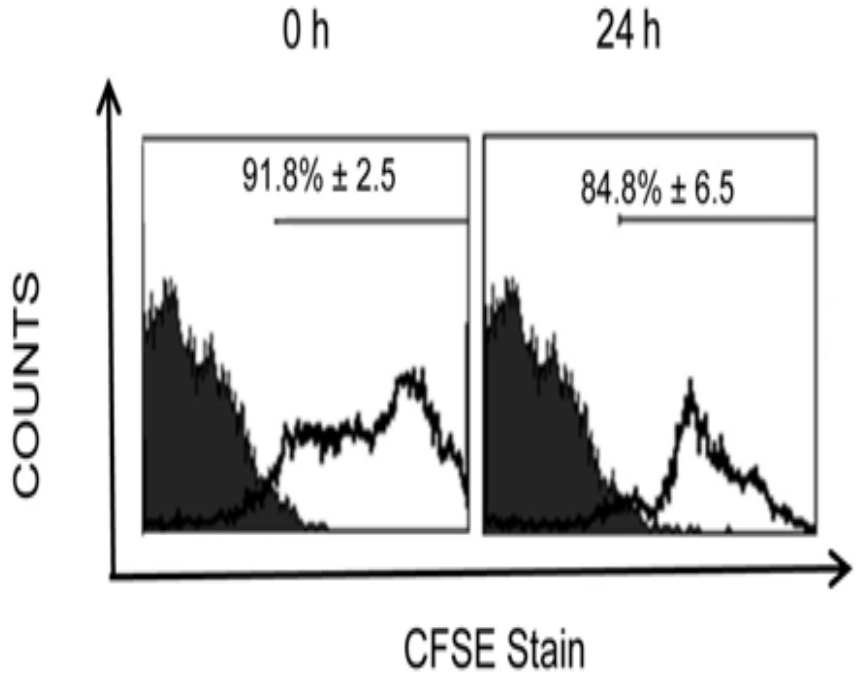

Figure 44: CFSE labelling of *L. donovani.* Promastigotes of *L. donovani* were labelled with CFSE and their stability was checked after 24 h. Closed grey histogram represents unstained *Leishmania* whereas line histogram represents stained *Leishmania.* Each point represents mean ± SEM of values obtained from three independent assays.

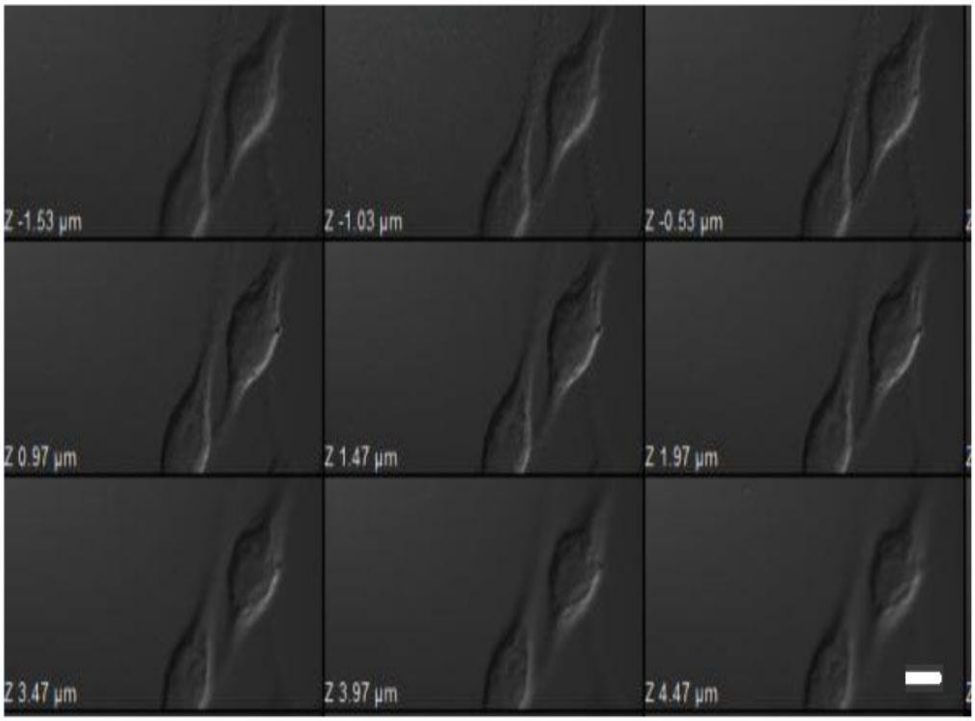

Figure 45: Uptake of *L. donovani by* RBL Mast Cells. CFSE labelled promastigotes were syringe separated and incubated with overnight cultured MCs at an MOI 1:10 in 48 well cell culture plate and were processed as discussed in materials and methods. RBL-2H3 were cultured on coverslips overnight. Cells were incubated with CFSE labeled *L. donovani* for 24 h and were processed as discussed in materials and methods. Cells were examined using a confocal laser scanning microscope *L. tropica* (magnification 100X). Scale bar represents 5µm. This is a representative image out of five images captured from at least three independent experiments.

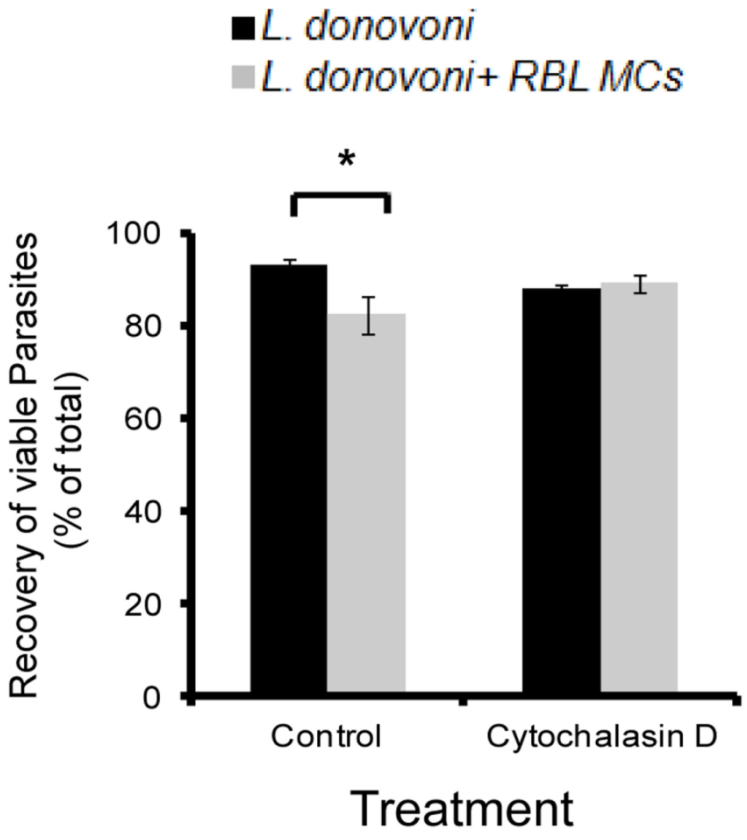

Figure 46: Cell viability of *L. donovani* on co-culture with MCs treated with Cytochalasin D. 0.1 million RBL Mast Cells were seeded in 48 well plate. Cells with pretreated with Cytochalasin D for three hours followed removing the medium and addition of fresh medium and adding *L. donovani* at MOI 1:10. % cell viability was assessed through trypan blue staining. Each point represents mean ± SEM of values obtained from three independent assays. *$p<0.05$ by Mann Whitney u test.

Table 2: Uptake of *L. donovani* with RBL Mast Cells

MCs cocultured with the *Leishmania*	**Time (h)**	***Leishmania* Uptake in MCs (% Gated)**	***Leishmania* Uptake in (MFI)**
Mast Cells alone	18	1.16±1.2	4.9±2.1
	24	1.8±0.8	4.8±1.6
Mast Cells+ L. donovani	18	1.9±2.6	5.2±2.3
	24	1.8±3.8	5.1±3.3

*P < 0.05between MC alone and MCs + *L. donovani* is given by Mann Whitney u test .

Invasion of Mast Cells by *C. albicans* and comparison with MΦs

Firstly in order to understand how WT *C. albicans* interact with RBL Mast Cells, RBL Mast Cells were co-cultured with *C. albicans* and their interaction was observed through live cell imaging. RBL Mast Cells and *C. albicans* were co-cultured for a period of 3 h at 37 °C under 5% CO2 condition. Images were captured at 1 min intervals for 3 h. Figure 47 is a sequence of still images from a representative live-cell video microscopy experiment showing interaction of RBL Mast Cells with *C. albicans*. RBL Mast Cells do not interact with the yeast form (white arrow) of *C. albicans*. After 30 min hyphae formation (black arrow) by *C. albicans* takes place. After 1 h hyphae started invading RBL Mast Cells creating membrane perturbation which is reversible as shown by *. When they were co-cultured with phagocytes such as Murine Alveolar macrophage cell line MH-S, yeast form (white arrow) of *C.* albicans were seen to make cell-cell contact and within 17 mins and after 30 mins hyphae (black arrow) of C. albicans started invading MH-S cells. Figure 48 is a sequence of still images from a representative live-cell video microscopy experiment showing a macrophage phagocytosing *C. albicans* cells, hypha growth with the macrophage and ultimately macrophage lysis. Hence it can be concluded that yeast form of *C. albicans* does not cause any remarkable changes in RBL Mast Cells but hyphal form are invades RBL Mast Cells.

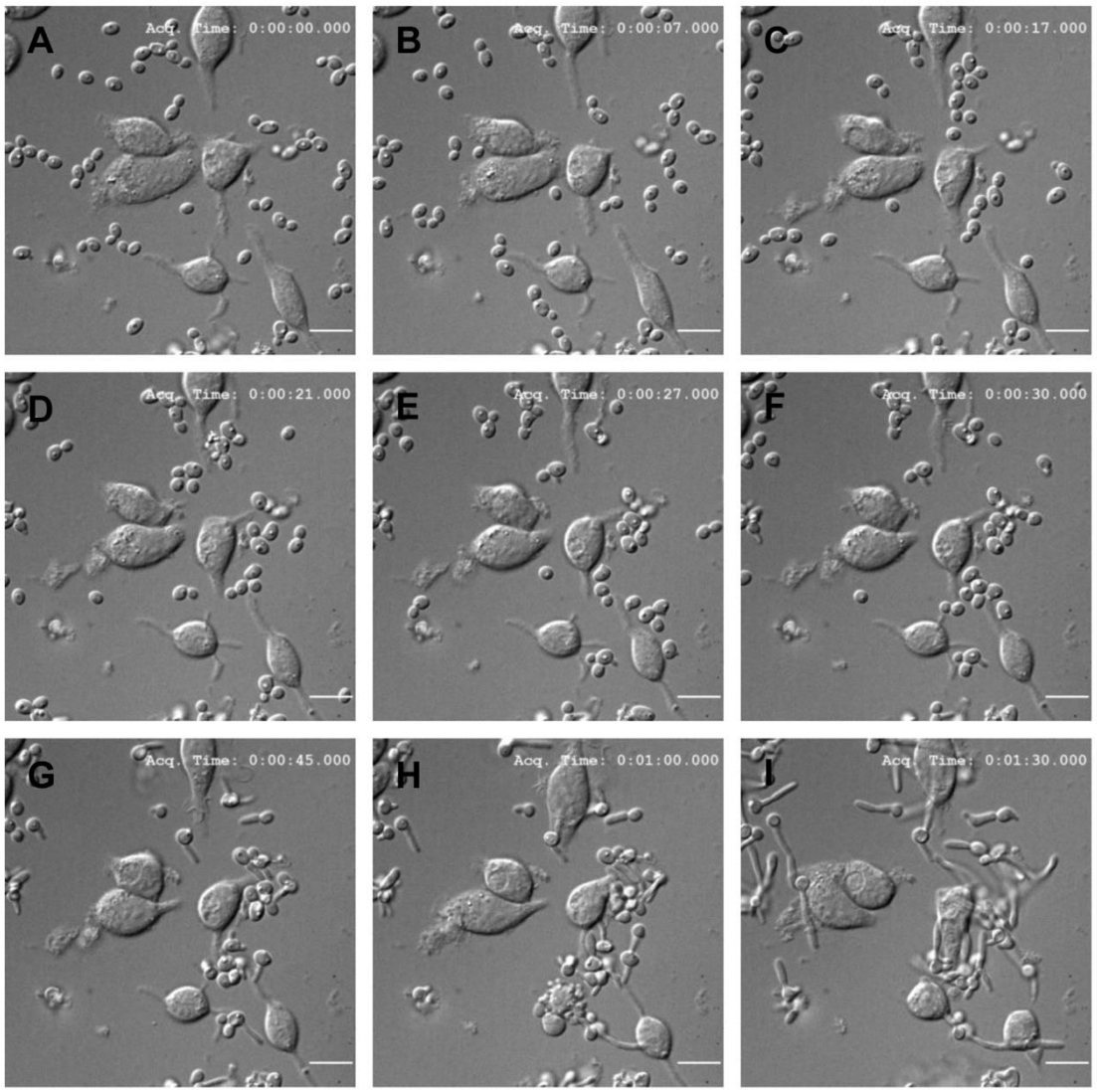

Figure 47: Live Cell Imaging of *C. albicans* with RBL Mast Cells. Shown are images of the indicated time points for *C. albicans* infected RBL Mast Cells. A, B, C, D, E, F, G, H and I represent still images at 0 min, 7 min, 17 min, 21 min, 27 min, 30 min, 45 min, 1 h and 1 h 30 min. Magnification is 60X and Scale bar is 10 μ m. White arrow represent yeast form, black arrow represents hyphal form and * is membrane perturbation.

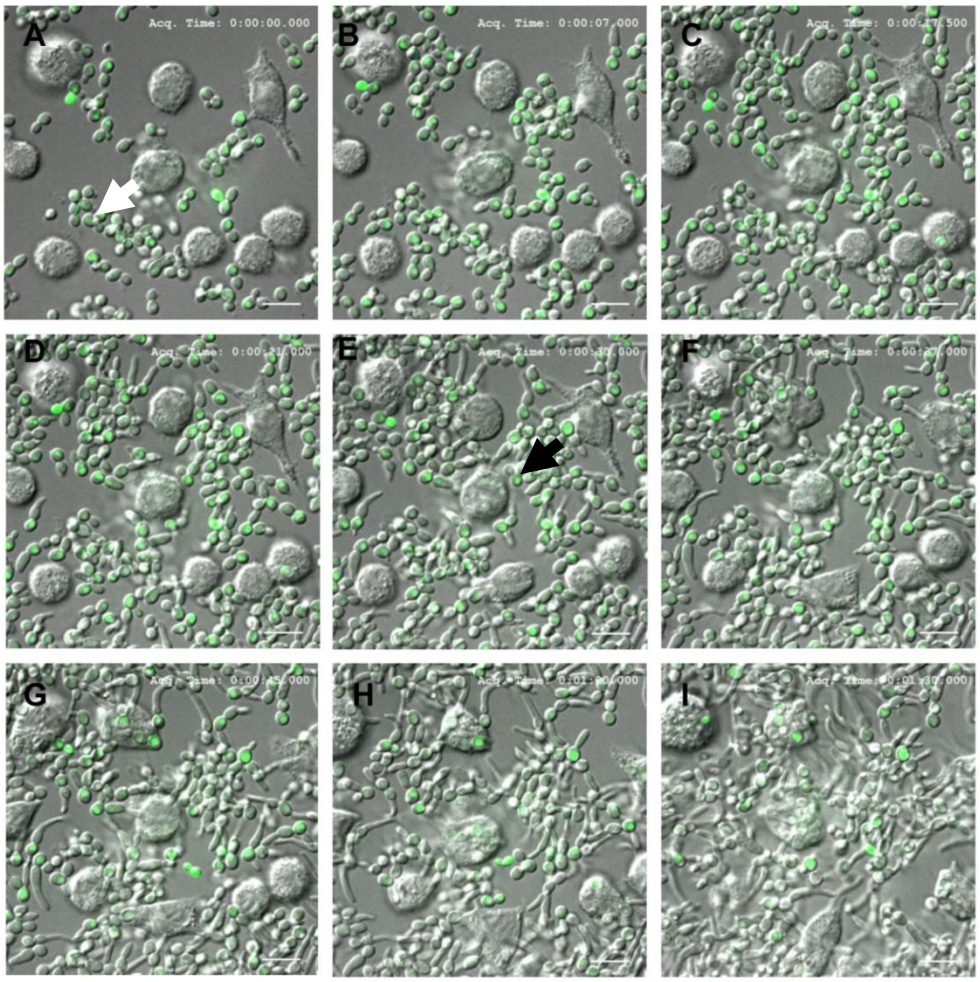

Figure 48: Live cell imaging of *C. albicans* with MH-S macrophages. Shown are images of the indicated time points for *C. albicans* infected MH-S macrophages. A, B, C, D, E, F, G, H and I represent still images at 0 min, 7 min, 17 min, 21 min, 27 min, 30 min, 45 min, 1 h and 1 h 30 min. Magnification is 60X and Scale bar is 10 μ m. White arrow represents yeast form and black arrow represents hyphal form.

Six heterozygote mutant of GPI-GnT subunits

For GPI biosynthesis, the first step is the formation of GPI-N-acetyl glucosaminyl phosphatidylinositol (GlcNAcPI) from uridinediphosphate-*N*-acetylglucosamine (UDP-GlcNAc) and phosphatidylinositol (PI). It consists of six different subunits Gpi1, Gpi2, Gpi3, Gpi15, Gpi19 and Eri1. Gpi2 and Gpi19 mutually coregulates with ergosterol biosynthesis. Gpi2 regulates hyphal morhphogenesis via Ras signaling and governs pathogenecity. Both Gpi2 and Gpi19 subunits negatively regulate each other (Yadav et al. 2014). So the virulence study was done for single allele disruption of six subunits of GPI-GnT complex such as Gpi1, Gpi2, Gpi3, Gpi15, Gpi19 and Eri1 and compared to the wild type strain BWP17. Firstly in order to distinguish them based on their size and granularity different mutants were run through flowcytometer. Through flowcytometer size parameter was obtained through Forward Scatter Mean (FSC) and granularity was obtained through Side Scatter Mean (SSC). The FSC mean of Cagpi2 is 144.38±5.8 which is statistically more significant compared to BWP17 which is 111.3±3.5. The SSC mean of Cagpi2 is 95.74±3.6 which is statistically more significant compared to BWP17 which is 87.6±3.4 (Table 3).

Table 3: Comparison of size and granularity of various heterozygous mutants of subunits of *C. albicans*by flow cytometric analysis

Heterozygote mutants of GPI-GnT subunits of *C. Albicans*	**Forward Scatter Mean (FSC Mean)**	**Side Scatter Mean (SSC Mean)**
BWP17 (Wild Type)	111.3±3.5	75.57±2.6
Cagpi1	121±3.6	87.6±3.4
Cagpi2	144.38±5.8***	95.74±3.6**
Cagpi3	120.16±4.8	92.74±2.8**
Cagpi15	114.53±2.4	85.52±3.2*
Cagpi19	114.99±2.2	76.82±2.8
Eri1	111.40±1.8	77.37±3.6

*p<0.05, **<0.005, ***p<0.0005 represent statistically significant difference between WT and their mutants by Student's t – test.

Effect of various heterozygous mutations in the first step of GPI biosynthesis in *C. albicans* on interaction with MCs and MΦs

Firstly to study the interaction of wild type strain BWP17 and heterozygote mutant of Gpi1, Gpi2, Gpi3, Gpi15, Gpi19 and Eri1 they were labelled with 10μM CFSE. By this labelling more than 90% of wild type as well as different heterozygote mutant were CFSE labelled and this labelling is retained even after 3 h when they switch to hyphal form. So CFSE labelled heterozygote mutant of six Gpi subunits and its Wild Type strain were co-cultured with RBL Mast Cells for 30mins. These cells were harvested and then cells were run through flowcytometer. Similarly, CFSE labelled heterozygote mutant of six Gpi subunits and its Wild Type strain were co-cultured with MH-S forcomparison for 30 mins at 37°C and 5% CO_2 incubator. It was found that neither yeast form of BWP17 nor heterozygote mutant of Gpi1, Gpi2, Gpi3, Gpi15, Gpi19 and Eri1 were being phagocytosed by RBL Mast Cells and this further reconfirmedwith the live cell imaging data which also indicated that even after 30 mins RBL Mast Cells were not able to phagocytose yeast form of WT strain of *C. albicans* (Table 4). But Macrophages showed different efficacy of phagocytosis of different heterozygous mutants of GPI-GnT complex and this phagocytosis is partially inhibited by Cytochalasin D (Table 4). Among all the mutants heterozygote mutant Gpi2 shows maximum uptake by MΦs which is 2 fold in comparison to that of WT BWP17.

Similarly, in order to study whether there is any difference in efficacy of invasion by hyphae of BWP17 and heterozygote mutant of GPI-Gnt complex, CFSE labelled yeast form of BWP17 and heterozygote mutant of GPI-Gnt complex at MOI 1:1 and MOI 1:5 were co-cultured with RBL Mast Cells and MH-S Macrophages Cells for 3 h at 37°C and 5% $C0_2$ incubator. The efficacy of invasion of Cagpi2 is significantly lesser compared to their wild type strain BWP17 at MOI 1:1 and MOI 1:5 in case of RBL Mast Cells (Table 4). Similarly, the efficacy of invasion by Cagpi19 and CaEri1 is significantly more as compared to wild type strain BWP17 at MOI 1:1 and MOI 1:5 in case of RBL Mast Cells. Now further to observe whether cytoskeleton of RBL Mast Cells play any role in invasion of either WT strain or heterozygous mutant of *C. albicans,* cells were pretreated with Cytochalasin D for 3 h followed by addition of yeast form of different strains by *C. albicans* for 3 h at 37°C and 5% CO_2 at MOI 1:5.

There was no significant difference in the invasion of wild type and heterozygote mutant by *C. albicans* in case of RBL Mast Cells. Similar results were also observed in case of RBL Mast Cells and MH-S Macrophages co-culture experiments of WT BWP17 and heterozygous mutant of GPI-GnT subunits where CaEri1 and Cagpi19 show maximum invasion and invasion was not affected much by Cytochalasin D (Table 5) Hence, these results conclude that different heterozygous mutants of GPI-GnT subunits affect the efficacy of invasion of *C. albicans* in both RBL MCs and MH-S MΦs. Thus invasion by RBL can be summarized as Cagpi2<BWP17=Cagpi3<Cagpi15<Cagpi1<Eri1<Cagpi19. Invasion by MH-S can be summarized as BWP17=Cagpi1=Cagpi15<Cagpi2<Cagpi3<Eri1<Cagpi19.

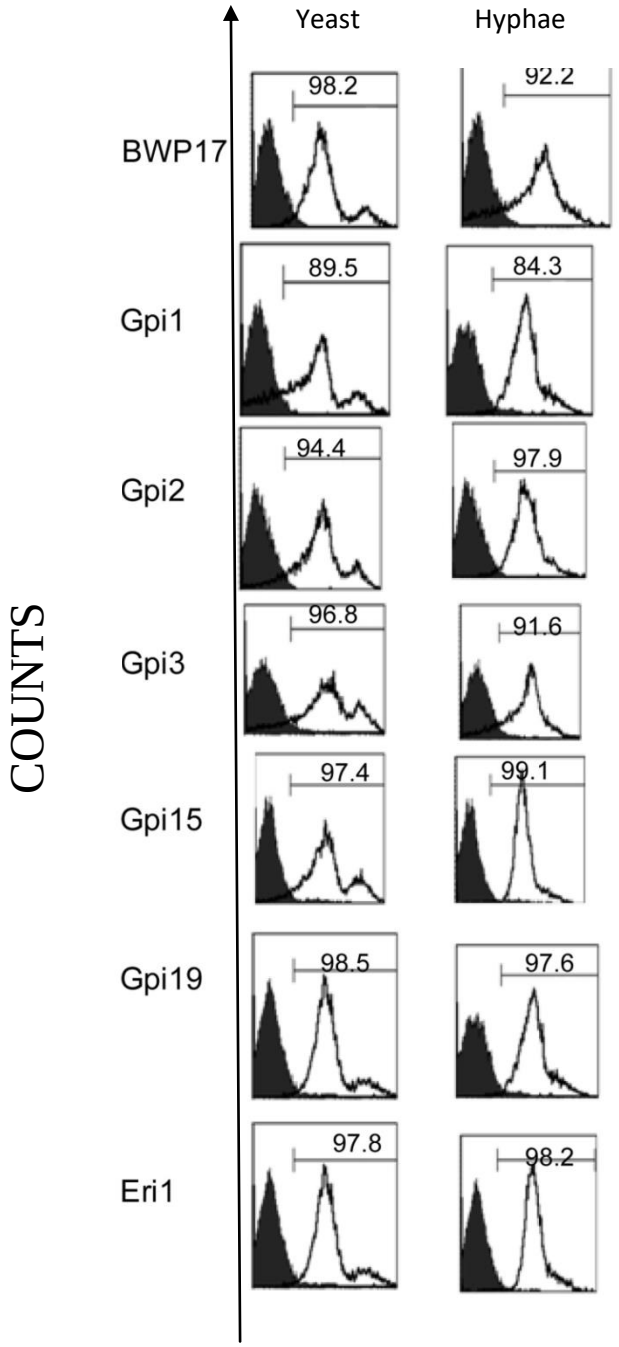

Figure 49: CFSE labelling of *C. albicans* BWP17 and its various mutants. *C. albicans* were labelled with 10 µM CFSE for 1 h at RT followed by washing with PBS. Values represent percent positive cells stained with CFSE. Grey filled histogram represent unstained whereas black line histograms represent *C. albicans* stained with CFSE

Table 4: Interaction of RBL Mast Cells and MH-S Macrophages with *C. albicans* (wild type and various mutants of GPI-GnT subunits) in yeast form at 30 mins

***C. albicans* Strain**	***C. albicans* uptake by RBL-2H3 (Percent of Total)**	***C. albicans* uptake by MH-S (Percent of Total)**	
		Control	**+Cytochalasin D**
BWP17	2.89±0.24	25.83±1.82	14.42±2.96*
Cagpi1	5.20±3.20	26.74±3.24	15.54±1.87*
Cagpi2	1.10±0.10	53.02±1.37**	36.71±3.28**
Cagpi3	2.90±1.70	19.18±2.25	13.66±2.34*
Cagpi15	2.20±1.24	22.18±3.28	12.81±1.57*
CaEri1	1.87±1.30	26.15±2.29	8.92±3.45**
Cagpi19	3.42±1.86	21.92±1.86	11.17±2.67*

*p<0.05 and **<0.005 represent statistically significant difference between firstly WT versus their mutant and secondly mutant versus respective mutant treated with Cytochalasin D by Student's t- test and ns= not significant

Table 5: Interaction of RBL Mast Cells and MH-S Macrophages with *C. albicans* (wild type and various mutants of GPI-GnT subunits) in hyphal form at 3 h

C. albicans Strain	**Invasion of RBL-2H3 by C. Albicans (Percent of RBL positive for C. albicans)**			**Invasion of MH-S by C. Albicans (Percent of MH-S positive for C. albicans)**		
	Control MOI (1:1)	Control MOI (1:5)	+Cytochalasin D MOI (1:5)	Control MOI (1:1)	Control MOI (1:5)	+Cytochalasin D MOI (1:5)
BWP17	15.42±2.12%	40.83±1.87%	39.41±2.46%^{ns}	28.64±2.52%	65.12±1.35%	65.31±1.57%^{ns}
Cagpi1	28.81±1.65%**	44.31±1.976%*	42.12±1.63%^{ns}	34.52±4.83%*	64.21±1.67%	63.22±1.62%^{ns}
Cagpi2	8.83±1.89%**	24.87±2.28%**	22.31±1.78%^{ns}	49.91±1.56%**	70.73±2.86%*	71.61±1.58%^{ns}
Cagpi3	18.61±1.97%*	40.92±2.55%	39.3±3.84%^{ns}	33.45±0.76%*	72.44±1.33%*	71.45±2.69%^{ns}
Cagpi15	22.95±2.18%*	42.96±2.67%*	43.2±1.26%^{ns}	22.72±4.64%*	66.11±2.57%	65.76±0.78%^{ns}
Eri1	31.02±2.93%**	52.43±1.84%**	53.1±3.65%^{ns}	48.21±2.53%*	85.72±1.89%**	84.31±2.82%^{ns}
Cagpi19	34.81±2.34%**	67.04±2.91%***	66.7±1.89%^{ns}	50.13±4.74%	91.21±2.02%***	90.15±2.85%^{ns}

*p<0.05, **p<0.005 and ***p<0.0005 represent statistically significant difference between firstly WT versus their mutant and secondly mutant versus respective mutant treated with Cytochalasin D by Student's t- test and ns=not significant

In order to determine what really happens to RBL Mast Cells and MH-S Macrophages on co-culture with wild type and various heterozygote mutants of GPI-GnT subunit of *C. albicans.* RBL Mast Cells and MH-S Macrophages were co-cultured with *C. albicans* (wild type and various mutants of GPI-GnT subunits) at MOI 1:1 and MOI 1:5 for 3 h at 37°C and 5% CO2. After that cells were harvested by trypsinization and recoveries of RBL MCs was calculated by counting using trypan blue exclusion method. A significant decrease in cell recovery was observed in RBL Mast Cells and MH-S Macrophages when they were co-cultured with Cagpi19 and Eri1 compared to BWP17 whereas a significant increase in cell recovery was observed in RBL Mast Cells and MH-S Macrophages when they were co-cultured with Cagpi2 and Cagpi15 (Table 6).

Table 6: Cell recovery of RBL Mast Cells and MH-S Macrophages after Co-culture with different mutants of *C. albicans*

Heterozygote mutants of subunits of GPI-GnT	**RBL Mast Cells**		**MH-S Macrophages**	
	MOI 1:1	MOI 1:5	MOI 1:1	MOI 1:5
Mammalian Cells Only	0.23±0.01	0.23±0.01	0.15±0.01	0.15±0.01
BWP17	0.15±0.02	0.11±0.01	0.1±0.02	0.08±0.02
Cagpi1	0.14±0.03	0.1±0.02	0.11±0.01	0.09±0.02
Cagpi2	0.16±0.01*	0.13±0.03*	0.12±0.03*	0.14±0.03**
Cagpi3	0.13±0.01*	0.09±0.02*	0.1±0.02	0.09±0.03
Cagpi15	0.16±0.02*	0.12±0.02*	0.11±0.02*	0.12±0.01*
Cagpi19	0.11±0.03*	0.07±0.02**	0.08±0.02*	0.05±0.01**
Eri1	0.12±0.01*	0.09±0.01*	0.09±0.03	0.07±0.01*

*p<0.05, **p<0.005 and ***p<0.0005 represent statistically significant difference between recovery pf mammalian cells co-cultured with WT versus their mutant.

Summary

This study leads to the conclusion that Mast Cells respond to different intracellular pathogens such as bacteria, parasite and fungi differentially. These differential responses have been summarized in Table 7.

Table 7: Initial interaction of Mast Cells with intracellular pathogens

Characteristics studied	***M. bovis* BCG**	***M. bovis* BCG derived antigen**	***L. tropica***	***L. donovani***	***C. albicans***
Phagocytosis	Yes	No	Yes	No	No
Macropinocytosis	ND	Yes	ND	ND	ND
Cytoskeleton dependence	Yes	Yes	Yes	NA	No
Augmentation of uptake by opsonisation	Yes	ND	ND	ND	ND
Augmentation of uptake by activation	ND	Yes	ND	ND	ND
Raft dependence of uptake	Yes	ND	ND	ND	ND
GPI anchor on pathogen dependence on uptake	NA	NA	ND	ND	Yes

ND=Not Studied and NA= Not Applicable.

Chapter-2

Role of Mast Cells In Direct Clearance of Intracellular Pathogens

As we saw in chapter 1 that MCs are involved involved in phagocytosis of BCG and *L. tropica.* They are invaded by both WT and various mutants of *C. albicans*. So our another objective was to explore whether MCs have any role in clearance of these intracellular pathogens. Basically pathogen clearance can be because of intracellular and extracellular immune responses. There are reports of pathogen clearance in MCs by phagocytosis (Malaviya et al. 1994). Similarly, extracellular responses such as degranulation and Mast Cell Extracellular Traps also affect viability of the pathogens.

Co-culture with MCs reduces the Colony Forming Units of *M. bovis* BCGSince large numbers of MCs are found in the lungs and their numbers increase on BCG infection, we wanted to check if co-culture had any role on viability of BCG. Co-culture followed by plating to determine colony forming units (cfu) led to a significant reduction in cfu. In order to explore whether direct interaction of BCG is required with MCs, we co-cultured *M. bovis* BCG with RBL Mast Cells in a transwell system. We found $11\pm2.5 \times10^6$ cfu when co-cultured with RBL-2H3 compared to $20.3\pm3.2 \times10^6$ in control whereas in transwell system there it is $17\pm1.3\times 10^6$ (Figure 59).

Mast Cell mediator release on co-culture with BCG

As MCs release various mediators on interaction with pathogens, we further co-cultured BCG with RBL Mast Cells for 1h, 3h and 6h. We found significant release of β-hexosaminidase and histamine (Figure 51). The release of these two mediators increased with time. It is interesting to see that more histamine content (% of Total) is released in comparison to β-hexosaminidase assay.This implies that it is the secretory granules which are released and not lysosomal granules. Further it was checked whether the release of various mediators that was obtained was because of cell lysis, LDH assay was done. It was found 10% - 20% LDH release occurs at 3 h and 6 h respectively (Figure 53). So at both these time points some component of secretion is attributed to cell lysis. But upto 1 h no cell lysis only degranulation takes place. By Transmission Electron Microscopy the ultrastructure of RBL-2H3 was visualized in control and infected cells with BCG. White arrows show intact granules in the control cells whereas black arrows show degranulated granules in the infected cells (Figure 53). Enlarged empty and partially filled granules are seen in the cells infected with BCG.

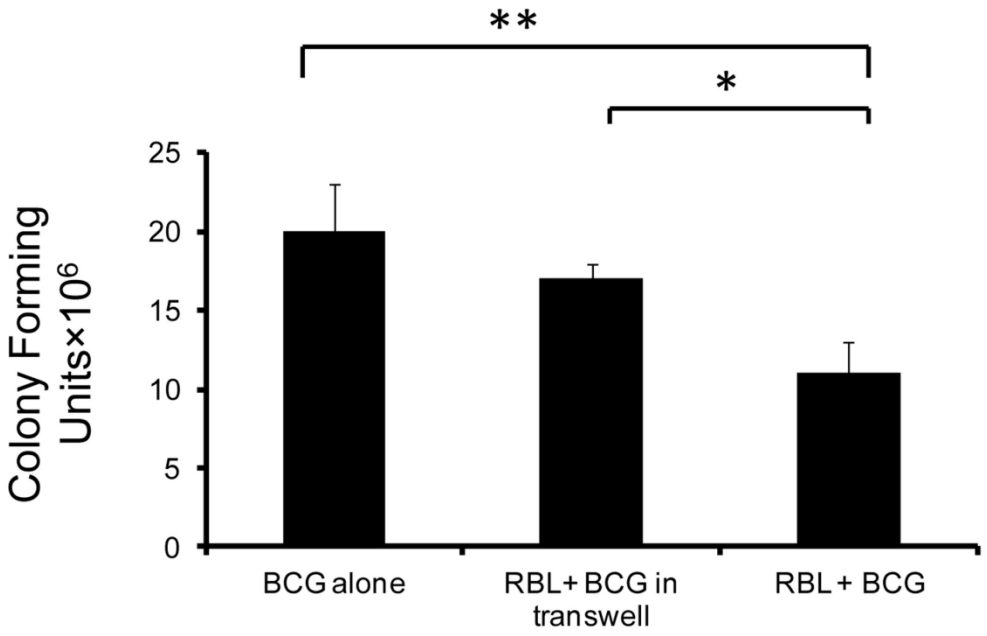

Figure 50: Effect of co-culture of RBL-2H3 on viability of BCG. 0.3×10^6 RBL-2H3 were seeded in 24 well plate overnight followed by addition of BCG at MOI (1:100) and incubating them for 24 h. Supernatant was serially diluted with PBS and plated on 7H11Agar OADC plates for 21 days at 37°C. Each data point represents mean ± SEM of values obtained from three independent assays. **$p<0.005$ and*$p<0.05$ represent statistically significant difference between control and treated samples.

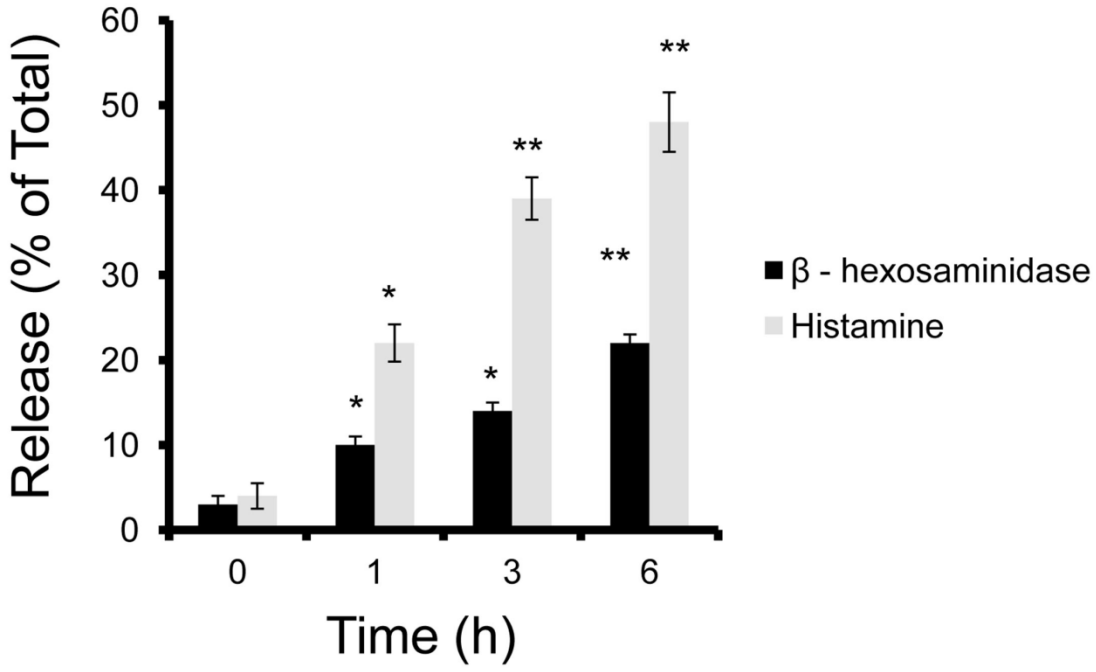

Figure 51: Response of RBL-2H3 MCs to Bacterial Challenge (*M. bovis* BCG): 0.3 million cells were co-cultured with BCG at MOI (1:100) for indicated time points and degranulation was assesed by β-Hexosaminidase assay and Histamine assay as described in Materials and Methods. Each data point represents mean ± SEM of values obtained from three independent assays. *<0.05, **P<0.005 ***<0.0005 are given by Student's t- Test.

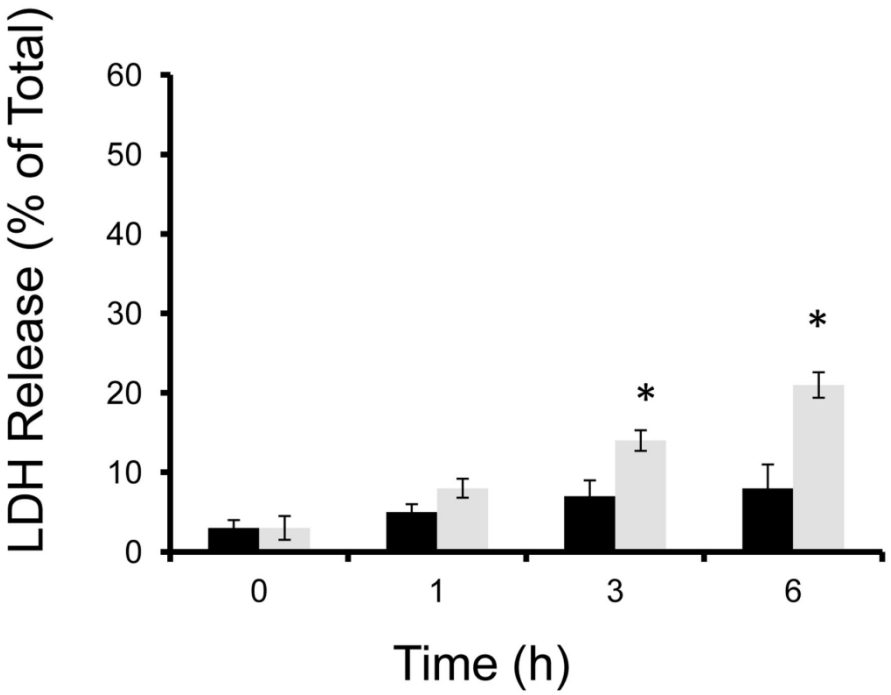

Figure 52: Cell toxicity assayed through LDH Assay. 0.3 million cells were co-cultured with BCG at MOI (1:100) for indicated time points and cell toxicity was assessed through LDH as described in Materials and Methods. Each data point represents mean ± SEM of values obtained from three independent assays. *<0.05 represent statistically significant difference between control and treated cells.

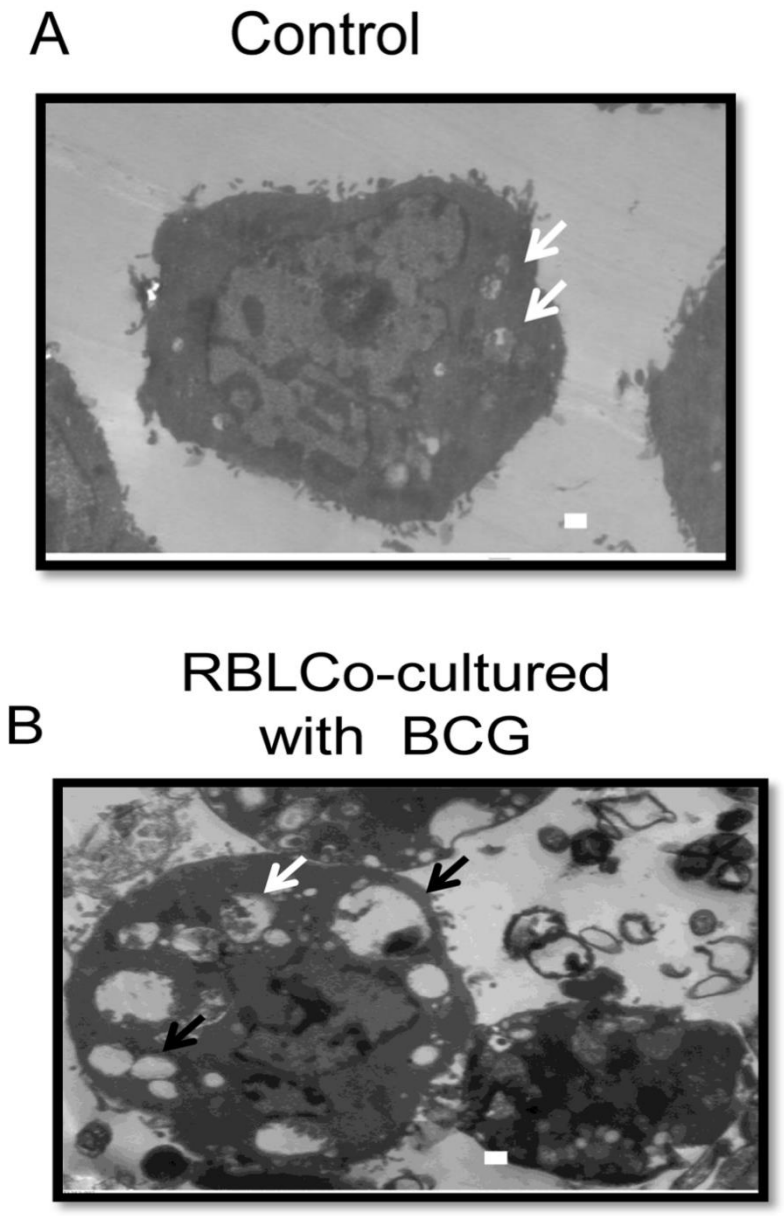

Figure 53: Effect of co-culture of BCG on ultrastructure of RBL-2H3 cells. 15 million RBL-2H3 were cultured in 225 cm^2 flask overnight and co-cultured with BCG at MOI 1:100 for 3 h followed by harvesting the cells and fixing with 2.5% glutaraldehyde and processed for Transmission Electron Microscopy. White arrows show intact granules where as black arrows show degranulated granules. Magnification is 2000X and scale bar is 500nm.

Production of ROS by MCs on interaction/co-culture with BCG

Immune Cells consume oxygen and generate ROS in response to specific stimuli especially phagocytosis of pathogens (Robinson 2008). 24h of co-culture with BCG resulted in ROS generation in RBL-2H3 as depicted by CMH2DCFDA staining (Figure 54). In untreated controls, 14.68±2.8% RBL MCs showed positive ROS generation. Co-culture of of BCG at Multiplicity of Infection (MOI) 1:10 after 24 h resulted in cells generating ROS 78.71±.3.6%(***P<0.0005, Student's t test). More than 5 fold increase has taken place in ROS generation.

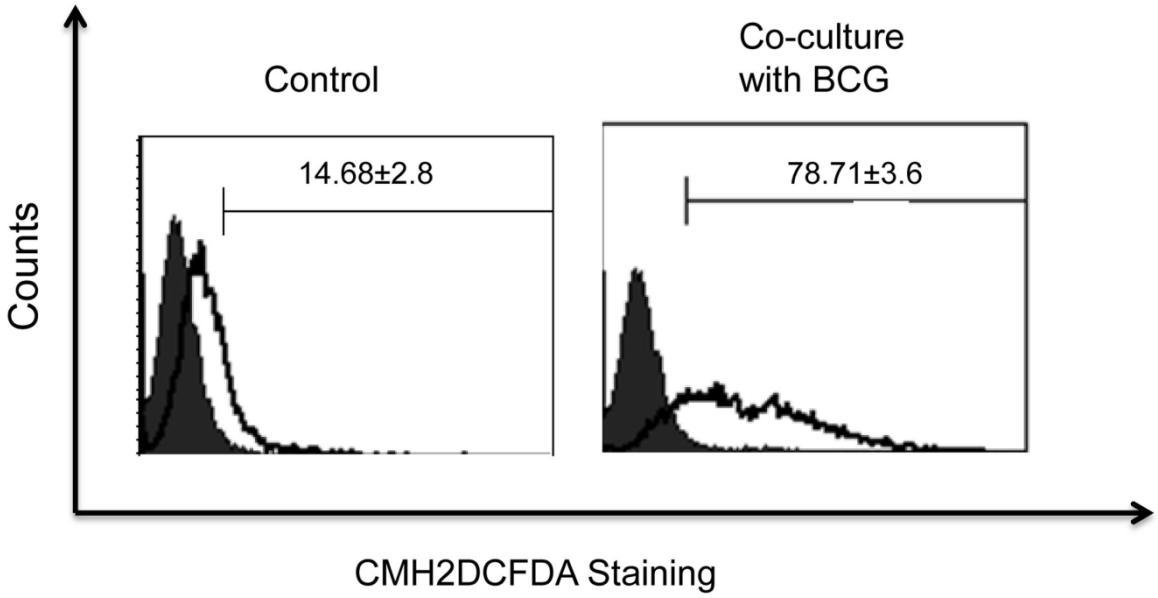

Figure 54: Generation of Reactive Oxygen Species. 0.3×10^6 MCs were seeded in 24 well cell culture plate and BCG were added at MOI 1:100 and were processed as discussed in materials and methods. Representative histograms (solid black line) in panels show CMH2DCFDA stained positive cells in comparison to unstained (filled grey). ROS generation in RBL MCs by BCG is shown. Each data point represents mean ± SEM of values obtained from three independent assays.

Role of Mast Cells in direct clearance of *Leishmnia*

Since large numbers of MCs are found in the superficial dermis (Maurer et al. 2006), where *Leishmania* is encountered after the bite of infected sand flies, they may have an important role in Leishmaniasis. To directly explore the interaction of *Leishmania* with MCs, promastigotes of *L. tropica* and *L. donovani* obtained from Indian clinical isolates were coincubated with Peritoneal Mast Cells (PMCs) and RBL MCs at 1:10 host/parasite ratio for indicated time points and their recoveries and viability were calculated. PMCs were isolated from mice BALB/c strain of mice that is susceptible to leishmaniasis (Sacks and Noben-Trauth 2002). Since MCs are the only terminally differentiated cells after haematopoiesis expressing CD45R and CD117 they can be isolated from the peritoneal lavage by sorting the cells staining with the antibodies of these receptors. The recovery was around 2% of pure MC population. The total number of cells (input) was 6 million cells whereas output was 1,20,000 cells.

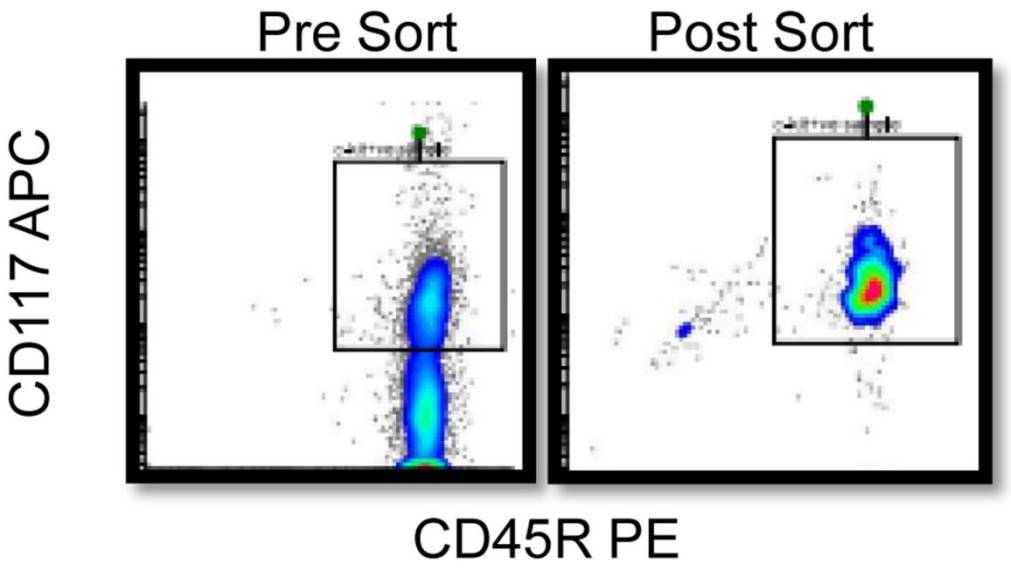

Figure 55: Isolation of PMCs from peritoneal lavage. PMCs were isolated from peritoneal lavage of female BALB/c mice and stained with CD117 APC and CD45R and sorted through BD FACS Melody Flow Sorter.

Co-culture with MCs reduces the recovery and viability of L. *tropica* (promastigotes) *in vitro.*

These PMCs were co-cultured with *L. tropica for* 24 h and viability was assessed through MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The viability has reduced to 86.44±2.5% in case of *L. tropica* (Figure 56) . This reduction in viability by MTT was significant**.** After 24 h in culture, the recoveries of viable promastigotes of *L. tropica* and when cultured alone were 89.3±1.8% (Figure 57). There was a marked decrease in the recovery of viable promastigotes of *L. tropica* (45.7±2.3% recovery) when cocultured with RBL MCs. Further to confirm that the death of promastigotes is because of direct contact with MCs and not due to some soluble mediator released by MCs, the respective promastigotes and MCs were co-cultured in transwell system and recoveries of viable promastigotes calculated..But recovery of viable promastigotes of *L. tropica* with MCs in the transwell system increased to 81.24±1.2%. The recovery of *L. tropica* has significantly reduced from 89.3±1.8% of parasite alone in transwell system to 81.24±1.2% of the parasite along with MCs in non transwell system**.** As promastigotes of *Leishmania* showed reduced viability on co-culture with MCs for 24 hour, Phosphatidylserine (PS) exposure on the external leaflet of the plasma membrane of promastigotes was checked to look for signs of death by apoptosis. After 24 h of coculture with RBL MCs, 80.8±2.4% of promastigotes of *L. tropica* confirming PS externalization by Annexin V staining (Figure 58).

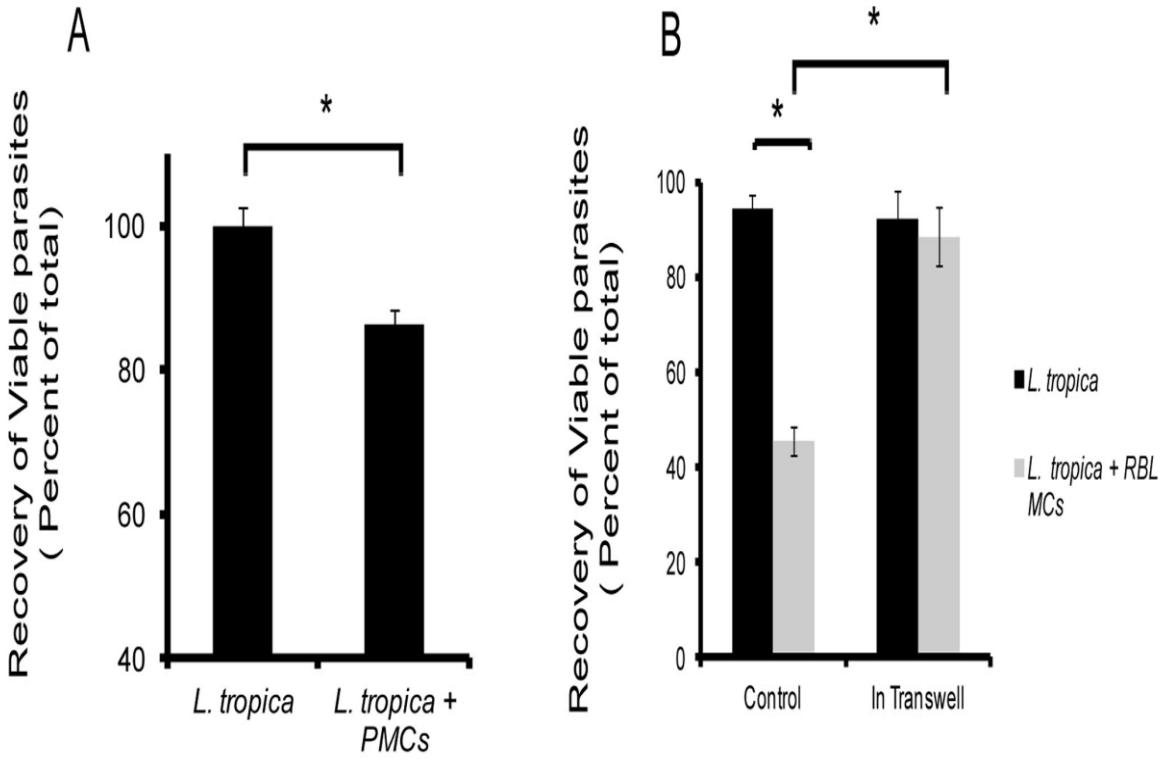

Figure 56: Cell death of *L. tropica* on co-culture with Mast Cells. (A) PMCs were co-cultured with *L. tropica in* 96 well plate for 24 h at MOI (1:10). MTT assay was done and The Y-axis represents the relative amount of viable cells after normalization to the *Leishmania control*. (B) 0.1×10^6 RBL MCs were seeded in 48 well cell culture plate and cultured overnight in CO_2 incubator. *L. tropica* were added at MOI 1:10. After indicated time points parasites were removed cell viability was counted using trypan blue exclusion method. Each point represents mean ± SEM of values obtained from three independent assays.*$p<0.05$ represent statistical significance by Mann Whitney u test.

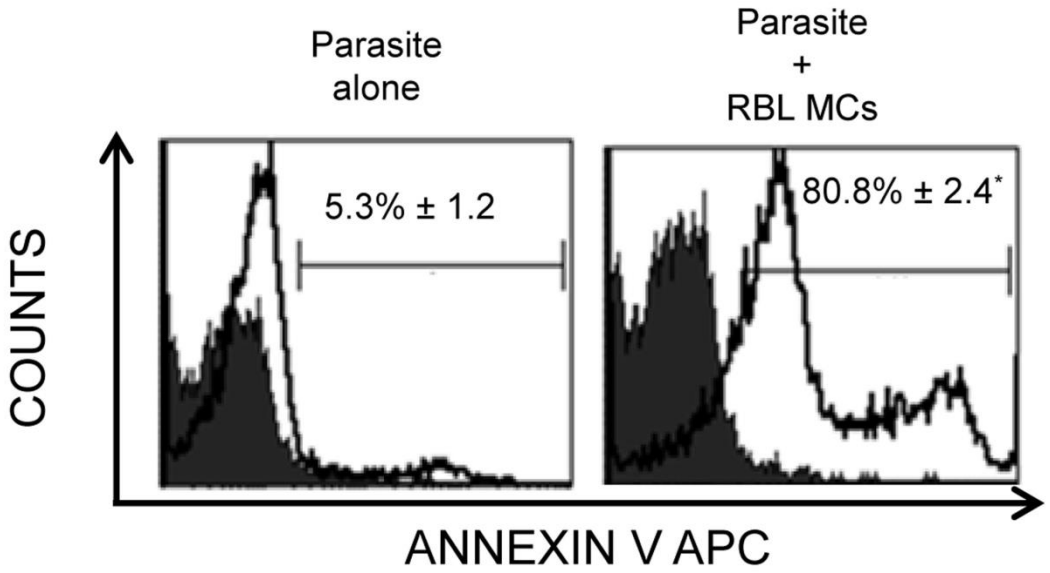

Figure 57: Annexin V staining of *L. tropica* after co-culture with RBL Mast Cells. 0.1 million cells were seeded in 48 well cell culture plate. *L. tropica* were syringe separated and added at MOI 1:10 for 24 h. Parasites were harvested washed followed by adding 50µl of Annexin Binding Buffer and were processed as mentioned in materials and method. Representative histograms (solid black line) in panels show Annexin APC positive cells in comparison to unstained (filled grey). Each point represents mean ± SEM of values obtained from three independent assays. .*$p<0.05$ represent statistical significance by Mann Whitney u test.

Generation of Reactive Oxygen Species (ROS) in response to direct interaction with promastigotes of *L. tropica* with Mast Cells

There are reports of killing of *Leishmania* by ROS (Channon and Blackwell 1985, Murray 1982, Pearson et al. 1983, Zarley, Britigan and Wilson 1991). Since we observed cell death in promastigotes we further checked whether ROS are generated in MCs upon co-culture with the promastigotes. ROS generation in control and *Leishmania* co-cultured RBL MCs were estimated by staining MCs with CMH2DCFDA. In untreated controls, 4.33+1.2% RBL MCs showed positive ROS generation. Co-culture of *L. tropica* at Multiplicity of Infection (MOI) 1:10 after 24 h resulted in 17.15%±2.4% cells generating ROS whereas using transwell system ROS generation was 8.79%±2.2% (Figure 58).To further determine the functional significance of ROS production catalase was used. Catalase converts hydrogen peroxide into H_2O and O_2. Previously it has been reported that the presence of exogenous catalases reduced NET formation in response to PMA activation (Fuchs et al. 2007). So MCs were cocultured with *L. tropica* in 96 well cell culture plate in the presence or absence of catalase. By MTT assay the absorbance at 595 nm of *L. tropica* co-cultured without or with MCs was 0.48±0.03 and 0.12±0.01 respectively as shown in figure 3b. Absorbance at 595 nm of *L. tropica* and when co-cultured with RBL MCs in the presence of catalase was around 0.34±0.015 (Figure 59 A). Similar studies were also carried out with PMCs The absorbance at 595 nm of *L. tropica* co-cultured without or with PMCs was around 0.51±0.01 and 0.59±0.014respectively (Figure 59 B). Absorbance at 595 nm of *L. tropica* when co-cultured with PMCs in the presence of catalase was around 0.55±0.01. This increase in OD units in *L. tropica* is statistically significant. The PMC results also validate the results obtained from RBL.

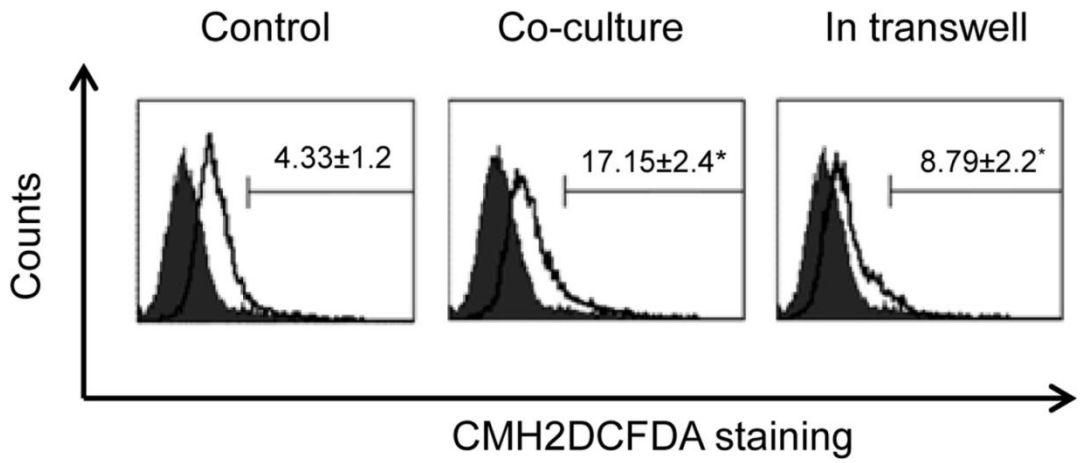

Figure 58: Generation of ROS by RBL MCs on co-culture of *L. tropica.* 0.3×10^6 MCs were seeded in 24 well cell culture plate and *L. tropica* were added at MOI 1:10 and were processed as discussed in materials and methods. Representative histograms (solid black line) in panels show CMH2DCFDA stained positive cells in comparison to unstained (filled grey). ROS generation in RBL MCs by *Leishmania* is shown. Each point represents mean ± SEM of values obtained from three independent assays.*$p<0.05$ represent statistical significance by Mann Whitney u test.

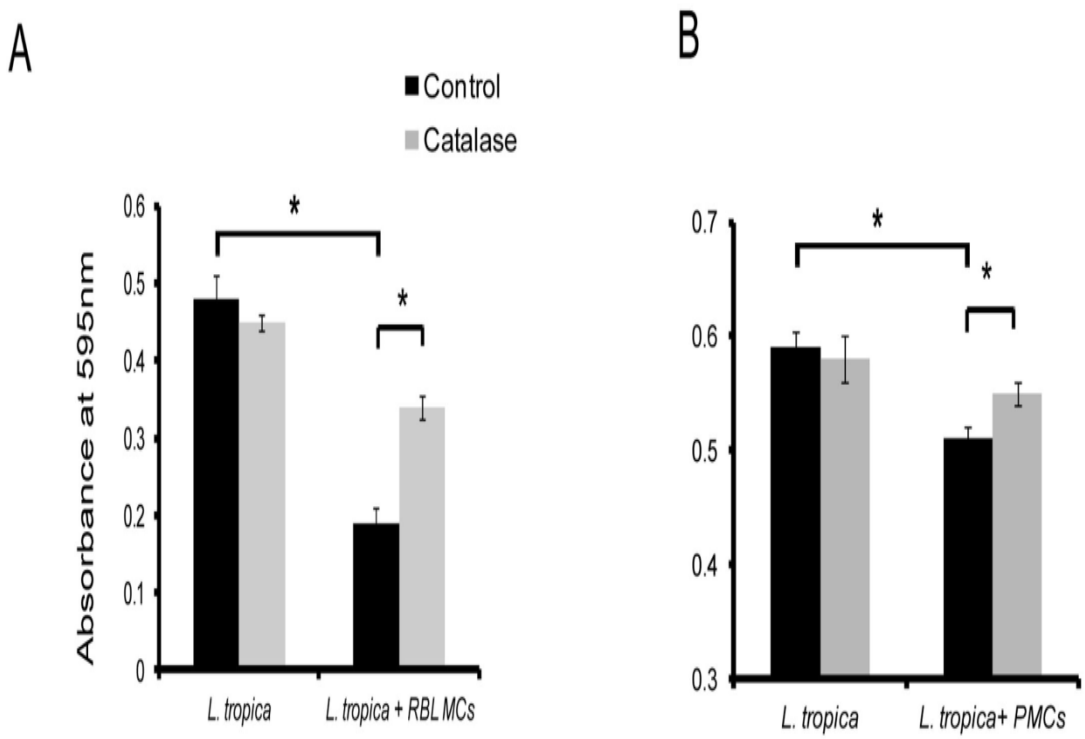

Figure 59: Effect of catalase treatment on the viability of *L- tropica* when co-cultured with RBL Mast Cells. RBL MCs (1×10^4 cells/well) in 100µl medium were cultured in a 96-well plate at 37 °C, and cocultured with *L. tropica* alone as well as with catalase for 24 h. Cells treated with medium only served as a negative control group. After removing the supernatant of each well and transferring to another 96-well plate 20 µl of MTT solution was then introduced and then processed as discussed in materials and methods. Panel b and c represent effect of catalase on viability of *L. tropica* , n=3. PMCs were cocultured with *L. tropica* alone as well as with catalase for 24 h. MTT assay was done to assess the viability of *L. tropica*. Each point represents mean ± SEM of values obtained from three independent assays.*$p<0.05$ represent statistical significance by Mann Whitney u test.

Mediator release from MCs in response to direct interaction with promastigotes of *L. tropica*

There are reports of release of MC mediators upon interaction with few species of *Leishmania* (Bidri et al. 1997), we checked whether the mediator release can be a reason of killing of promastigotes. We checked the release of β-hexosaminidase, as an indicator of MC mediator release on co-culture of both the species. We found low but statistically significant release of β-hexosaminidase by MCs on co-culture with *L. tropica* compared to resting MCs alone (Figure 60).

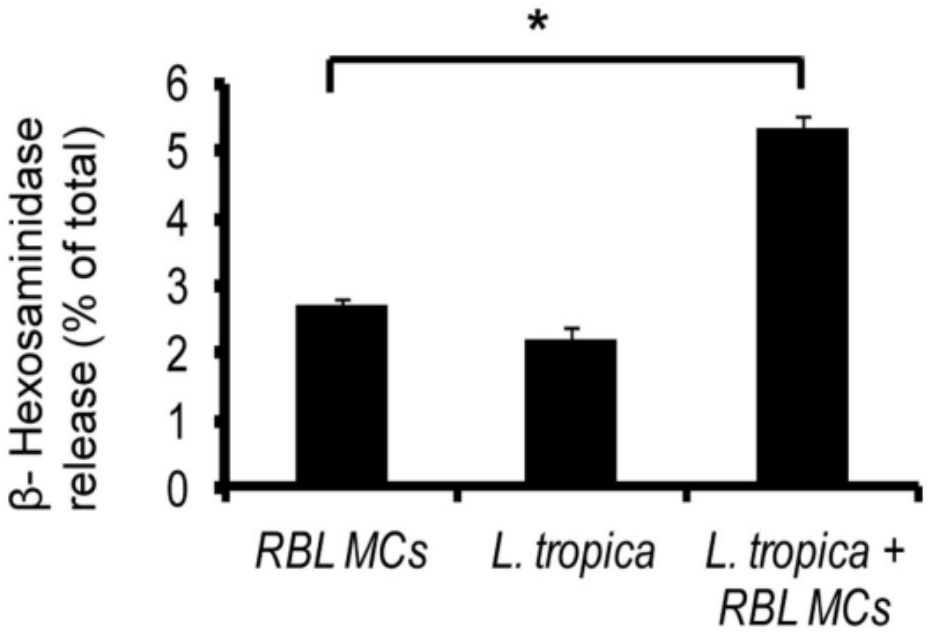

Figure 60: Release of β-hexosaminidase by RBL Mast Cells on co-culture with *Leishmania tropica.* 0.1× 10^6 cells suspended in 1.0 ml medium were co-cultured with different MOI *Leishmania tropica* for 1h and 3h at 37°C. Supernatants were collected and lysates were prepared by treating the adherent cells with 0.2% Triton X. The release of β-hexosaminidase in the cell supernatant was assayed as described in materials and methods. Each point represents mean ± SEM of values obtained from five independent assays. *$p<0.05$ represent statistically significant difference between control and treated cells by Mann Whitney u test.

Co-culture with MCs reduces the recovery and viability of *L. donovani* (promastigotes) *in vitro.*

PMCs were co-cultured wit *L. donovani* for 24 h and viability which was done through MTT assay has reduced to 91±1% in case of *L. donovani* (Figure 61 A). This reduction in viability by MTT was significant. After 24 h in culture, the recoveries of viable promastigotes *L. donovani*, when cultured alone was 93.3±2.6% (Figure 61 B). There was a small but significant decrease in the recovery of viable promastigotes of *L. donovani* (82.3±2.5% recovery) in presence of MCs (Figure 61 B). Further to confirm that the death of promastigotes is because of direct contact with MCs and not due to some soluble mediator released by MCs, promastigotes and MCs were co-cultured in transwell system and recoveries of viable promastigotes calculated. Recoveries of promastigotes alone in the transwells were similar as before *L. donovani* was 91.2±2.4% Also, the recovery of viable promastigotes of *L. donovani* cocultured with MCs in the transwell system was same as recovery of *L. donovani* alone. Hence, *L. donovani* showed better recoveries when co-cultured with MCs in the transwell system (Figure 61 B). As promastigotes of *Leishmania* showed reduced viability on co-culture with MCs for 24 hour, Phosphatidylserine (PS) exposure on the external leaflet of the plasma membrane of promastigotes was checked to look for signs of death by apoptosis. After 24 h of coculture with RBL MCs, 25.2±1.6% of promastigotes of *L. donovani* showed binding to Annexin V, confirming PS externalization (Figure 62).

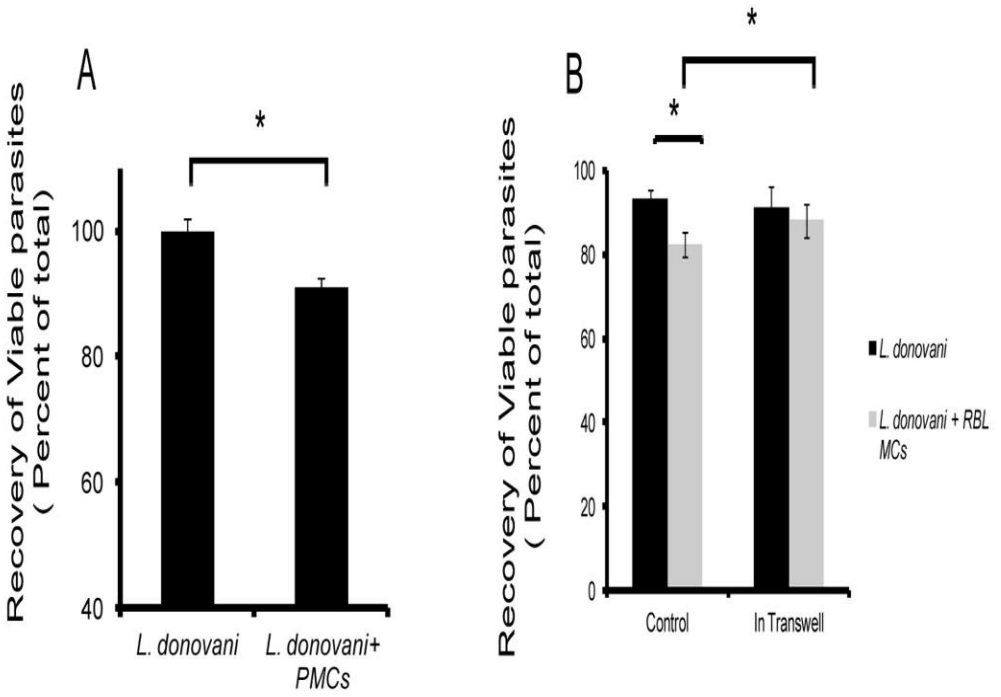

Figure 61: Cell death of *L. donovani* on co-culture with Mast Cells. (A) PMCs were co-cultured with *L. tropica in* 96 well plate for 24 h at MOI (1:10). MTT assay was done and The Y-axis represents the relative amount of viable cells after normalization to the *Leishmania control*. (B) 0.1×10^6 RBL MCs were seeded in 48 well cell culture plate and cultured overnight in CO_2 incubator. *L. tropica* were added at MOI 1:10. After indicated time points parasites were removed cell viability was counted using trypan blue exclusion method. Each point represents mean ± SEM of values obtained from three independent assays. *$p<0.05$ represent statistically significant difference between control and treated cells by Mann Whitney u test.

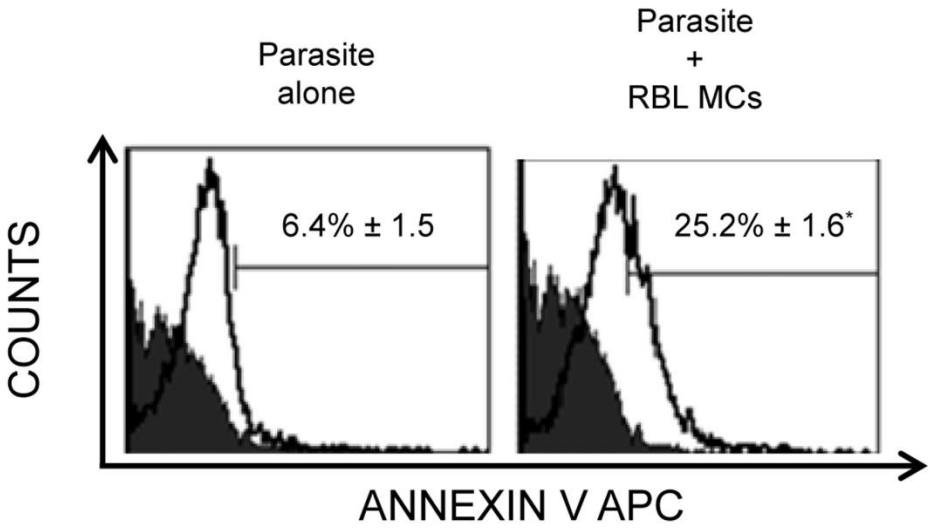

Figure 62: Annexin V staining of *L.donovani* after co-culture with RBL Mast Cells. 0.1 million cells were seeded in 48 well cell culture plate. *L. donovani* were syringe separated and added at MOI 1:10 for 24 h. Parasites were harvested washed followed by adding 50µl of Annexin Binding Buffer and were processed as mentioned in materials and method. Representative histograms (solid black line) in panels show Annexin APC positive cells in comparison to unstained (filled grey). Each point represents mean ± SEM of values obtained from three independent assays. . *$p<0.05$ represent statistically significant difference between control and treated cells by Mann Whitney u test.

Generation of Reactive Oxygen Species (ROS) and mediator release from MCs in response to direct interaction with promastigotes of *L. donovani*.

Since we observed cell death in promastigotes we further checked whether ROS are generated in MCs upon co-culture with the promastigotes. ROS generation in control and *Leishmania* co-cultured RBL MCs were estimated by staining MCs with CMH2DCFDA. In untreated controls, 4.33+1.2% RBL MCs showed positive ROS generation. We found statistically more significant ROS generation in MCs upon co-culture with *L. donovani* compared to their controls (Figure 63). Co-culture of *L. donovani* at MOI 1:10 after 24 h resulted in 26.43%±1.8% MCs generating ROS whereas using transwell system ROS generation was 4.16%±3.6%. To further determine the functional significance of ROS production catalase was used. Catalase converts hydrogen peroxide into H_2O and O_2. Previously it has been reported that the presence of exogenous catalases reduced NET formation in response to PMA activation (Fuchs et al. 2007). So MCs were cocultured with *L. donovani* in 96 well cell culture plate in the presence or absence of catalase. Similarly, absorbance at 595 nm of *L. donovani* co-cultured with or without MCs was 0.45±0.02 and 0.23±0.025 respectively (Figure 64 A). Absorbance at 595 nm of *L. donovani* when co-cultured with MCs in the presence of catalase was around 0.33±0.023. This increase in OD units in *L. donovani* is statistically significant (Figure 64 A). Similar studies were also carried out with PMCs. Similarly, absorbance at 595 nm of *L. donovani* co-cultured with or without PMCs was around 0.52±0.007 and 0.57±0.016 respectively (Figure 64 B). Absorbance at 595 nm of *L. donovani* when co-cultured with PMCs in the presence of catalase was around 0.55±0.012. This increase in OD units in *L. donovani* is statistically significant (figure 64 B). The PMC results also validate the results obtained from RBL.

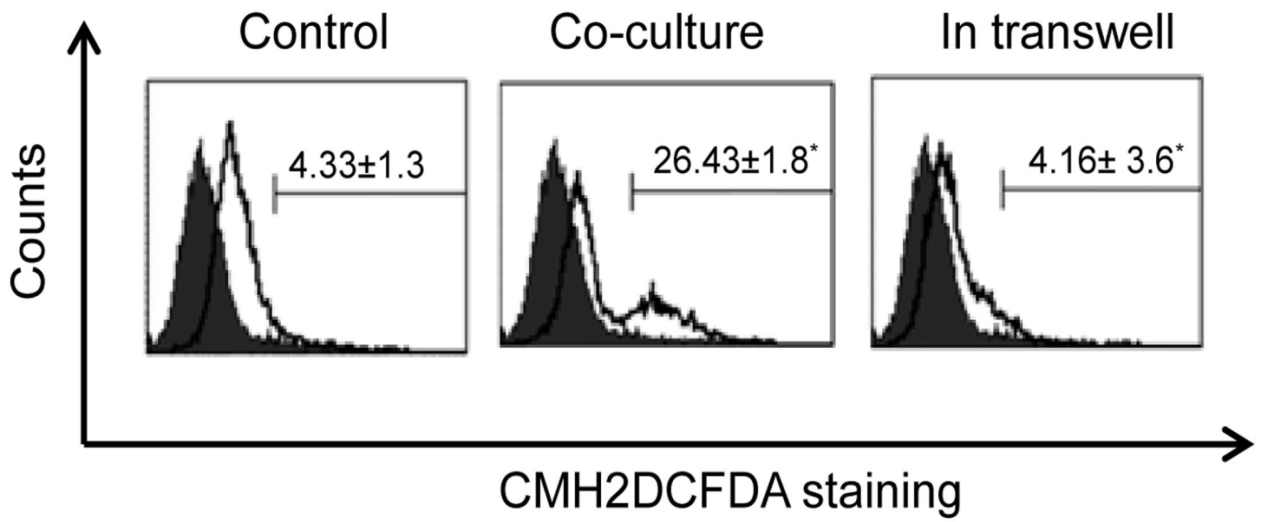

Figure 63: Generation of ROS by RBL MCs on co-culture of *L. donovani*. 0.3 × 10^6 MCs were seeded in 24 well cell culture plate and *L. donovani* were added at MOI 1:10 and were processed as discussed in materials and methods. Representative histograms (solid black line) in panels show CMH2DCFDA stained positive cells in comparison to unstained (filled grey). Each point represents mean ± SEM of values obtained from three independent assays. *$p<0.05$ represent statistically significant difference between control and treated cells by Mann Whitney u test.

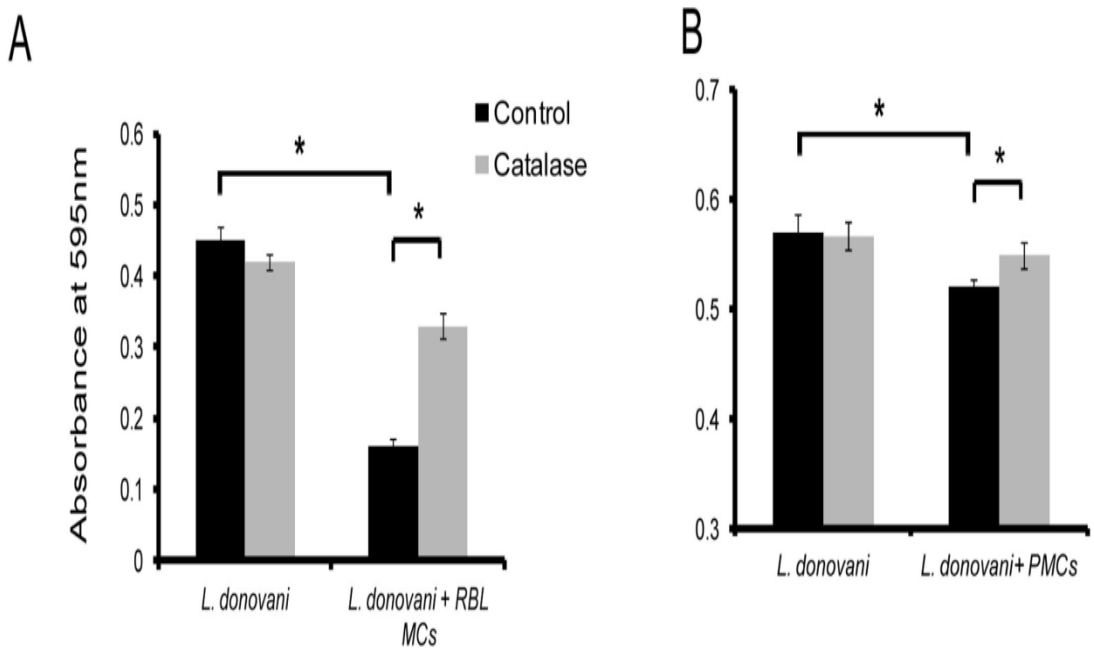

Figure 64: Effect of catalase treatment on the viability of *L. donovani* when co-cultured with RBL mast cells. RBL MCs (1×10^4 cells/well) in 100µl medium were cultured in a 96-well plate at 37 °C, and cocultured with *L. donovani* alone as well as with catalase for 24 h. Cells treated with medium only served as a negative control group. After removing the supernatant of each well and transferring to another 96-well plate 20 µl of MTT solution was then introduced and then processed as discussed in materials and methods. Panel b and c represent effect of catalase on viability of *L. donovani*, n=3. PMCs were cocultured with *L. tropica* alone as well as with catalase for 24 h. MTT assay was done to assess the viability of *L. donovani*

Mediator release from Mast Cells in response to direct interaction with promastigotes *L. donovani*

Since there are also reports of release of MC mediators upon interaction with few species of *Leishmania* (Bidri et al. 1997), we checked whether the mediator release can be a reason of killing of promastigotes. We checked the release of β-hexosaminidase, as an indicator of MC mediator release on co-culture of *L. donovani*. We found low but statistically significant release of β-hexosaminidase by MCs on co-culture with *L. donovani* compared to resting MCs alone (Figure 65).

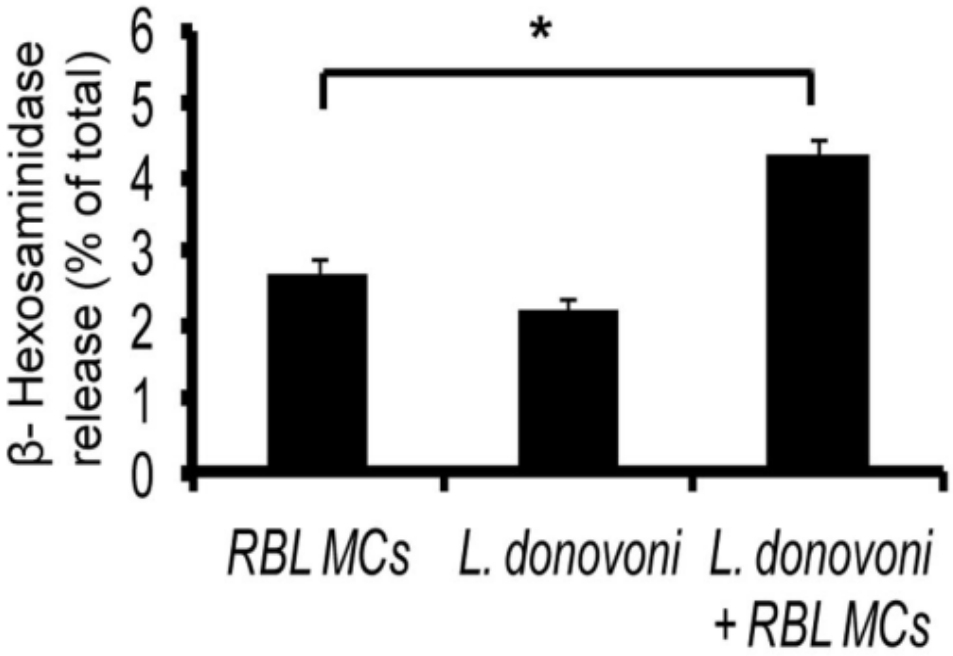

Figure 65: Release of β-hexosaminidase by RBL Mast Cells on co-culture with *Leishmania donovani.* 0.1×10^6 cells suspended in 1.0 ml medium were co-cultured with different MOI *Leishmania donovani* for 1h and 3h at 37°C. Supernatants were collected and lysates were prepared by treating the adherent cells with 0.2% Triton X. The release of β-hexosaminidase in the cell supernatant was assayed as described in materials and methods. Each point represents mean ± SEM of values obtained from five independent assays. *$p \leq 0.05$ represent statistically significant difference between control and treated cells by Mann Whitney test.

Co-culture with MCs reduces the Colony Forming Units of *C. albicans*

As we found that *C. albicans* and their GPI-GnT mutants were able to invade RBL Mast Cells, we wanted to check if the co-culture had any effect on viability of *C. albicans.* To accomplish this co-culture followed by plating was done to determine CFU. As it was also confirmed that the yeast form of *C. albicans* were phagocytosed by Macrophages and the hyphal form were invaded by RBL Mast Cells, CFU assay was done. Table 8 shows CFU of Wild Type as well as various GPI-GnT mutants of *C. albicans* on coculture with RBL Mast Cells and MH-S Macrophages. The CFU of CaGpi2 on co-culture with RBL Mast Cells is 0.25±0.04 compared to BWP17 which is 0.52±0.02 (Figure 66). This reduction in CFU is statistically significant, $p<0.005$, Student's t test. The CFU of CaGpi2 on co-culture with MH-S Macrophages is 0.23±0.02 compared to BWP17 which is 0.52±0.02 (Table 8). This reduction in CFU is statistically significant, $p<0.005$, Student's t test.

In case of RBL Mast Cells the decrease in cfu of *C. albicans* can be represented as

Cagpi19 = Eri 1< Cagpi2 < Cagpi1=Cagpi3<BWP17<Cagpi15

In case of MH-S the decrease in cfu of *C. albicans* can be represented as

Cagpi2 < Cagpi1 < Cagpi15 < Cagpi19 < Eri1 = BWP17

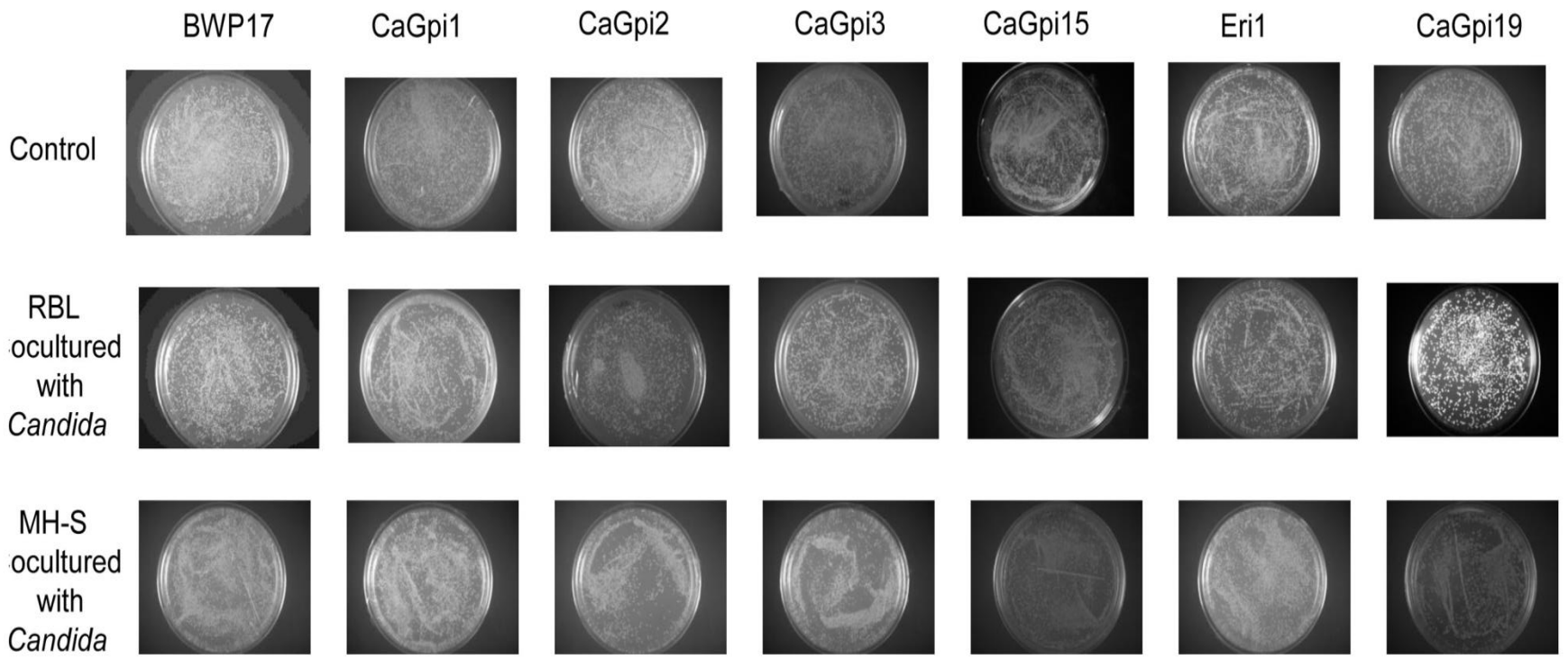

Figure 66: Mammalian cell mediated *C. albican* killing. To determine the extent of killing and/or phagocytosis, 0.1 million RBL-2H3 and MH-S cells were seeded in 48 well cell culture plate and kept for adherence followed by addition of *C. albicans* at MOI 1:5 for 18h followed by lysing the mammalian cells using 0.2% Triton-X-100 (Sigma, MO, USA) and plating on the Yeast Extract Peptone Dextrose (YPD) plates and kept at 30°C for 24h. The number of *C. albicans* was determined as CFU/ml. The formula used is cfu/ml = (no. of colonies x dilution factor) / volume of culture plate

Table 8: Virulence study of various mutants

Different Mutants	***C. albicans*** **CFU × 10^6**	**RBL +** ***C. albicans*** **CFU × 10^6**	**MH-S +** ***C. albicans*** **CFU × 10^6**
BWP17	0.45 ± 0.02	0.35 ± 0.06*	0.40 ± 0.03*
Cagpi1	0.49 ± 0.03	0.30 ± 0.01*	0.32 ± 0.04*
Cagpi2	0.52 ± 0.02	0.25 ± 0.04**	0.23 ± 0.02**
Cagpi3	0.41 ± 0.07	0.30 ± 0.03*	0.33 ± 0.03*
Cagpi15	0.48 ± 0.08	0.40 ± 0.03*	0.32 ± 0.02*
Cagpi19	0.42 ± 0.05	0.24 ± 0.01**	0.35 ± 0.03*
Eri1	0.46 ± 0.07	0.24 ± 0.04**	0.4 ± 0.01*

Summary

This study leads to the conclusion that Mast Cells respond to clearance of different intracellular pathogens such as bacteria, parasite and fungi differentially. These differential responses have been summarized in Table 9.

Table 9: Role of Mast Cells in Direct Clearance of Intracellular Pathogens

Characteristics studied	***M. bovis* BCG**	***L. tropica***	***L. donovani***	***C. albicans***
Clearance	Yes	Yes	Yes	Yes
Degranulation	Yes	Yes	Yes	ND
GPI anchor on pathogen dependence clearance	NA	ND	ND	Yes

Chapter-3

Role of Mast Cell Extracellular Traps in Clearance of Intracellular Pathogens

Another mechanism of extracellular killing of pathogens by immune cells is by formation of extracellular traps (ETs) (Brinkmann et al. 2004). Mast Cells (MCs) on interaction with bacteria such as *Streptococcus pyogens*, *Staphylococcus pneumonia, Pseudomonas aeroginosa* (von Kockritz-Blickwede et al. 2008), *Enterococcus faecalis* (Scheb-Wetzel et al. 2014), *Listeria monocytogens* (Campillo-Navarro et al. 2017) and fungus such as *Candida albicans* (Lopes et al. 2015) release Mast Cell Extracellular Traps (MCETs). There is also one report of MCETs formation in psoriasis (Lin et al. 2011). MCETs were found to be responsible for the extracellular killing of clumped bacteria that were not efficiently phagocytosed. Those MCET fibers were composed of DNA, histones, the MCs specific protease tryptase, and AMPs such as the cathelicidin AMP LL-37 (von Kockritz-Blickwede et al. 2008). So it was investigated whether such phenomenon occurs when Mast Cells were co-cultured with intracellular pathogens.

Mast cell death by Etosis and release of Mast Cell Extracellular Traps on interaction with *M. bovis* BCG.

As it has already been discussed that Mast Cells are involved in extracellular trap formation, it was checked whether the MCs die on co-culture with *M. bovis* BCG. The recovery was unaffected at MOI 1:1 and MOI 1:10 till 24 h. It was found in control cells which were not co-cultured with *M. bovis* BCG the cell recovery was around $0.47\times10^{6}\pm0.001$ whereas after 24 h at MOI 1:100 the cell recovery was around 0.18×10^{6} ±0.005. So cell recovery was reduced by about 62% (Figure 67). So it was then investigated the reason behind the cell death. Firstly it was tested whether the cells die due to apoptosis or necrosis. So staining was done through Annexin V and 7AAD. It was found that RBL Mast Cells co-cultured with BCG for 12 h were 11.53±2.6% Annexin V positive compared to 4.13±1.8% in control which is statistically significant. Similarly, it was found that RBL Mast Cells co-cultured with BCG for 24 h were 14.40±21% Annexin V positive compared to 4.74±1.2% in control which is statistically significant (Figure 68). So there is some other mechanism of cell death and it may be etosis.

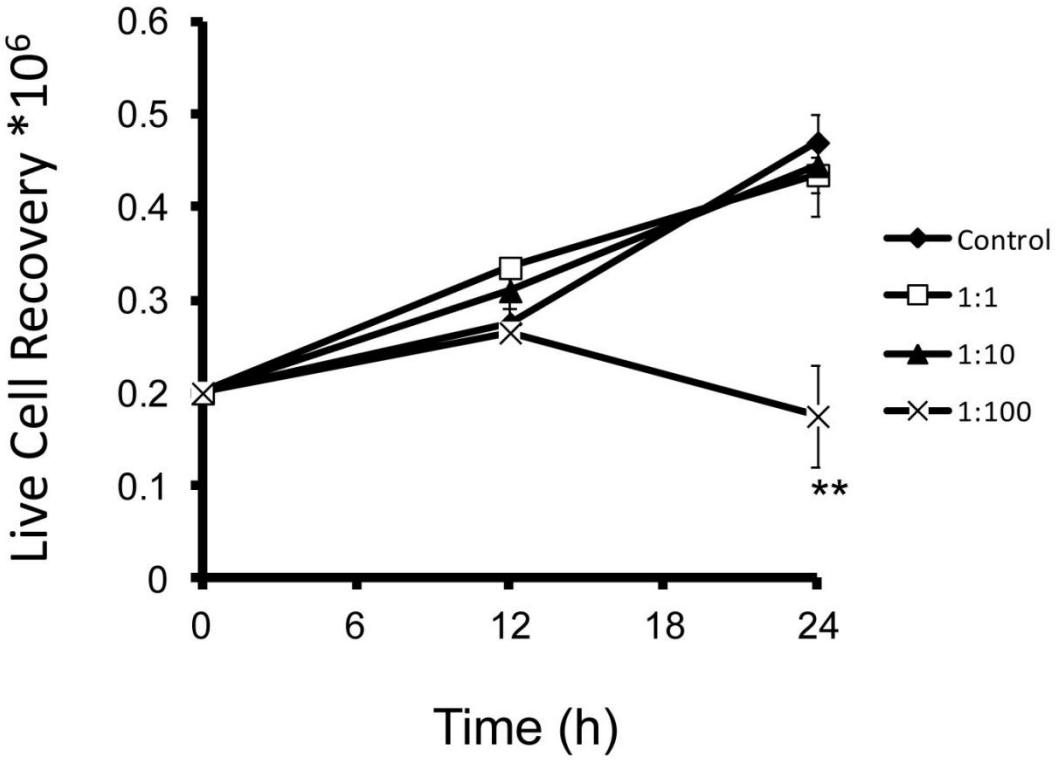

Figure 67: Live cell recovery of RBL Mast Cells on co-culture with BCG. Recovery of RBL-2H3 and MH-S cells after BCG infection: RBL-2H3 cells were cultured in a 48-well culture plates for overnight. BCG was added at different MOI as indicated and incubated for different time points. Recoveries of viable cell numbers were assessed by trypan blue exclusion method using a hemocytometer. Each data point represents the mean ± SEM of results obtained from three independent assays. *p≤0.05, **≤0.005, ***p≤0.0005 represent statistically significant difference between recoveries of control and infected cells

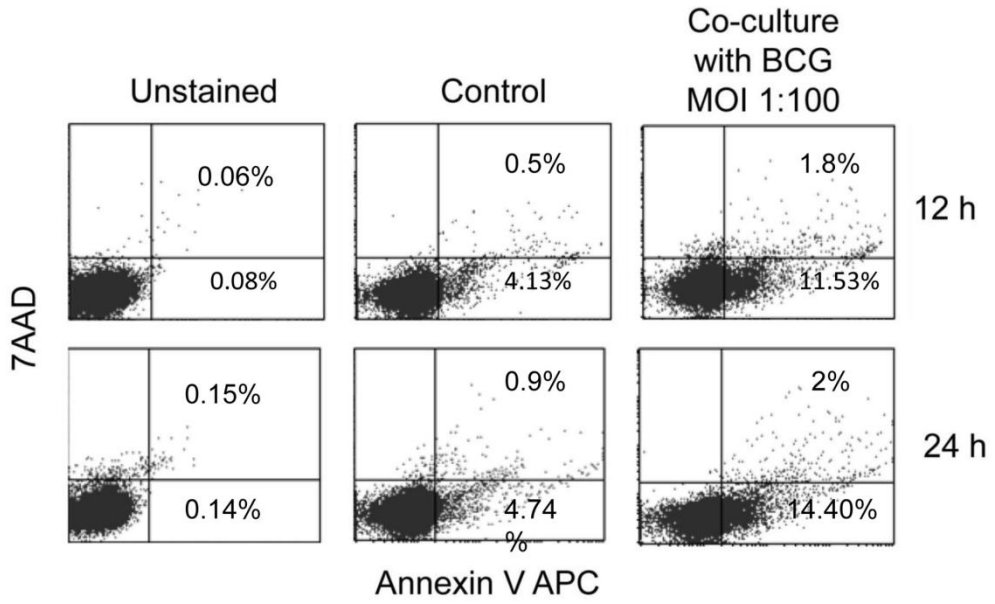

Figure 68: Effect of co-culture of BCG on viability of RBL Mast Cells. 0.1 million RBL cells were seeded in 48 well plate and kept in the incubator for overnight. After 24 h, cells were stained with 7AAD and Annexin V and analyzed by flow cytometry. Each data point represents the mean ± SEM of results obtained from three independent assays. *p≤0.05, **≤0.005, ***p≤0.0005 represent statistically significant difference between control and infected cells.

So in order to investigate whether cell death of RBL Mast Cells is due to etosis, RBL Mast Cells were co-cultured with *M. bovis* BCG for 24 h followed by fixing with 2.5% glutaraldehyde and then processed for Scanning Electron Microscopy. Figure 69) shows no extension formation or release of DNA by non-co-cultured MCs, whereas in RBL Mast Cells co-cultured with *M. bovis* BCG some extensions of around 0.1µm diameter were seen. These extensions may be DNA. Images in row 2 are 10000X magnified images and images in row 1 are 1000X magnified images (at least 10 images were visualized in each case). *M. bovis* BCG is not seen entrapped in these structures.

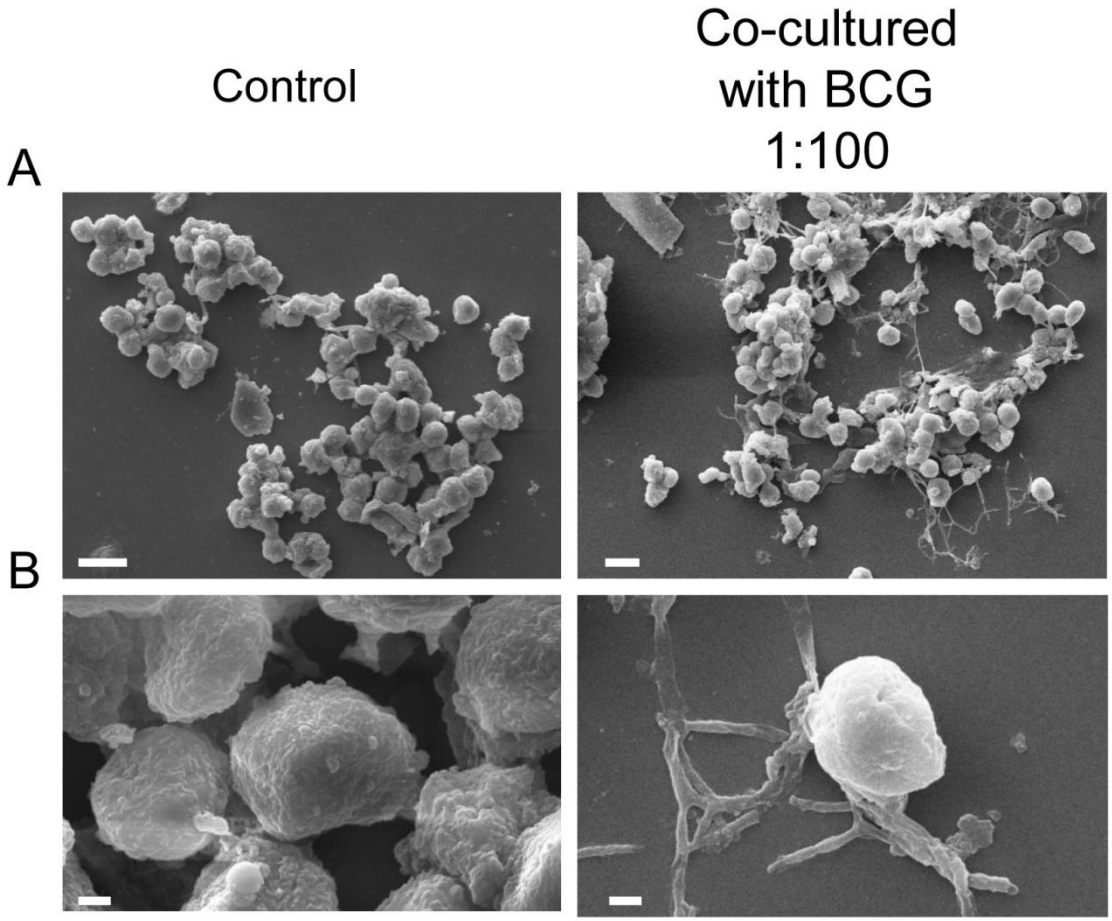

Figure 69: Visualization of Mast Cell Extracellular Traps by Scanning Electron Microscopy: 2.5× 10^6 MCs were seeded in 6 well plate for overnight. *M. bovis* BCG were syringe separated and were added at MOI 1:100. After 24 h, bacteria were removed and the cells were harvested followed by fixing with 2% glutaraldehyde for 1h and were processed for Scanning Electron Microscope Zeiss EV040 in Advanced Instrumentation Research Facility. Magnification in panel A is 1000X and scale bar is 10 µm and magnification in panel B is 10000X and scale bar is 2 µm.

Visualization of mast cell extracellular traps containing histones and tryptase in response to *M. bovis* BCG

There are reports that MCs undergo etosis and form extracellular traps called Mast Cell Extracellular Traps (MCETs), which comprise of DNA along with histones and tryptase (Campillo-Navarro et al. 2017, Lin et al. 2011, Lopes et al. 2015, Scheb-Wetzel et al. 2014, von Kockritz-Blickwede et al. 2008). RBL MCs were seeded on cover slides and co-cultured with CFSE labelled *M. bovis* BCG for 24 h, thereafter DNA was visualized by DAPI staining, tryptase and histones were stained with fluorescently tagged specific antibodies. As is seen in (Figure 70) blue coloured DAPI stained DNA with red coloured histones are visible in the nucleus as well as in the extracellular regions. Green coloured *M. bovis* BCG are seen trapped in the DAPI stained chromatin along with red coloured histone. Figure shows red coloured tryptase and green coloured histone with extracellular DNA which is blue coloured on co-culture with *M. bovis* BCG which can be seen in DIC by arrow. The nucleus of RBL Mast Cells has lost their shape and the eu- and heterochromatin homogenize(Figure 71). The nuclear envelope and the granule membrane has disintegrated allowing the mixing of components of extracellular traps as shown by *.

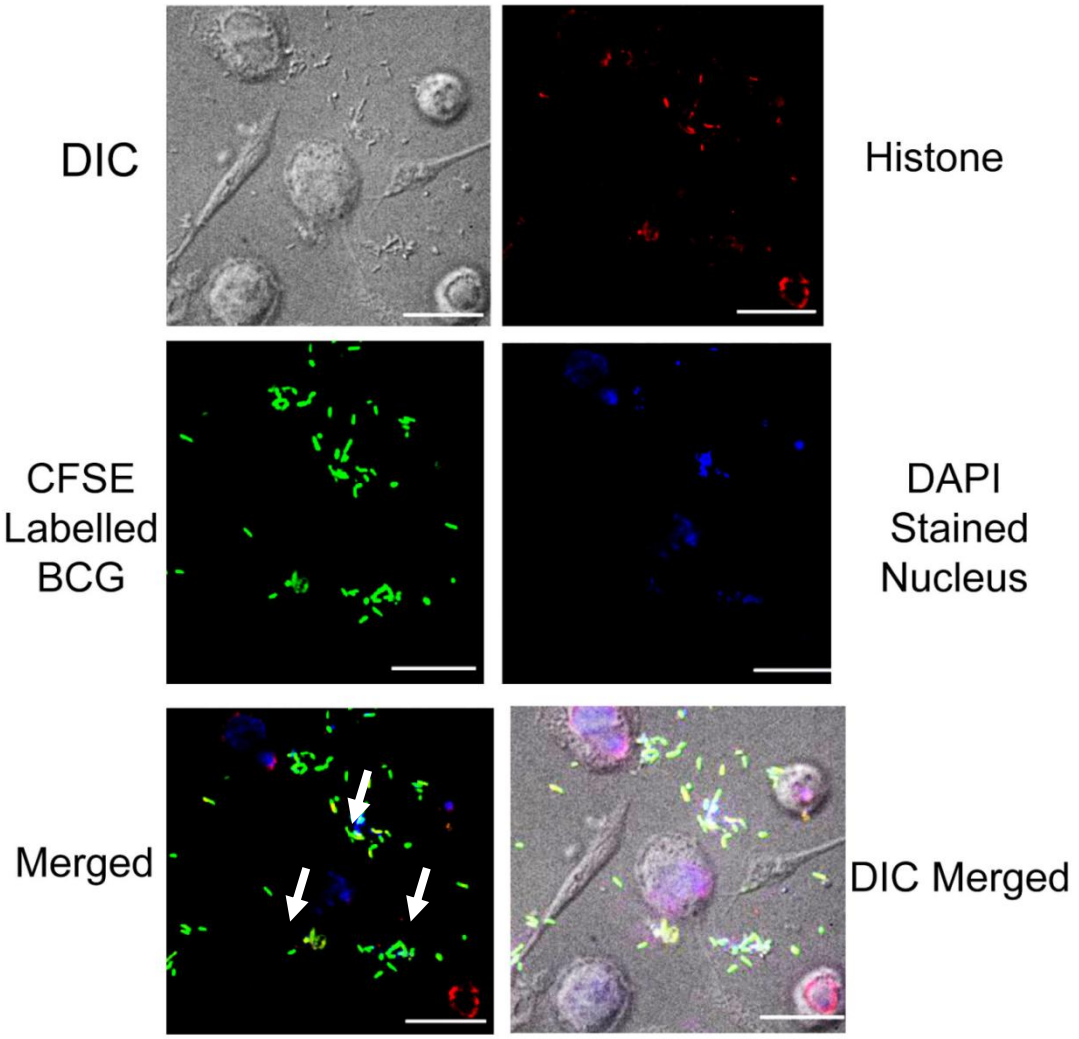

Figure 70: Immunostaining of MCETs with histone on *in vitro* interaction of RBL MCs with BCG. Cells cultured on a cover glass slip for overnight incubated with BCG (green) for 24h at an MOI of (1:100) in RBL complete medium. Cells were washed with PBS thrice, fixed with PFA, quenched with Ammonium Chloride, anti histone H2a raised in rabbit (1:250) was added for 1h followed by washing in 3% goat serum and adding secondary antibody anti rabbit Alexa 546 followed by washing and mounted in medium having DAPI (blue). The samples were visualized under confocal microscope. Magnification is 100X and scale bar is 20 µm.

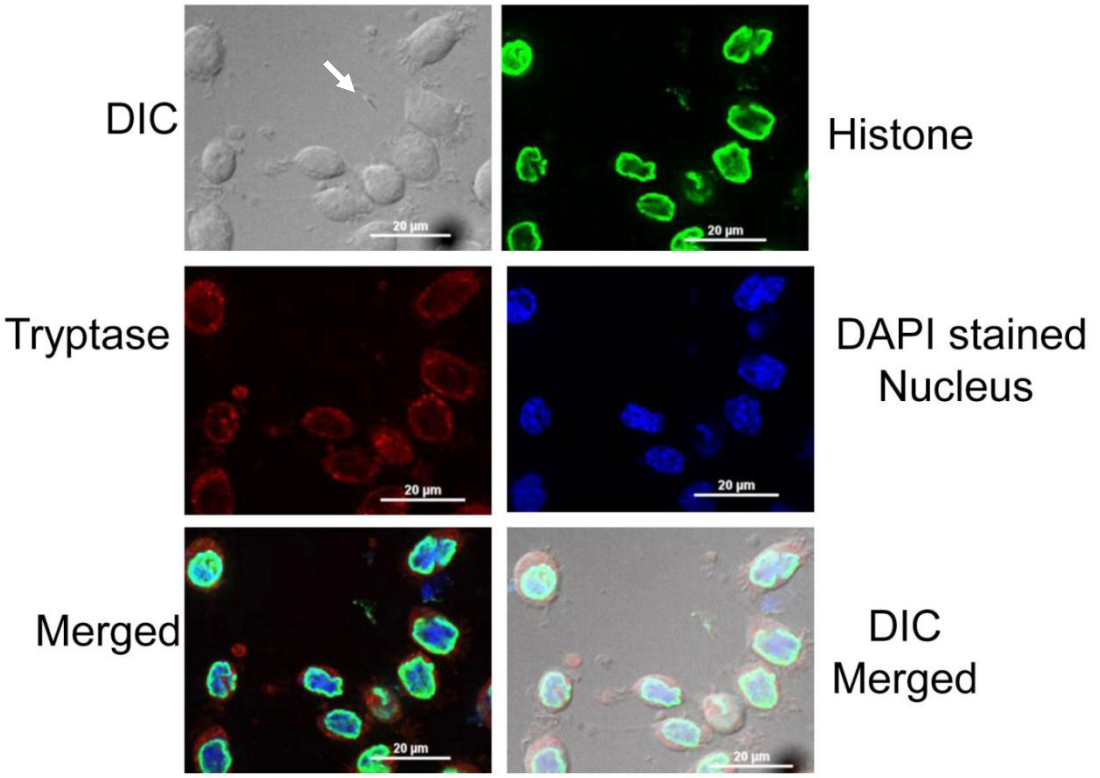

Figure 71: Immunostaining of MCETs with histone and tryptase on *in vitro* interaction of RBL MCs with BCG. Cells cultured on a cover glass slip for overnight incubated with BCG (green) for 24h at an MOI of 1:100 in RBL complete medium. Cells were washed with PBS thrice, fixed with PFA, quenched with Ammonium Chloride, anti histone H2a raised in rabbit (1:250) anti tryptase raised in mouse was added for 1h followed by washing in 3% goat serum and adding secondary antibody anti rabbit Alexa 546 and anti mouse Alexa 488 followed by washing and mounted in medium having DAPI (blue). The samples were visualized under confocal microscope. Magnification is 60X and scale bar is 20 µm.

Mast cell death by Etosis and release of mast cell extracellular traps on interaction with *L. tropica*

It was checked whether MCs die on co-culture with promastigotes of *L. tropica*. In control RBL MCs the viability was around 96.2%±3% at 18h and 90.5%±2.5% at 24 h. The viability of MCs upon co-culture with promastigotes of *L. tropica* fell to 89.5%±2.5% at 18h and 79.3%±3.5% at 24 h which is a statistically significant decrease compared to control (Figure 72). There was a concomitant decrease in the recovery of viable MCs at 24 h of co-culture with promastigotes of *L. tropica*.

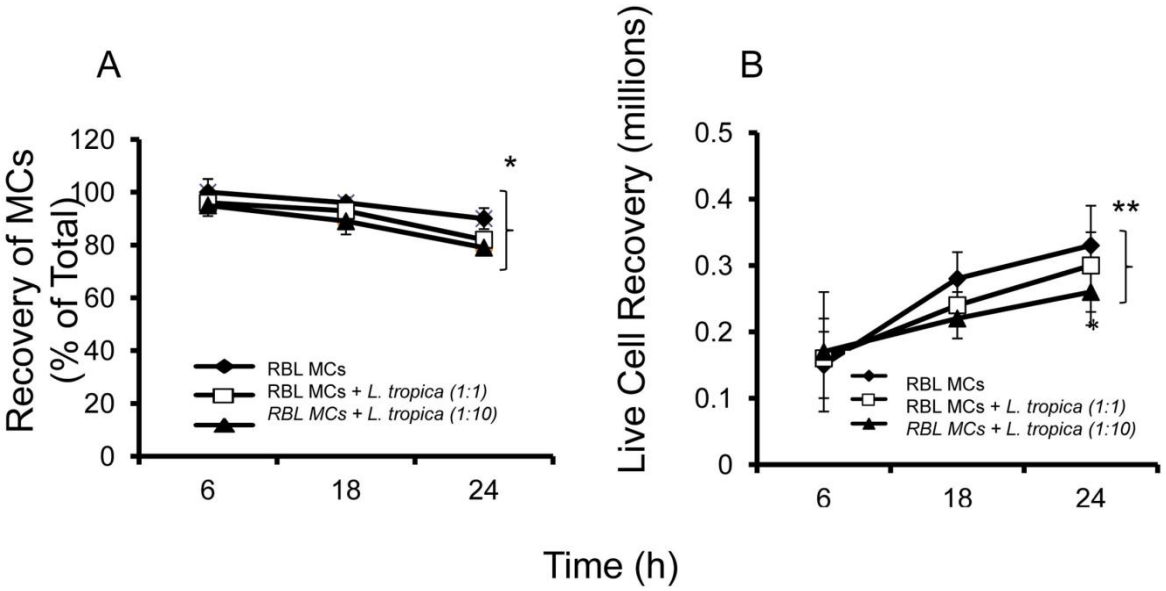

Figure 72: Cell viability of RBL MCs upon interaction with *L. tropica.* 0.1×10^6 MCs were seeded in 48 well cell culture plate and *L. tropica* were added at MOI 1:10. After specified time points parasites were removed and MCs were harvested by trypsinization and cell viability was counted using trypan blue exclusion method n=3. *p<0.05 and **p<0.005 represent statistically significant difference between control and treated cells by ANOVA.

Visualization of mast cell extracellular traps containing histones and tryptase in response to *L. tropica*.

As we saw MCs die by releasing DNA on infection with promastigotes of these *Leishmania* species, we wanted to visualize this phenomenon by microscopy. RBL MCs cultured alone, or co-cultured with promastigotes of *L. tropica* were fixed, their DNA stained with DAPI (4, 6-diamidino-2-phenylindole), and visualized by confocal microscope. MCs cultured alone showed DNA staining indicating compact intact chromatin in nuclei, MCs co-cultured with *Leishmania* promastigotes on the other hand showed disrupted/disintegrated chromatin with long extensions as expected in case of MCETs (Figure73). MCs treated similarly were also examined by Scanning Electron Microscopy to confirm release of DNA on infection by promastigotes of these *Leishmania* species. (Figure 74 A) shows no extension formation or release of DNA by non-co-cultured MCs whereas in MCs cells co-cultured with *L. tropica* (Figure 74 B) some extensions of around 0.1µm diameter were seen. These extensions may be DNA. Images in row 2 are 10000X magnified images and images in row 1 are 2000X magnified images (at least 10 images were visualized in each case). In a few images no *L. tropica* or 1±1 is seen in total 10 images.

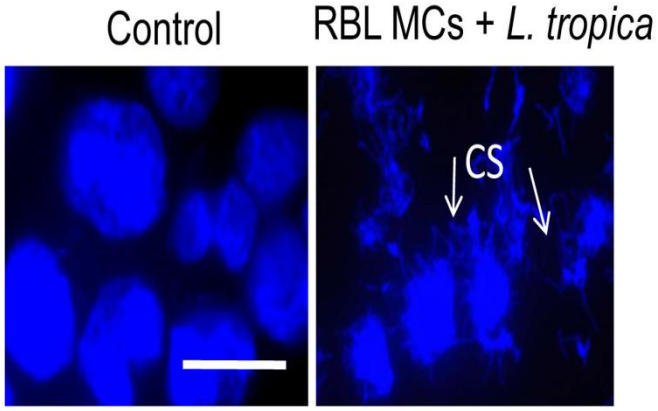

Figure 73: Visualization of Mast Cell Extracellular Traps on co-culture with *L. tropica* by Confocal Microscopy. RBL MCs cultured on a cover glass slip for overnight incubated with *L. tropica* for 24h at an MOI of 10:1 in RBL complete medium. Cells were washed with PBS thrice, fixed with methanol mounted in medium having DAPI (blue) and visualised under confocal laser scanning microscope. Magnification is 100X and scale bar is 10 µm.

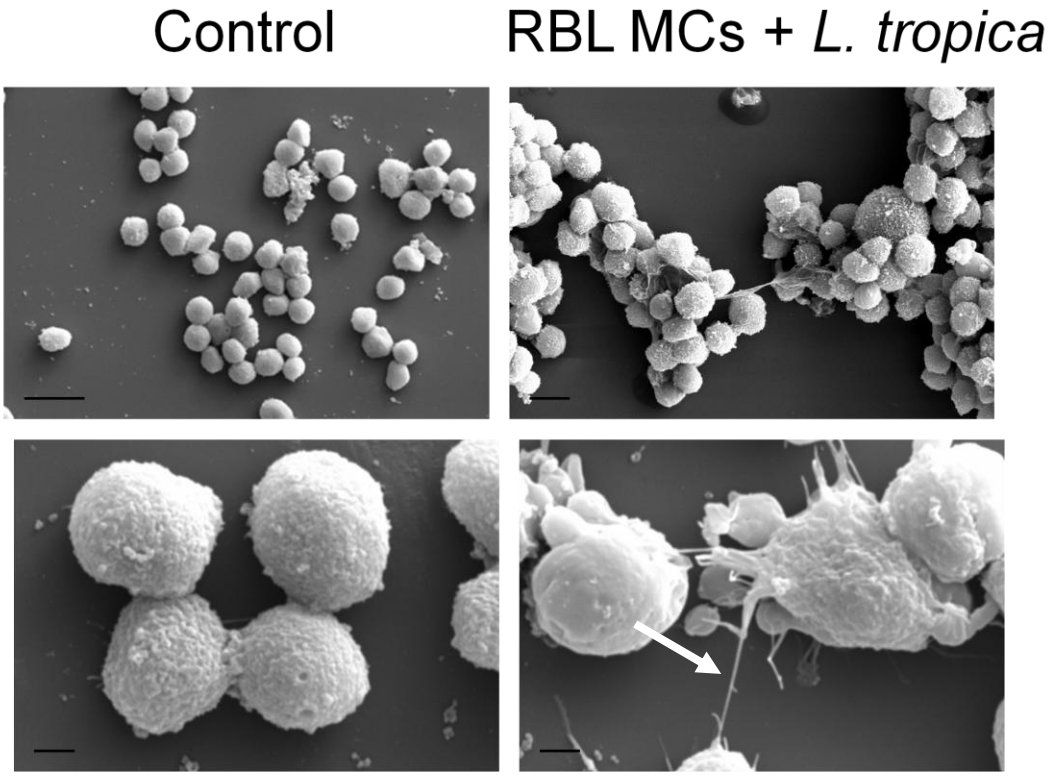

Figure 74: Visualization of Mast Cell Extracellular Traps on co-culture with *L. tropica* by Scanning Electron Microscopy. 2.5× 10^6 MCs were seeded in 6 well plate for overnight and *L. tropica* were added at MOI 1:10. After 24h parasites were removed cells were harvested and were fixed with 2% glutaraldehyde for 2h and were processed for Scanning Electron Microscopy. N= 3. Scale bar in top panel of is 10µm and magnification is 2000X and scale bar in bottom panel of 5b is 2µm and magnification is 10000X, n=3. White arrows could be DNA fibre.

To further visualize MCETs RBL MCs were seeded on cover slides and co-cultured with CFSE labelled promastigotes of *L. tropica* for 24 h, thereafter DNA was visualized by DAPI staining, tryptase and histones by staining with fluorescently tagged specific antibodies. As is seen in (Figure 75, and 76), blue coloured DAPI stained DNA with red coloured tryptase or histone are visible in the extracellular regions. Tryptase staining is extensive in cytosol region of MCs. Thus, fluorescence images depict release of tryptase (red), histone (red), and DNA (blue) with extracellular trapped promastigotes of *L. tropica* (green).

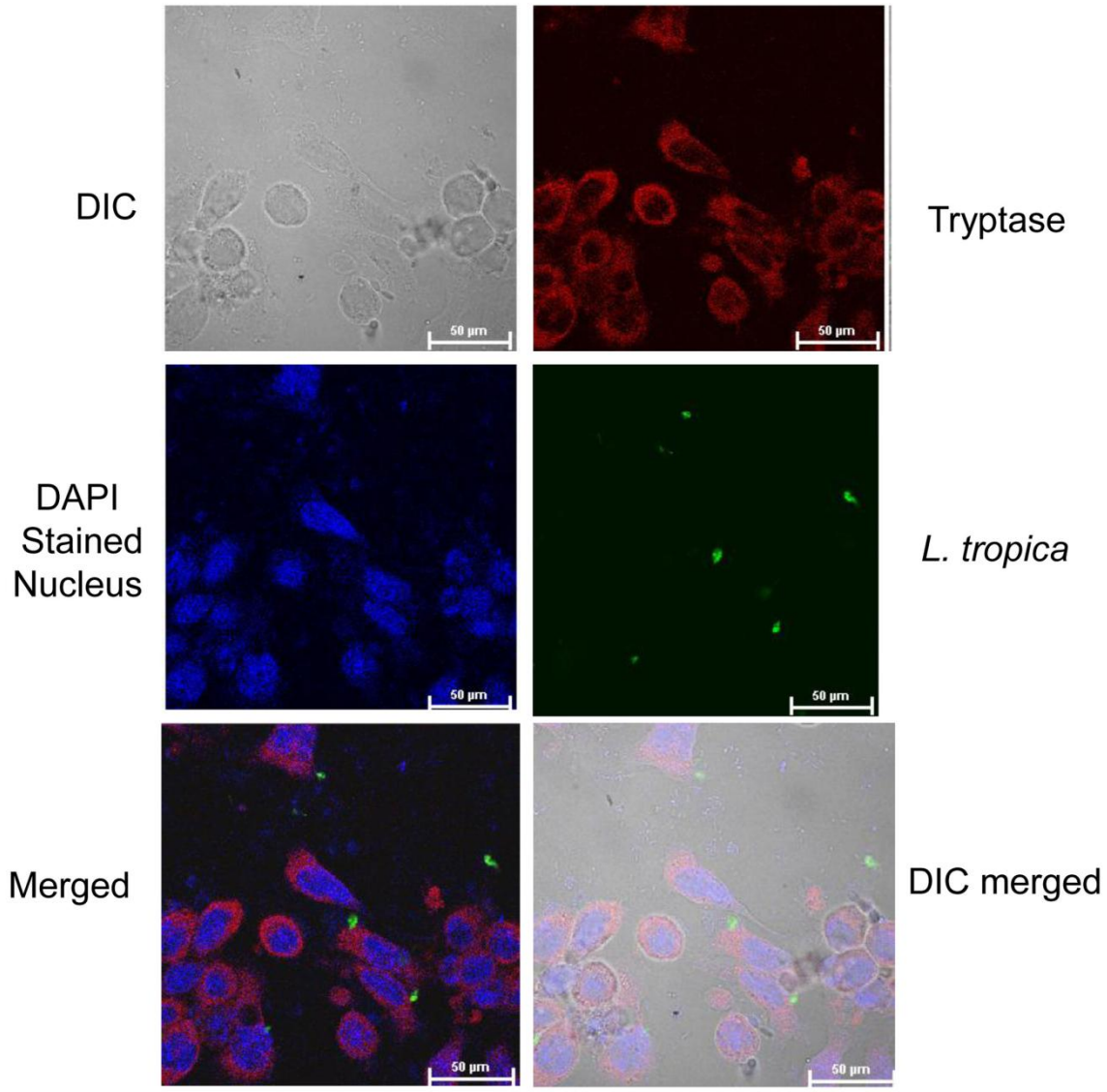

Figure 75: Immunostaining of MCETs with tryptase on *in vitro* interaction of RBL MCs with *L. tropica*. Cells cultured on a cover glass slip for overnight incubated with *L. tropica* (green) for 24h at an MOI of 10:1 in RBL complete medium. Cells were washed with PBS thrice, fixed with PFA, quenched with Ammonium Chloride, anti tryptase raised in mouse (1:50) was added for 1h followed by washing in 3% goat serum and adding secondary antibody anti mouse Alexa 546 followed by washing and mounted in medium having DAPI (blue) visualized under Nikon Real Time Laser Scanning Confocal Microscope Model A1R with motorized inverted microscope having Live Cell and Spectral Imaging – Model Ti-E at 60X. Scale bar is 50µm.

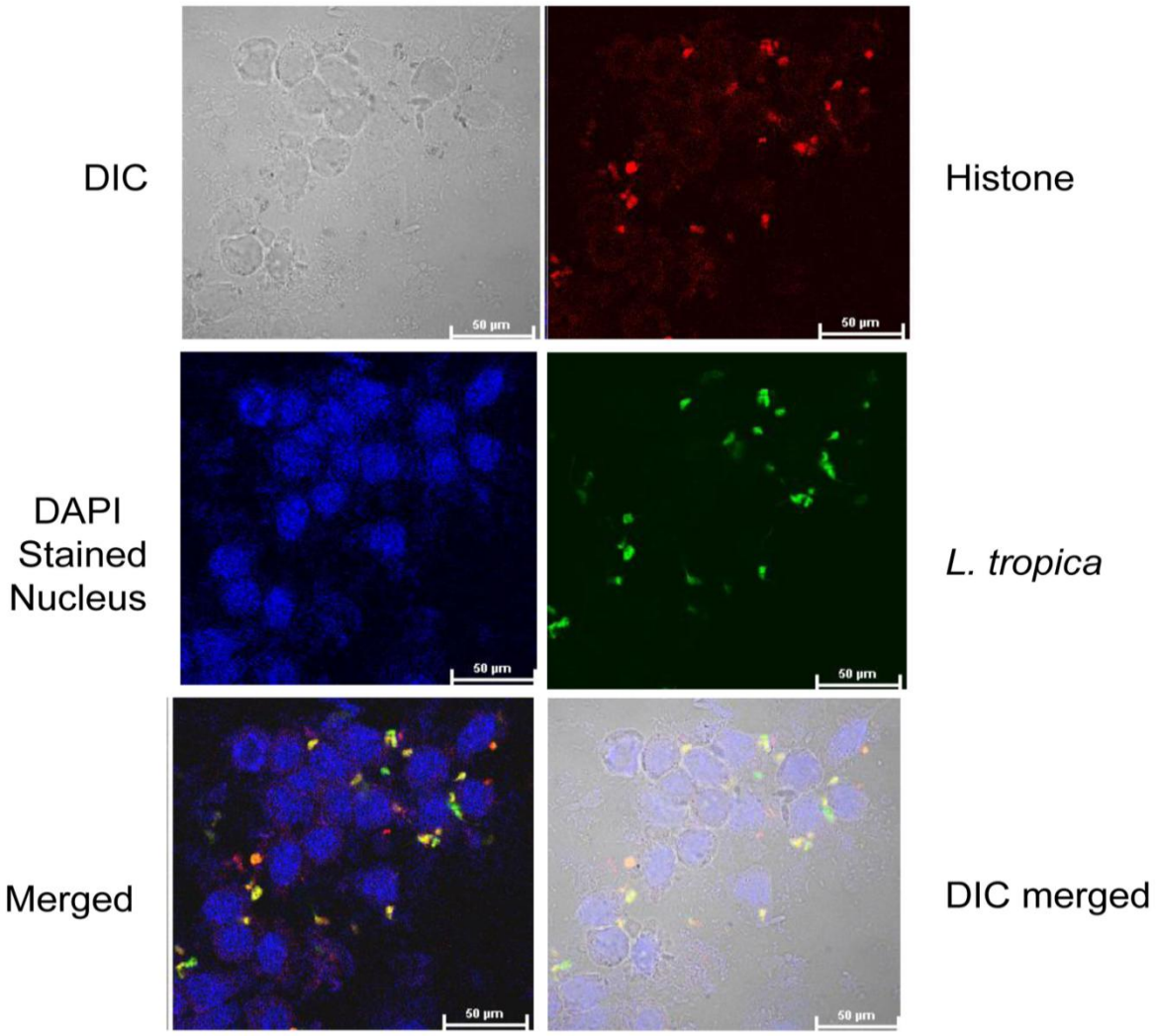

Figure 76: Immunostaining of MCETs with histone on *in vitro* interaction of RBL MCs with *L. tropica.* Cells cultured on a cover glass slip for overnight incubated with *L. tropica* (green) for 24h at an MOI of 10:1 in RBL complete medium. Cells were washed with PBS thrice, fixed with PFA, quenched with Ammonium Chloride, anti tryptase raised in mouse (1:50) was added for 1h followed by washing in 3% goat serum and adding secondary antibody anti mouse Alexa 546 followed by washing and mounted in medium having DAPI (blue) visualized under Nikon Real Time Laser Scanning Confocal Microscope Model A1R with motorized inverted microscope having Live Cell and Spectral Imaging – Model Ti-E at 60X. Scale bar is 50µm.

Evidence of extracellular killing of *L. tropica* by Mast Cell Extracellular Traps.

The results so far indicated release of DNA with histones and tryptase forming MCETs in response to promastigotes of *L. tropica*. Further to confirm that the MCETs had a role in killing of these promastigotes it was necessary to check whether disrupting DNA could affect the viability of promastigotes. The cells in co-culture were treated with DNase and the viability of promastigotes of *L. tropica* showed a significant increase from 45.7%±2.3% to 66.7%±4.4%. Similarly, when PMCs were co-cultured with *L. tropica* for 24 h viability by MTT assay was reduced to 86.44±2.5% in case of *L. tropica.* Further, to confirm the similar effector mechanisms are operating in PMCs as well, DNase treatment was carried out during PMC *Leishmania* co-culture. The viability by MTT assay has significantly increased from 86.44±2.5% to 96±0.8% in case of *L. tropica* (Figure 77). So we conclude that MCET formation is important for killing of promastigotes of *L. tropica*.

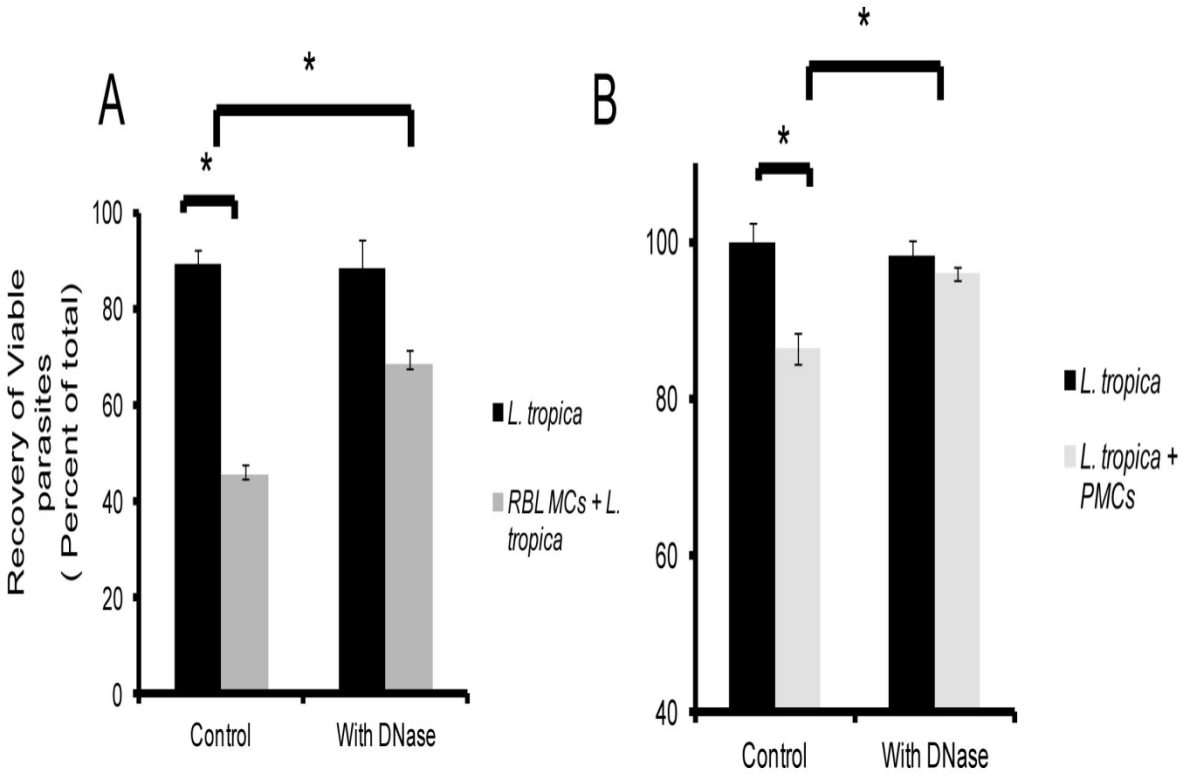

Figure 77: Evidence of extracellular killing of *L. tropica* by Mast Cell Extracellular Traps. 0.1×10^6 MCs were seeded in 48 well cell culture plate and cultured overnight in CO_2 incubator. Panel A and B represents recovery of mast cells (% of total) on co-culture with *L.tropica* pretreated with DNAase on co-culture with RBL-2H3 and PMCs.

Mast cell death by Etosis and release of mast cell extracellular traps on interaction with *L. donovani.*

It was checked whether MCs die on co-culture with promastigotes of *L. donovani.* The viability of MCs upon co-culture with promastigotes of *L. donovani* was 73.6%±5% at 24 h which is a statistically significant decrease compared to control (Figure 78). There was a concomitant decrease in the recovery of viable MCs at 24 h of co-culture with promastigotes of *L. donovani* (Figure 78). Further, to confirm if MC death was by ETosis, we looked for the release of extracellular DNA by Sytox Green staining after co-culturing MCs with the promastigotes of both species. MCs cultured alone showed very low levels of extracellular DNA (2.3%±1.5% at 18h and 3.2%±2.3% at 24 h). Significantly higher release of DNA was seen by MCs on co-culture with *L. tropica* for 18h (6.5%±0.5%), and 24 h (21.6%±1.2%) respectively (Figure 4c), and with *L. donovani* for 24 h (11.7%±0.6%) (Figure 79)

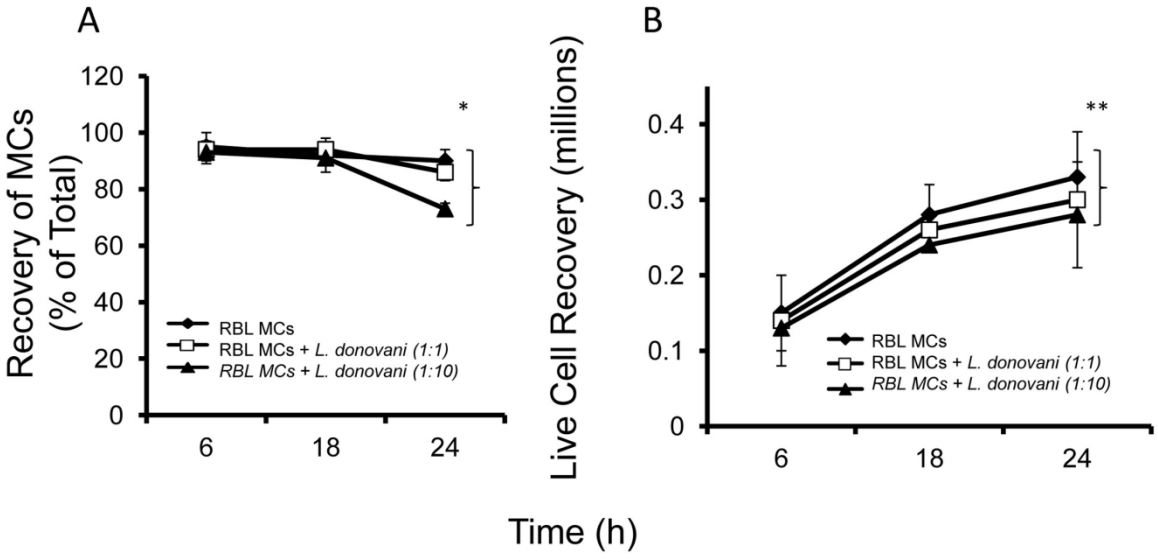

Figure 78: Cell viability of RBL MCs upon interaction with *L. donovani.* 0.1×10^6 MCs were seeded in 48 well cell culture plate and *L. donovani* were added at MOI 1:10. After specified time points parasites were removed and MCs were harvested by trypsinization and cell viability was counted using trypan blue exclusion method n=3. *p<0.05 and **< 0.005 represent statistically significant difference between control and treated cells by ANOVA.

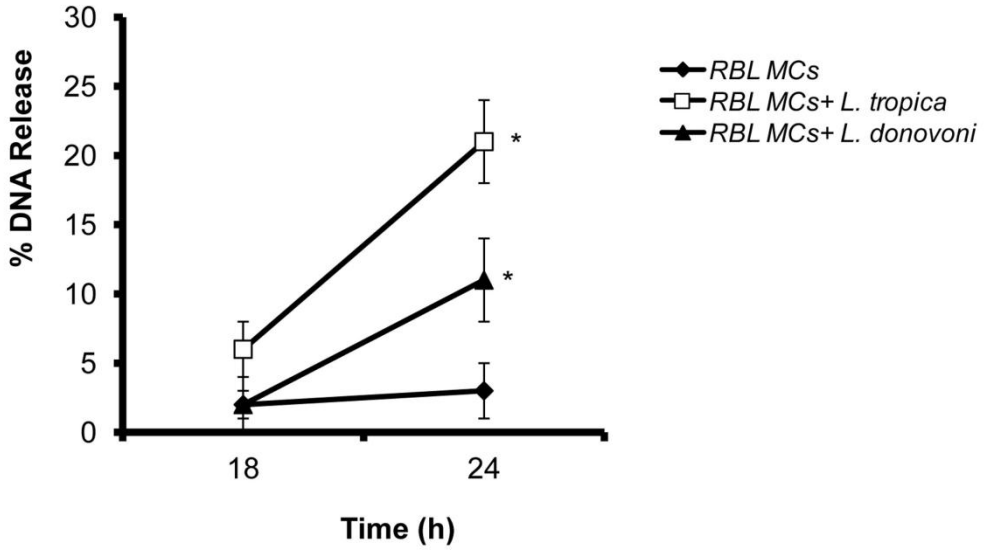

Figure 79: DNA release by RBL Mast Cells by Sytox Green Assay. 0.05 million RBL MCs were seed in 96 well plate for overnight. Promastigotes of *Leishmania* were added 2.5 µM Sytox Green was added and kept at 37°C and 5% CO_2 for 24 h. Taking MC lysis control as 100% DNA release was estimated. *$p<0.05$ represent statistically significant difference between control and treated cells by Mann Whiteney u test.

Visualization of mast cell extracellular traps containing histones and tryptase in response to *L. donovani*.

As we saw MCs die by releasing DNA on infection with promastigotes of *L. donovani* we wanted to visualize this phenomenon by microscopy. RBL MCs cultured alone, or co-cultured with promastigotes of *L. donovani* were fixed, their DNA stained with DAPI (4, 6-diamidino-2-phenylindole), and visualized by confocal microscope. MCs cultured alone showed DNA staining indicating compact intact chromatin in nuclei, MCs co-cultured with *Leishmania* promastigotes on the other hand showed disrupted/disintegrated chromatin with long extensions as expected in case of MCETs (Figure 80). MCs treated similarly were also examined by Scanning Electron Microscopy to confirm release of DNA on infection by promastigotes of these *Leishmania* species. Figure shows no extension formation or release of DNA by non-co-cultured MCs, whereas in MCs cells co-cultured with *L. donovani.* We see some extensions of around 0.1µm diameter. These extensions may be DNA. Images in row 2 are 10000X magnified images and images in row 1 are 2000X magnified images (at least 10 images were visualized in each case). 20±5 *L. donovani* are seen in total 10 images (Figure 81). Both these microscopic studies reveal the formation of more extensive chromatin extensions or traps by MCs in response to *L. donovani* in comparison to the ones in response to *L. tropica*.

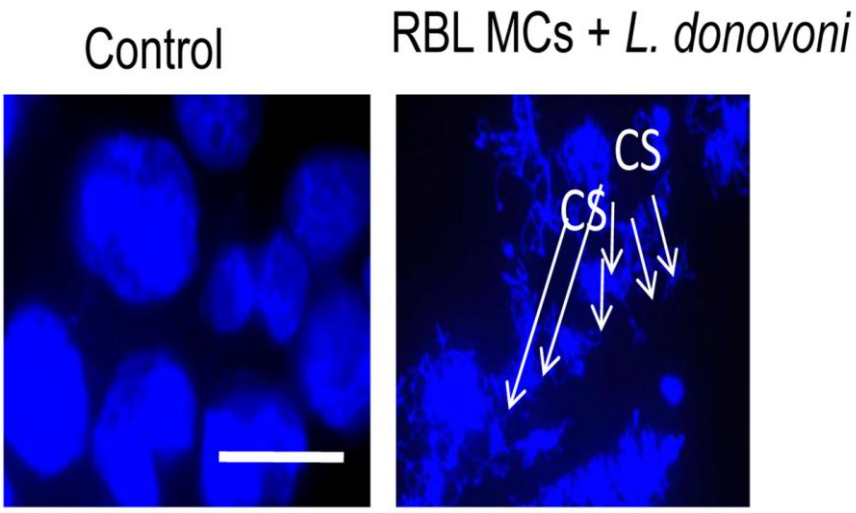

Figure 80: Visualization of Mast Cell Extracellular Traps on co-culture with *L. donovani* by Confocal Microscopy. RBL MCs cultured on a cover glass slip for overnight incubated with *L. donovani* for 24h at an MOI of 10:1 in RBL complete medium. Cells were washed with PBS thrice, fixed with methanol mounted in medium having DAPI (blue) and visualised under confocal laser scanning microscope. Magnification is 100X and scale bar is 10 µm.

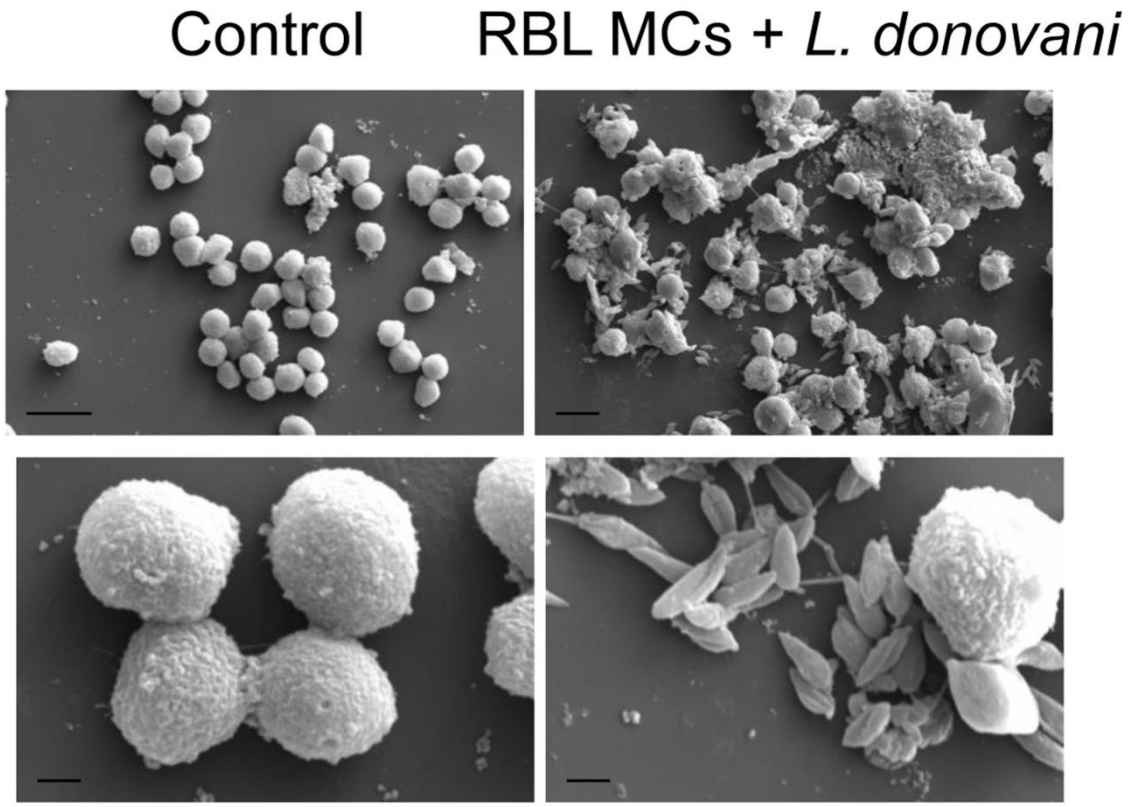

Figure 81: Visualization of Mast Cell Extracellular Traps on co-culture with *L. donovani* by Scanning Electron Microscopy. 2.5× 10^6 MCs were seeded in 6 well plate for overnight and *L. donovani* were added at MOI 1:10. After 24h parasites were removed cells were harvested and were fixed with 2% glutaraldehyde for 2h and were processed for Scanning Electron Microscopy. N= 3. Scale bar in top panel of is 10µm and magnification is 2000X and scale bar in bottom panel of is 2µm and magnification is 10000X, n=3. White arrows could be DNA fibre.

RBL MCs were seeded on cover slides and co-cultured with CFSE labelled promastigotes of *L. donovani* for 24 h, thereafter DNA was visualized by DAPI staining, tryptase and histones by staining with fluorescently tagged specific antibodies. As is seen in (Figure 82, and 83), blue coloured DAPI stained DNA with red coloured tryptase or histone are visible in the extracellular regions. Tryptase staining is extensive in cytosol region of MCs. Thus, fluorescence images depict release of tryptase (red), histone (red), and DNA (blue) with extracellular trapped promastigotes of *L. donovani* (green) (Figure 82, and 83). The number of promastigotes of *L. donovani* in the extracellular regions are much more than the number of promastigotes of *L. tropica* entrapped in MCETs in all the fields observed.

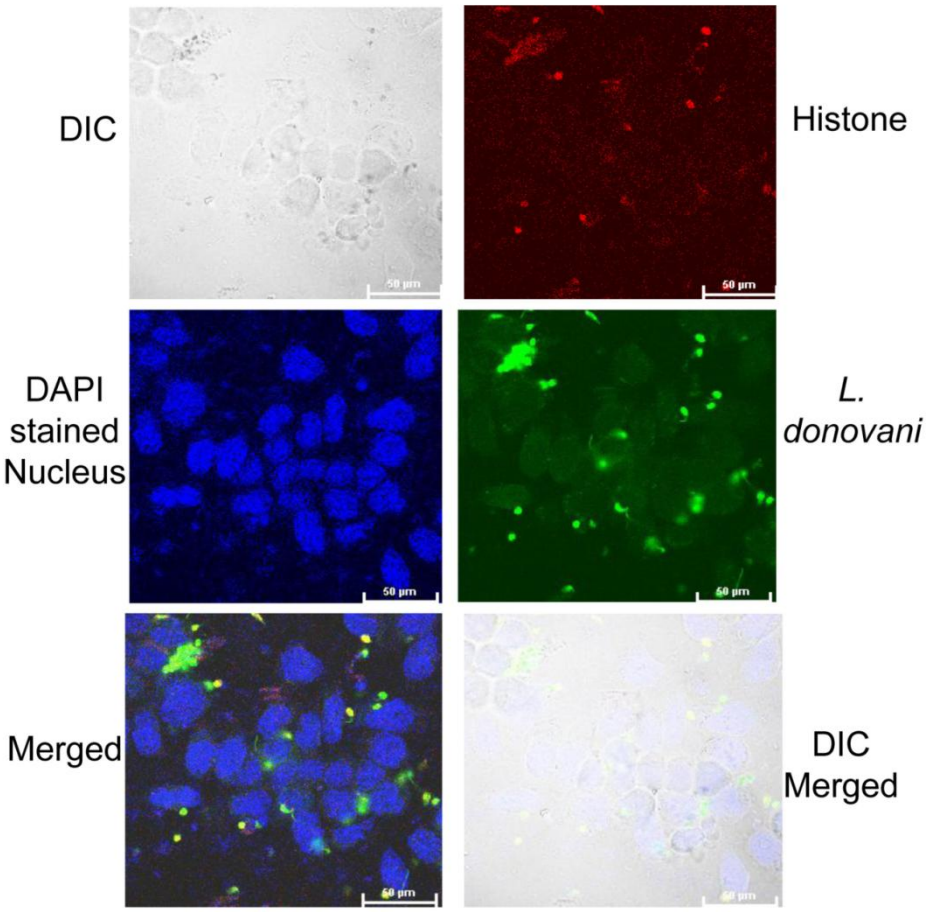

Figure 82: Immunostaining of MCETs with histone on *in vitro* interaction of RBL MCs with *L. donovani.* Cells cultured on a cover glass slip for overnight incubated with *L. donovani* (green) for 24h at an MOI of 10:1 in RBL complete medium. Cells were washed with PBS thrice, fixed with PFA, quenched with Ammonium Chloride, anti tryptase raised in mouse (1:50) was added for 1h followed by washing in 3% goat serum and adding secondary antibody anti mouse Alexa 546 followed by washing and mounted in medium having DAPI (blue) visualized under Nikon Real Time Laser Scanning Confocal Microscope Model A1R with motorized inverted microscope having Live Cell and Spectral Imaging – Model Ti-E at 60X. Scale bar is 50µm.

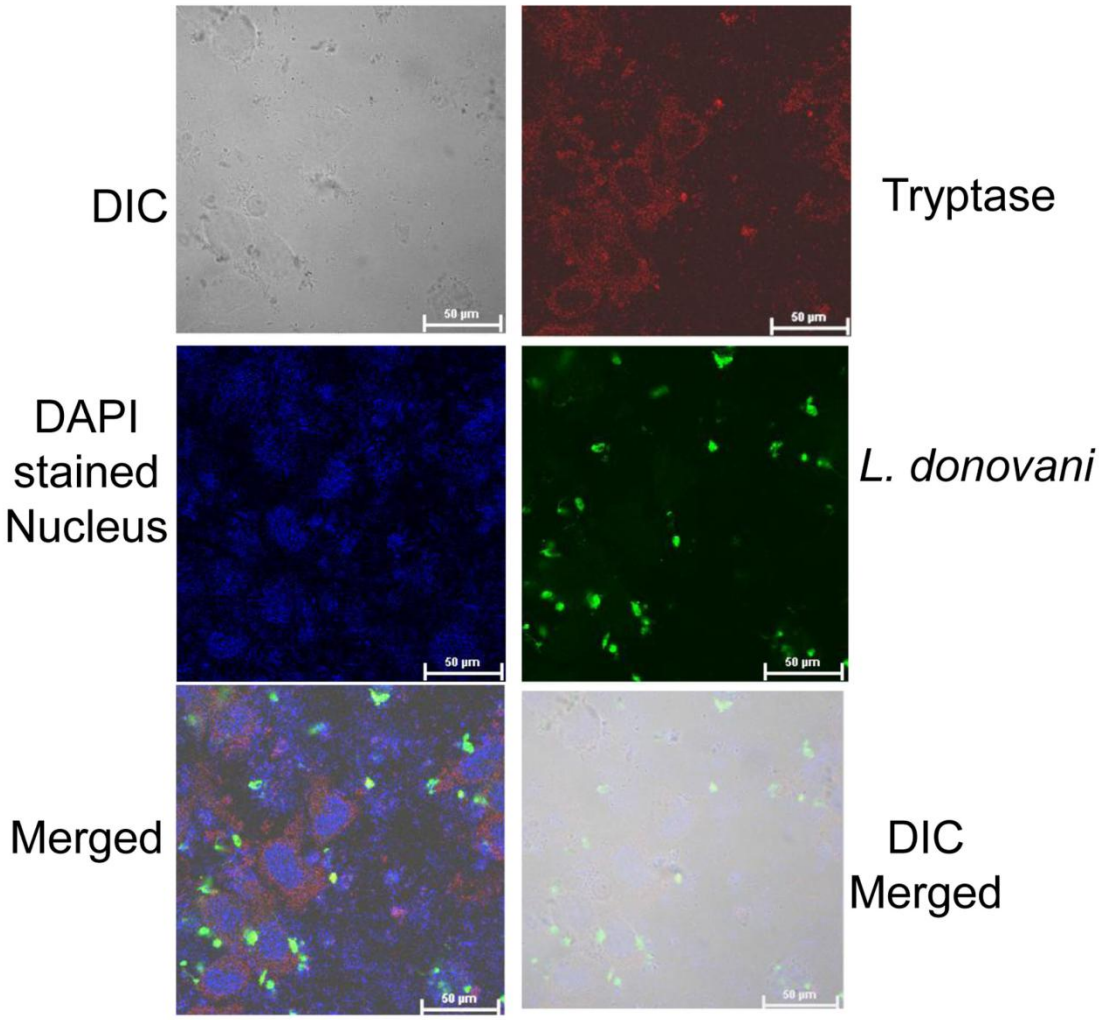

Figure 83: Immunostaining of MCETs with tryptase on *in vitro* intreraction of RBL MCs with *L. donovani.* Cells cultured on a cover glass slip for overnight incubated with *L. donovani.* (green) for 24h at an MOI of 10:1 in RBL complete medium. Cells were washed with PBS thrice, fixed with PFA, quenched with Ammonium Chloride, anti tryptase raised in mouse (1:50) was added for 1h followed by washing in 3% goat serum and adding secondary antibody anti mouse Alexa 546 followed by washing and mounted in medium having DAPI (blue) visualized under Nikon Real Time Laser Scanning Confocal Microscope Model A1R with motorized inverted microscope having Live Cell and Spectral Imaging – Model Ti-E at 60X. Scale bar is 50µm.

Evidence of extracellular killing of *L. donovani* by Mast Cell Extracellular Traps.

The results so far indicated release of DNA with histones and tryptase forming MCETs in response to promastigotes of *L. donovani*. Further to confirm that the MCETs had a role in killing of these promastigotes it was necessary to check whether disrupting DNA could affect the viability of promastigotes. The cells in co-culture were treated with DNase and the viability of promastigotes of *L. donovani* showed a significant increase from 82.43%±1.2% to 90.16%±2.4%, respectively (Figure 84). So we conclude that MCET formation is important for killing of promastigotes of *L. donovani* .Similarly, when PMCs were co-cultured with *L. donovani* for 24 h viability by MTT assay was reduced to 91±1% in case of *L. donovani* (Figure 84A). Further, to confirm the similar effector mechanisms are operating in PMCs as well, DNase treatment was out during PMC *Leishmania* co-culture. The viability by MTT assay has also increased significantly from 91±1% to 94±1.2% in case of *L. donovani* (Figure 84B). So we conclude that MCET formation is important for killing of promastigotes of *L. L. donovani*

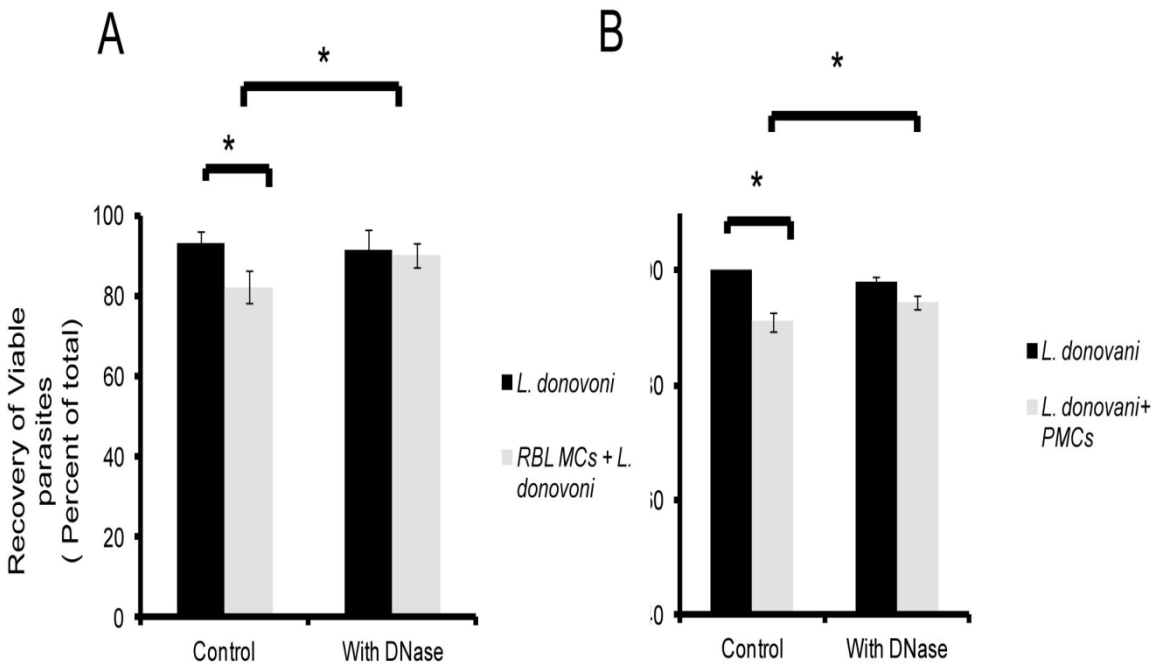

Figure 84: Evidence of extracellular killing of *L. donovani* by Mast Cell Extracellular Traps. 0.1×10^6 MCs were seeded in 48 well cell culture plate and cultured overnight in CO_2 incubator. Panel A and B represents recovery of mast cells (% of total) on co-culture with *L.donovani* pretreated with DNAase on co-culture with RBL-2H3 and PMCs.

Discussion

The initial interactions between a pathogen and its host set the stage for the development of long-term immunity or lack thereof. Among the earliest interactions of a pathogen with the immune system will be those involving MCs as they are part of the resident cells at the interface of the host and its environment (Abraham and Malaviya 1997). Mast Cells are present in the area that are the first one to interact with the invading pathogens (Abraham and St John 2010). They are equipped with various receptors (Abraham and St John 2010). Because of these two attributes they are the first one to interact with the invading pathogens. There are reports of Mast Cells interaction with bacteria, parasites and fungus and mounting of their immunological responses (Abraham and St John 2010). The vital role of MCs in triggering the innate immune response has been demonstrated following infections with *Escherichia coli*, *Klebsiella pneumonia*, and *Bordetella pertussis*. MCs have also been suggested to contribute to protective immunity against nonbacterial pathogens, especially intestinal parasites (Saha et al. 2004).

But so far there are no detailed and comparative studies have been carried out related to interaction of Mast Cells specifically with intracellular pathogens. We did a comparative study related to interaction of Mast Cells with *M. bovis* BCG, *Leishmania tropica*, *Leishmania donovani* and WT and mutants of *C. albicans. M. bovis* BCG lung infection in mice is a self-limiting infection, similar to *M. tuberculosis* infection in healthy human beings. For this reason, the mouse BCG infection model has been used extensively for studying the interactions between *Mycobacteria* and immune cells in the lung (Kumari and Saxena 2011). BCG is the most widely used vaccine in preventing TB especially in childhood (Fine 1995). Although there are reports of interaction of Mast Cells with *M. tuberculosis*, triggering the release of several prestored mediators and cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-6 (Munoz et al. 2003, Munoz, Rivas-Santiago and Enciso 2009) as well as *in vivo* report suggesting Mast Cells participation in host defense against *M. tuberculosis* infection through the production and secretion of cytokines such as tumor necrosis factor-α, interleukin-1 and chemokines such as keratinocyte-derived chemokine, monocyte chemotactic protein-1 that play a role in the recruitment and activation of inflammatory cells in Balb/c mice infected with *M. tuberculosis* (Carlos et al. 2007), there is no such study related to response of Mast Cells on interaction with *M. bovis* BCG. It is very interesting to

study how Mast Cells respond after BCG infection. So in order to accomplish this we first established BCG infection in C57BL/6 mice with an established protocol (Saxena et al. 2002). The infection was depicted by massive inflammatory cells and granuloma formation which was visualised by Hematoxylin and Eosin staining and Acid Fast Bacterial Staining. Since we were interested in looking for response of Mast Cells to BCG infection we did Toluidine Blue staining. We found there was an increase in the number of Mast Cells. So it is important to study response of Mast Cells on interaction to BCG.

Secondly, we chose to study the initial interaction of Mast Cells with parasites such as *L. donovani* and *L. tropica.* Only one study so far has looked at the direct interaction between Leishmania promastigotes and MCs, and also the specific MC effector responses (Bidri et al. 1997). Specific genetic deletion of mast cells *in vivo* had different impact on the course of infection with various species of *Leishmania* (Paul et al. 2016, Maurer et al. 2006). There are reports stating *L. major*-infected MC-deficient $Kit^{(W)}/Kit^{(W\text{-}v)}$ mice developed larger skin lesions than did normal $Kit^{+/+}$ mice (Maurer et al. 2006) whereas C57BL/6 Cpa^{Cre} or BALB/c Cpa^{Ce} had no effect when infected with *L. major* (Paul et al. 2016). Some others have compared the lesion sizes in presence or absence of MCs, focusing more on recruitment of other cells or the inflammatory responses (Saha et al. 2004)**.** Also a distinct dichotomy of opinions that MCs can either help or not in the pathogen replication exists, we were therefore, encouraged to discover through this study, the very initial events when MCs encounter *Leishmania* parasites in the *in vitro* conditions. Their interaction with RBL MCs was used as an *in vitro* model to study interaction as would happen in skin just after sand fly bite.

L. tropica causes Cutaneous Leishmaniasis (CL). CL is the most common form of leishmaniasis and causes skin lesions, mainly ulcers, on exposed parts of the body, leaving life-long scars and serious disability. *L. donovani* causes Visceral Leishmaniasis (VL). Visceral leishmaniasis (VL), also known as kala-azar is fatal if left untreated in over 95% of cases. It is characterized by irregular bouts of fever, weight loss, enlargement of the spleen and liver, and anaemia. The molecular basis of this pathogenicity has not been very well understood. There is a polymorphism in LPG structure. The LPG of *L. donovani* has no side substitution in the peptidoglycan and therefore remains linear (Sacks et al. 1995). The LPGs of *L. tropica* are

glycosylated at the C3 position of the galactose in the linear peptidoglycan (Soares et al. 2002). Both species may lead to differential signalling in different host immune cells, as has been shown for *L. major* and *L. donovani* effects on macrophage gene regulation/expression in the past (Gregory et al. 2008). Differential signalling may lead to different pathogenic outcomes.

We also studied interaction of Mast Cells with *C. albicans* which is a commensal, colonizing predominantly in skin and mucosal surfaces. Mast cells are present in tissues prone to fungal colonization. So they are among the first immune cells to get into contact with *C. albicans*. The GPI in eukaryotes anchors a variety of proteins to the cell surface which include virulence factors, enzymes involved in host immune response evasion, cell wall biogenesis, and maintenance proteins as well as hyphaspecific proteins. The pathway affects the functioning and viability of the organism. The pathway is of 10–12 steps that generates the precursor GPI anchor core in the endoplasmic reticulum. The enzymatic subunit for each step has been identified, and its role in the catalytic step has been specified based on yeast and mammalian studies. The role of the accessory subunits in these steps is unclear. The accessory proteins provide a regulatory control on the pathway (Victoria et al. 2012, Yadav et al. 2014). So we also looked at the role of accessory proteins of Glycosylphosphatidylinositol-N-acetylglucosaminyltransferase (GPI-GnT) complex in pathogenesis of *C. albicans.* The first step is the formation of GPI-N-acetyl glucosaminyl phosphatidylinositol (GlcNAcPI) from uridinediphosphate- N-acetylglucosamine(UDP-GlcNAc) and phosphatidylinositol (PI). It is catalyzed by GPI-N-acetylglucosaminyltransferase complex which consists of six different subunits Gpi1, Gpi2, Gpi3, Gpi15, Gpi19, and Eri1. Gpi2 and Gpi19 mutually coregulates with ergosterol biosynthesis. Gpi2 regulates hyphal morhphogenesis via Ras signaling and governs pathogenecity. Both Gpi2 and Gpi19 subunits negatively regulate each other (Yadav et al. 2014). By observing phenotypic effects it was found that the single allele disruption of GPI15 (GPI15 heterozygote) makes the strain azole sensitive and hypofilamentous compared to wild type (Kalpana Pawar 2015). It is also found that the chitin content of GPI2 is less (Yadav et al. 2014) and it is because of this reason the size of GPI2 is comparatively more compared to other mutants as depicted by Forward Scatter.

RBL-2H3 cell line was used as a model for MCs as it is adherent, easy to cultivate and express same receptors as MCs like FcεRI, c-Kit and other receptors known to interact with bacterial pathogens e.g. CD48 (Lin et al. 1999, Munoz et al. 2003). RBL-2H3 is a rodent mast cell line that has been extensively used to study MC biology in general and MC-pathogen interactions in particular (de Bernard et al. 2005, Mollerherm, von Kockritz-Blickwede and Branitzki-Heinemann 2016, Munoz et al. 2003, Sakurai, Yamaguchi and Sonoyama 2012, Wesolowski, Caldwell and Paumet 2012). It has also been confirmed by our group that RBL cells respond to similar physiological triggers as MCs like IgE cross-linking or increase in intracellular calcium levels releasing same mediators (secretory granule cargo – histamine and lysosomal β-hexosaminidase) as primary MCs with similar downstream signalling mechanisms (Naskar and Puri 2017, Puri and Roche 2008). There are reports suggesting role of mast cells in infection with *L. major* and *L. donovani* in rats (Fulton and Joyner 1948, Nolan and Farrell 1987). We therefore decided to study the direct interaction of RBL MCs with *L. tropica* and *L. donovani*.

To visualize these intracellular pathogens they were stained with CFSE. More than 90% of cells were stained with CFSE and labeling was stable till 24 h in case of BCG and *Leishmania.* The labeling was stable in transition from yeast to hyphal form in case of *C. albicans*. Again MCs were able to take up BCG especially at higher MOI of BCG indicating higher pathogen load. Confocal microscopy showed BCG is internalized and reaches to late endosome after 24h. The uptake was inhibited by Cyt D showing involvement of cytoskeleton. Cell membranes are majorly composed of sphingolipids comprising of acyl chains forming quasi gel membranes with reduced mobility, phospholipids that contains saturated fatty acids in the inner leaflet and cholesterol which specifically partitions the bilayer that contain saturated fatty acid promoting the transition from the quasi gel phase to a more liquid ordered conformation with substantial lateral mobility (Manes, del Real and Martinez 2003). These membranes in the liquid ordered conformation are known as rafts. Bacteria such as *Mycobacterium* spp, Fim H expressing *Eschericia coli, Salmonella typhimurium* and *Brucella* spp. exploit host rafts to generate phagosomes that allow them to survive in professional and non professional phagocytic cells (Baorto et al. 1997, Garner, Hayward and Koronakis 2002, Gatfield and Pieters 2000, Watarai et al. 2002). We found that uptake of BCG by RBL MCs was also further inhibited with

MβCD, Nyastatin and Filipin which disrupts raft. When the body is challenged by pathogen, the pathogen is coated with opsonin before it interacts with the immune cells. Just to mimic this physiological condition BCG was opsonised with anti-BCG serum and phagocytosis assay was performed. Although RBL-2H3 does not express FcγR, there was a dramatic increase in phagocytosis. Tuberculosis patients have an increased serum Mannose Binding Lectin (MBL) level compared to control (Bonar, Chmiela and Rozalska 2004). It was further proved that MBL plays a role in promoting *M. tuberculosis* access to phagocytic cells (Bonar et al. 2005). As RBL-2H3 cells express CD48 bearing high-mannose N-linked carbohydrate (Malaviya et al. 1999), dramatic uptake shown by these cells on opsonisation of BCG can be because of this reason.

There are two routes of killing of pathogen by RBL Mast Cells such intracellular killing by phagocytic route as well as extracellular killing by degranulation or by Etosis. So this gives rise to micro-organism derived antigens which can be taken up by macropinocytosis. Live BCG were sonicated and were taken up by RBL Mast Cells. This may mimic phenomenon of macropinocytosis. RBL also take up fluid phase marker known as Lucifer Yellow which further reconfirms RBL can undergo macropinocytosis (Naqvi 2012). Macropinocytosis is characterized by active uptake involving actin cytoskeleton. So, sBCG uptake was performed at different temperatures. SBCG uptake was partially inhibited at 4°C confirming the active nature of uptake. Optimal saturable uptake was seen only at 37°C. Cyt D was also used as an inhibitor of cytoskeleton. Cyt D treatment lead to 30% inhibition in sBCG uptake. It has been seen that generation of macropinosomes increases upon a variety of stimuli inducing activation of growth factor receptors, non-receptor tyrosine kinases, protein kinase C and small G proteins (Falcone et al. 2006). These signalling mechanism lead to an increase in level of Ca^{2+}. Ionomycin lead to increase the intracellular Ca^{2+} level to mimic mast cell activation under physiological conditions by activating protein kinase C (Hafler et al. 1989) and then studied macropinocytosis in them. Thus, this is the first report where MCs undergo macropinocytosis. This also gives an insight that on activation MCs can take up antigen more efficiently and may present to APCs or to T cells directly as non – professional APCs (Carroll-Portillo et al. 2015, Stelekati et al. 2009).

When *L. tropica* and *L. donovani* were cocultured with RBL Mast Cells, only *L. tropica* were taken up by RBL Mast Cells which was confirmed both by microscopy and flowcytometry. Previous study with neutrophils also report that uptake of *L. donovani* by neutrophils was an infrequent event (Gabriel et al. 2010)**.** A previous study has reported that Bone Marrow Derived Mast Cells (BMMCs) can bind and internalize promastigotes of *L. major* and *L. infantum* (Bidri et al. 1997) which causes Cutaneous Leishmaniasis. Mast Cells' involvement seems to depend on *Leishmania* spp studied and also seems to vary between strains studied.

Further, we also looked at the initial interaction of Mast Cells with *C. albicans* and compared to Macrophages. Yeast form of wild type of *C. albicans* were able to make cell to cell contact in RBL which is slow compared to Macrophages which was depicted by live cell imaging. A range of environmental factors affect *C. albicans* morphology for example, at low pH (< 6), low cell densities, starvation *C. albicans* cells predominantly grow in the yeast form, while at a high pH (> 7) hyphal growth, the presence of serum or N-acetylglucosamine, physiological temperature and CO_2 promote the formation of hyphae.

We found that yeast form of *C. albicans* were not phagocytosed by RBL Mast Cells whereas hyphal form invades RBL Mast Cells which is similar to earlier reports (Lopes et al. 2015). *C. albicans* after invasion were able to cause membrane protusions known as blebs in RBL Mast Cells. Spherical membrane protrusions that are formed when regions of the plasma membrane separate from the underlying cell cortex, which comprises a layer of actin, myosin and associated proteins (Charras et al. 2008). When the cortex is separated from the plasma membrane, cytoplasmic pressure leads to formation of blebs. Although blebbing occurs during apoptosis, there are also reports of occurrence of blebs in healthy cells during cytokinesis, cell spreading and in some cases of cell migration (Yanase et al. 2009). Blebbing based motility has been observed in *Dictyostelium*, primordial germ cells from fish amphibians and amoebae but studies in mammalian cells are restricted to tumors only (Yanase et al. 2009). There is also report stating that Mast Cells form reversible blebs on stimulation with antigen (Yanase et al. 2009).

C. albicans study with Mast Cells and Macrophages have shown that different mutants of GPI - GnT pathway affect the efficacy of phagocytosis, invasion of RBL

Mast Cells. This also gives an indication of virulence of different mutants of GPI–GnT pathway. Among all the mutants heterozygous CaGpi2 mutant show maximum phagocytosis in yeast form which is cytoskeleton dependent in Macrophages compared to wild type as well as other mutants. Invasion by CaGpi2 is less compared to other mutants which is similar both in Mast Cells and Macrophages. CaGpi19 and CaEri1 show maximum invasion in both Mast Cells and Macrophages which concludes that they are more virulent compared to other sub units of Gpi-GnT.

When we co-cultured BCG with RBL-2H3 for 24 h we found reduction in CFU compared to control as well as in the transwell system which clearly depicted that direct contact is required for killing of BCG. Moreover, co-culture of BCG with RBL cells lead to degranulation leading to release of β-hexosaminidase and histamine accompanied by releasing of LDH at higher time points. The secretion was further confirmed by Transmission Electron Microscopy showing empty granules after co-culture with BCG. Many other pathogens are also known to activate MCs to secrete pre-formed as well as *de novo* synthesized mediators (Munoz et al. 2003, Munoz et al. 2009). The increase LDH secretion or loss of cell membrane integrity may indicate activation of Programmed Cell Death pathways leading to either death by apoptosis or death of cells may be due to Etosis in which traps may be generated to ensnare the pathogen for further destruction (Brinkmann et al. 2004)

When we co-cultured *L. tropica* and *L. donovani* with PMCs of BALB/c mice, which is a strain susceptible to leishmaniasis (Sacks and Noben-Trauth 2002) , we found reduction in their viabilities. Significant decrease was seen in viability of both strains more specifically *L. tropica,* on co-culture with MCs. By using transwell during co-culture studies, we found significant increase in viability of *L. tropica,* as well as *L. donovani.* This indicated that direct cell-cell contact between MCs, and promastigotes of both *Leishmania* species was required for the observed killing of *Leishmania* promastigotes. Compared to *L. tropica, L. donovani* is more viable on co-culture with MCs in 24 h. Moreover, the recovery of *L. tropica* has itself significantly reduced in transwell system along with MCs in nontranswell system. This reduction may be due to some soluble mediators. Previous knockout studies with mice have shown that TLR2, TLR4 and TLR9 may be important for cytokine response, and inducible nitric oxide synthase (iNOS) mRNA expression and expressions of TLR1, TLR2 and TLR3 in response to *Leishmania* infection (Charmoy et al. 2007, Murray et al. 2013, Pandey

et al. 2015). Also, in macrophages, the complement receptors, fibronectin receptor, and the mannose-fucose receptor (MR) on the surface of macrophages play import roles in promastigote binding/attachment (Kane and Mosser 2000). Since all these receptors are expressed on MCs as well, they may be the ones involved in direct interaction, and signalling involved in MC response to *Leishmania.*

Killing of *Leishmania* promastigotes by MCs can either be by phagocytosis or by extracellular means by mediator release or Etosis and Mast cell extracellular traps. MCs have been shown to internalise pathogens causing reduction in viability which was dependent on ROS (Malaviya et al. 1994). By our flow cytometric, and microscopic studies with CFSE-labeled promastigotes, a significant phagocytosis of *L. tropica* but not of *L. donovani* by MCs was observed. By Cytochalasin D treatment of MCs before co-culture, a significant increase in cell viability of *L. tropica* in presence of MCs was observed. This further validates, that reduction in viable number of promastigotes of *L. tropica* may partially be through phagocytosis by MCs. A previous study has reported that Bone Marrow Derived Mast Cells (BMMCs) can bind and internalise promastigotes of *L. major* and *L. infantum* (Bidri et al. 1997).

Once inside the MCs, the promastigotes may be killed by ROS generated inside host MCs. In fact, we did observe significant ROS production in co-cultured MCs, and also apoptosis of extracellular *Leishmania* parasites, confirmed by Annexin V binding assay. One recent study states that ROS from macrophages is required for killing of promastigotes of *L. amazonensis* (Roma et al. 2016), whereas in another study ROS is shown to regulate only the inflammatory response probably by controlling neutrophils numbers in lesions (Carneiro et al. 2016). Another study has also shown that ROS can also precipitate apoptosis of the *L. donovani* parasites (Das, Mukherjee and Shaha 2001). Treatment of catalase increased viability of *L.tropica* and *L. donovani* when they were co-cultured with PMCs as well as RBL-2H3. So, overall ROS generation by MCs in response to interaction with *Leishmania* may be important in the direct killing of pathogens by MCs. The pore size of the membrane of the transwell that we used was 0.4µm whereas the size of exosomes is 40nm to 100nm (Raposo and Stoorvogel 2013). So in transwell system the exosomes could have interacted with MCs, but as ROS generation is much reduced, we can conclude that direct interaction between cells is required and there does not seem to be much contribution of exosomes from Leishmania to this process.

One interesting observation of our study is that MCs are unable to phagocytose promastigotes of *L. donovani*, but still co-culture with these promastigotes leads to ROS production in MCs. Since MCs are not able to take up *L. donovani*, we can rule out the intracellular killing mechanism by MCs. We also observed that the viability of *L. donovoni* after co-culture with MCs was unaffected by Cytochalasin D treatment of MCs, therefore, we predicted that the killing can be by some extracellular means. We saw generation of significant amounts of ROS and release of β-hexosaminidase indicating degranulation. This can also be the reason of reduction in viability of *L. donovani*. We have observed release of β-hexosaminidase during initial hour of interaction with *L. tropica* and *L. donovani.* There are earlier reports also which depict that infection with live, virulent promastigotes of *L. major* leads to the release of preformed β-hexosaminidase and TNF-α, and to *de novo* synthesis of TNF-α in BMMCs (Bidri et al. 1997).

Co-culture of different GPI-GnT mutants of *C. abicans* with Mast Cells and Macrophages showed difference in virulence. GPI2 is the least virulent mutant among all the GPI-GnT mutant of *C. albicans* as the CFU formed after co-culturing with RBL Mast Cells and MH-S Macrophages. Cell death of RBL Mast Cells and Macrophages by Cagpi2 is lesser than wild type. Cell death of RBL Mast Cells and Macrophages by Cagpi19 and Eri1 is more than wild type. These studies lead to conclusion that GPI-GnT mutants of *C. albicans* do affect their virulence showing minimum virulence in Cagpi2 and maximum in Cagpi19 and Eri1 confirmed both in Mast Cells and Macrophages.

Further, we also found significant cell death of RBL Mast Cells when co-cultured with BCG. Cell death was due to apoptosis and Etosis. There are reports of release of tryptase as well as histones along with the DNA by MCs in a mechanism of extracellular killing of pathogens known as Etosis (von Kockritz-Blickwede et al. 2008). In our study, the interaction of MCs with both *Leishmania* species led to significant death of MCs and release of DNA as confirmed by fluorescence microscopy and electron microscopy. This is the first report stating MCET formation in case of BCG.

Further, significant MC death is observed in our study at 24 h after infection with *Leishmania*. In our study, the interaction of MCs with both *Leishmania* species led to

significant death of MCs and release of DNA as confirmed by fluorescence microscopy, electron microscopy, and Sytox green staining. Thus, we found the formation of MCETs in response to both *L. tropica* and *L. donovani*. We found reduction in killing of promastigotes of these parasites by DNase treatment on co-culture with PMCs as well as RBL-2H3. This leads to the conclusion that promastigotes of both species are susceptible to MCETs. Thus MCETs may not only physically restrict them, but also able to kill them to some extent. Thus, this is the first report showing direct interaction of MCs with either *L. donovani* or *L. tropica* by direct contact leading to generation of ROS and MCETs. ROS is required to initiate Neutrophil Extracellular Traps (NETs) (Papayannopoulos et al. 2010, Stoiber et al. 2015), and similar mechanism may also be operational in MCs. Although *L. donovani* is not phagocytosed by MCs but still causes ROS generation, which may be important for releasing MCETS.

Numerous factors influence disease severity, but the most important determinant of the form of Leishmaniasis is the species of *Leishmania* involved. During this study we were also able to compare the interactions of *L. tropica* and *L. donovani* with MCs and found that the response is different for *L. tropica* and *L. donovani. L. tropica* shows greater cell death on interaction with MCs, and is susceptible to phagocytosis but *L. donovani* is not. Secondly, although both *Leishmania* species seem susceptible to MCETs, as shown by reduction in cell death by DNAse treatment, but in all our images of MCETs relatively higher number of promastigotes of *L. donovani* in comparison to those of *L. tropica* are seen. These differences could be due to uptake of a proportion of promastigotes of *L. tropica* by MCs, and in addition, may be *L. tropica* promastigotes are more susceptible to killing by MCETs, in comparison to those of *L. donovani*. Previous reports suggest that viability of *L. donovani* is less affected to NETs due to an abundant surface virulence glycolipid lipophosphoglycan (LPG) anchored into the promastigote membrane (Spath et al. 2003). Although larger amount of DNA is released on coculture with *L. donovani* but killing is not to that extent rather it is able to evade killing which is further reconfirming the previously published report (Gabriel et al. 2010). Both species may lead to differential signalling in different host immune cells, as has been shown for *L. major* and *L. donovani* effects on macrophage gene regulation/expression in the past (Gregory et al. 2008). Differential signalling may lead to different pathogenic outcomes.

Also, although MCETs and NETs share common characteristics, there are several cell type-specific differences. MCs release less MCETs and at longer time points stimulated with the same stimuli in comparison to neutrophils, which also correlates with our study. Our study with MCs it takes 24 h for MCET generation. Another important difference between MCs and neutrophils are their components, which are embedded in the DNA structures. In MCs it is possible that MC-specific tryptase and histones, which were both shown to be present in extracellular regions, co-localizing with extracellular DNA and trapped promastigotes by our confocal fluorescence microscopic study, which have also been shown as a component of MCETs previously may have similar antimicrobial functions (Mollerherm et al. 2016). Viability of *L. tropica* increases upto only ~66%, which is far less than ~89% when cultured alone, this can be because of phagocytosis or release of toxic antigens of ETosis mainly ROS after digesting the DNA lump, which adversely affects *L. tropica* survival (Stoiber et al. 2015).

While the *Candida* study was carried on MCETs were discovered by Lopes et. al. They discovered that Mast Cells have temporal response on interaction with *C. albicans* which is divide into three categories in which the immediate response is depicted by degranulation by Mast Cells. Intermediate response depicted by MCET formation and delayed response depicted by cytokine secretion (Lopes et al. 2015).

As Mast Cells are located in the respiratory tract, they can interact with BCG. We found Mast Cells are recruited more in lungs after infection. They can phagocytose BCG. Mast Cells degranulate in presence of BCG. They can generate MCETs to ensnare BCG. They can generate ROS. The combined effect of Mast Cell responses lead to killing of BCG (Figure 85).

Since MCs are already present in skin and are one of the first immune cells to encounter *Leishmania* promastigotes, they are the ones that respond early. Our study is the first one to show that they do so, by phagocytosing promastigotes of *L. tropica*, generating ROS causing the apoptosis of promastigotes (Figure 86). The interaction also causes release of pre formed mediators, which can initiate an inflammatory response. At about 24h some of the MCs die by Etosis; again ROS may be important for this, form MCETs containing chromatin, and decorated with MC tryptase and histones to ensnare promastigotes of *L .tropica*. MCs therefore, seem to play an

important part in early innate immune response to control *L. tropica* infection, by limiting their numbers by direct killing by phagocytosis and extracellularly by MCETs. They also initiate inflammatory response by mediator release as has been shown by others. In case of *L. donovani*, though the phagocytosis by MCs does not happen, but the interaction does cause ROS production leading to some apoptosis in promastigotes of *L. donovani* (Figure 86). The formation of MCETs is also seen, and they are able to kill promastigotes to some extent, and definitely able to trap them at early time points. So, overall it can be concluded that MCs play a very important direct role in early innate immune response to *L. tropica* and *L. donovani* and it will be important to factor in their interaction and effector responses during *Leishmania* infection for success of any vaccine or therapeutic approach to Leishmaniasis.

Mast Cells also interact with *C. albicans* as Mast Cells are mostly present in those location where *C. albicans* infect. Yeast form of *C. albicans* is not phagocytosed by Mast Cells whereas Macrophages can phagocytose it. Different mutants of GPI-GnT show different responses in Mast Cells and Macrophages (Figure 87)

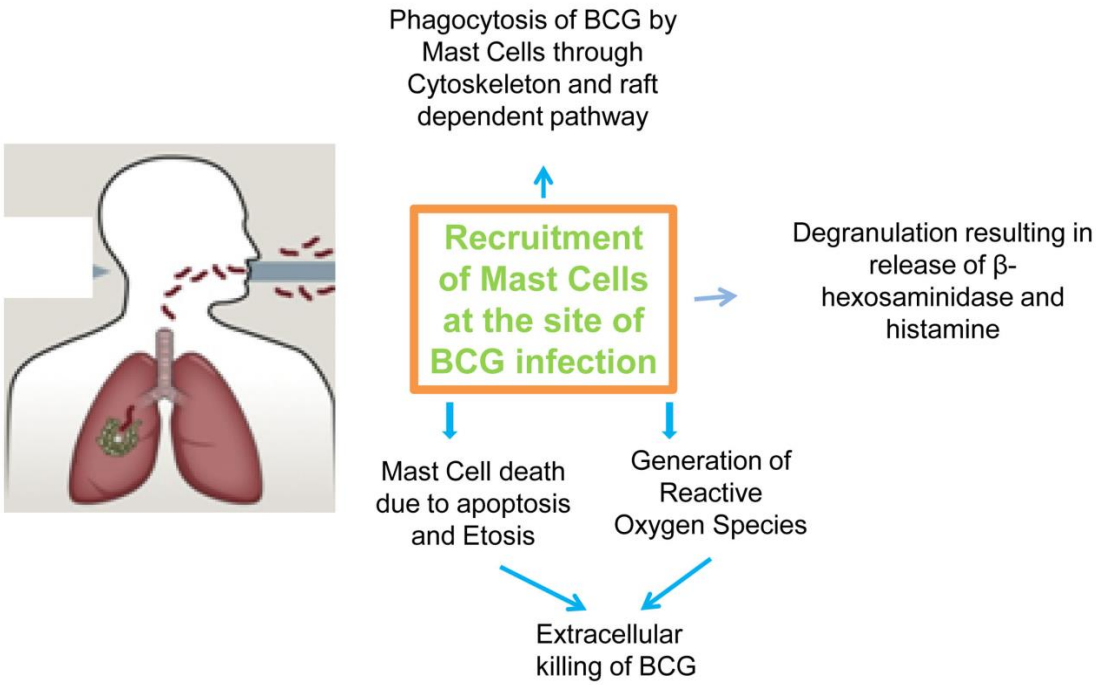

Figure 85: Summary of Mast Cell and BCG interactions. Our findings indicate that Mast Cells respond to BCG infection by recruiting at the site of infection. Mast Cells phagocytose BCG through cytoskeleton and raft dependent pathway. Immediate response include degranulation and phagocytosis with in 1h. In delayed response Mast Cell die through Etosis generate Reactive Oxygen Species which ultimately leads to killing of BCG.

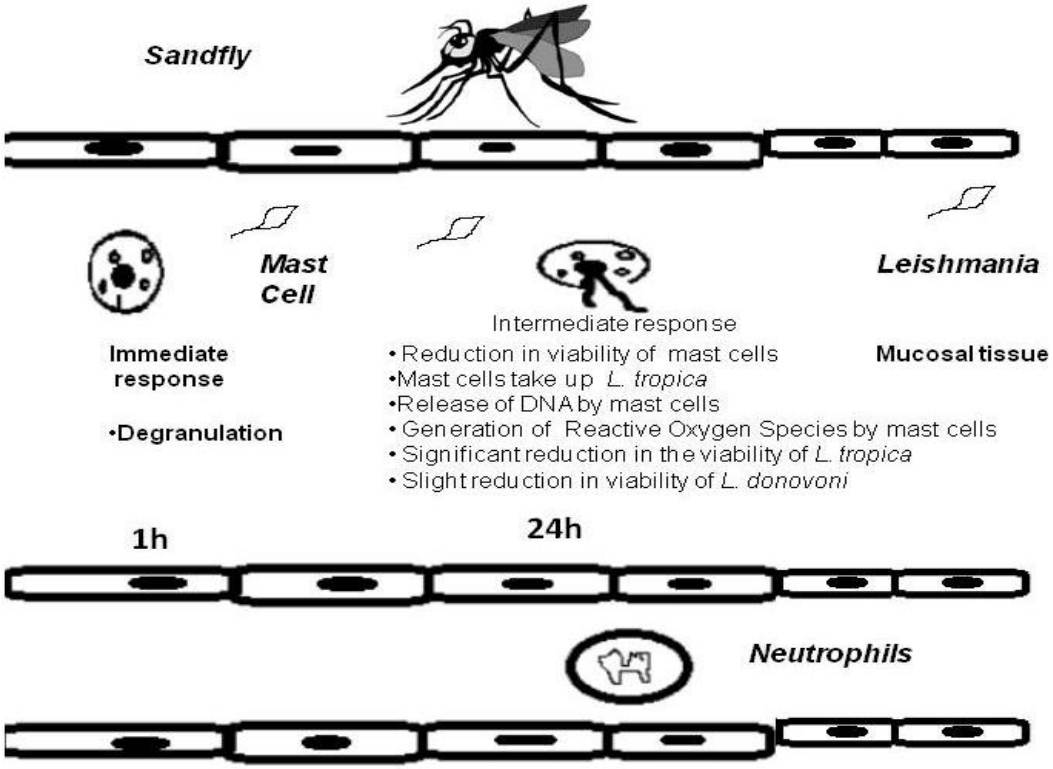

Figure 86: Our findings indicate that Mast Cells respond to *L. tropica* and *L.donovani* by degranulation which can be categorized under immediate response within 1h. Viability of *L. tropica* and *L.donovani* is also reduced at 24h on coculture with mast cells. Internalization of BCG, *L.tropica* but not *L. donovani* and generation of Reactive Oxygen Species accompanied by release of DNA and trapping of *Leishmania* in MCETs also take place at later time points.

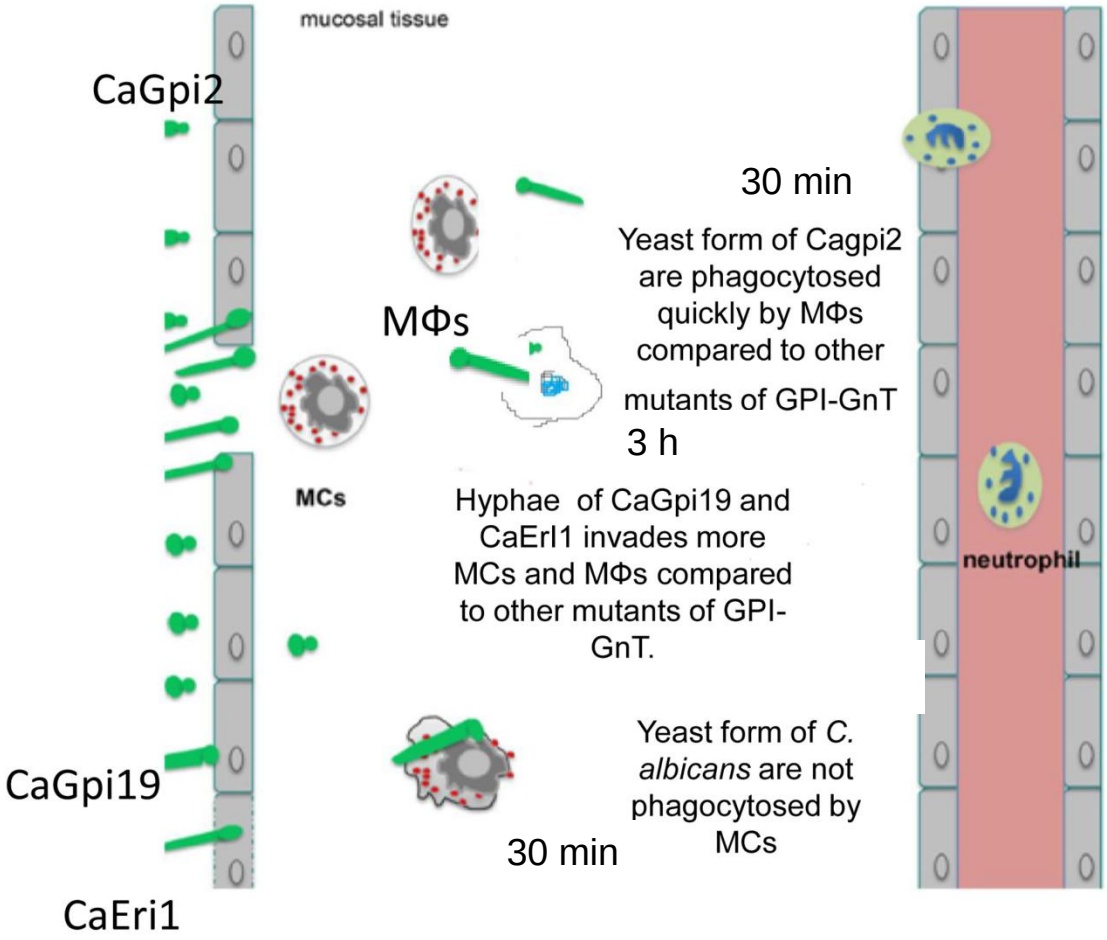

Figure 87: Summary of Mast Cell and *C. albicans* interactions. Our findings indicate that neither yeast form of WT *C. albicans* nor any of the GPI-GnT mutants are phagocytosed by Mast Cells whereas Macrophages can phagocytose yeast form of CaGpi2 to a greater extent compared to other mutants of GPI-GnT. Hyphae of CaGpi19 and CaEri1 are able to invade quickly both Mast Cells And Macrophages. MCs= Mast Cells and MΦs= Macrophages.

MCs act as initiators of immune responses in most intracellular pathogens such as BCG, *Leishmania,* Candida. Not only do they affect other cells by their secretions but they themselves play an active direct role in pathogen clearance – phagocytosis, ROS production and MCET formation. They can be recruited also can proliferate unlike some other immune cells.

Summary

1. Mast Cells (MCs) are specialized 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).

2. In addition to exocytosis, and participation in inflammation, mast cells also have the ability to phagocytize diverse micromolecular, particulate materials and pathogens. Mast cells are found in large numbers beneath the skin, the mucosa of the genitourinary, gastrointestinal and respiratory tracts. So they are the first immune cells to interact with the invading pathogens or environmental antigens (Marshall 2004).

3. To function as immune surveillance cells, MCs have three mechanisms of pathogen recognition: mainly through pathogen associated molecular pattern (PAMP) receptors located on the mast cell surface; complement receptors or immunoglobulin receptors; or recognition of endogenous peptides (Hofmann and Abraham 2009).

4. The various responses of Mast Cells include secretion of various mediators, initiation of inflammation, recruitment and activation of other immune cells, direct phagocytosis, production of many antimicrobial peptides, lymph node potentiation through TNFα granules and Mast Cell Extracellular Traps (MCETs).

5. Two other more recent studies have shown that MCs are capable of direct recognition, and uptake of *M. tuberculosis* through lipid raft microdomains, and are activated by these bacteria to release presynthesized and *de novo* synthesized mediators (Munoz et al. 2003, Munoz, Rivas-Santiago and Enciso 2009).

6. Infections with intracellular pathogens like *Mycobacterium tuberculosis* and *Leishmania* species continues to be one of the major global health threats estimating around 2 million lives annually all over the world. *M. tuberculosis* basically attack through respiratory route.
7. it has been found T lymphocytes and alveolar and interstitial macrophages mostly respond to *M. tubercculosis*. However, other cell types, such as dendritic cells, type II

lung epithelial cells, endothelial cells, and fibroblasts also phagocytose *M. tuberculosis*, suggesting that many other cell types may also respond to the immunological control of *M. tuberculosis* in the lung (Munoz et al. 2003).

8. Mast cells range from 500 to 4,000 per mm^3 in the lungs (Abraham and Malaviya 1997). Because of their location and capacity to release mediators one early study has reported MCs increment and degranulation in the lungs of infected animals during early phase of infection with *M. tuberculosis* (Ratnam et al. 1977).

9. Large numbers of MCs ranging from 7,000 to 12,000 per mm3 are found in the skin (Abraham and Malaviya 1997), predominantly in the superficial dermis, where *Leishmania* is encountered after the bite of infected sand flies (Maurer et al. 2006).

10. Mast Cells are capable of sensing the two phases of *C. albicans*, and it was suggested that MCs participate as an early inductor of inflammation during the early innate immune response to this fungus (Lopes et al, 2015).

11. The GPI anchor in eukaryotes anchors various proteins to the cell surface. The first step in GPI biosynthesis is the formation of GPI-N-acetyl glucosaminyl phosphatidylinositol (GlcNAcPI) from uridinediphosphate- N-acetylglucosamine(UDP-GlcNAc) and phosphatidylinositol (PI) . It is catalyzed by GPI-N-acetylglucosaminyltransferase (GPI-GnT) complex which consists of six different subunits Gpi1, Gpi2, Gpi3, Gpi15, Gpi19, and Eri1.

12. The role of GPI anchor in immune cell interactions was studied by making use of mutants of *C. albicans* lacking each of these subunits individually and thereby having a defect in GPI biosynthesis.

13. The major aim of this study was to explore the direct role for MCs in clearance of these pathogens by both intracellular and extracellular mechanisms. The study highlights the importance of MCs in the disease outcome and vaccine or therapeutic strategy development for these infections.

14. We did a comparative study related to interaction of Mast Cells with *M. bovis* BCG, *Leishmania tropica*, *Leishmania donovani* and WT and mutants of *C. albicans.*

15. So in order to accomplish this we first established BCG infection in C57BL/6 mice with an established protocol (Saxena et al. 2002). The infection was depicted by massive inflammatory cells and granuloma formation which was visualised by Hematoxylin and Eosin staining and Acid Fast Bacterial Staining. Since we were interested in looking for response of Mast Cells to BCG infection we did Toluidine Blue staining. We found there was an increase in the number of Mast Cells. So it is important to study response of Mast Cells on interaction to BCG.

16. RBL-2H3 cell line was used as a model for MCs as it is adherent, easy to cultivate and express same receptors as MCs like FcεRI, c-Kit and other receptors known to interact with bacterial pathogens e.g. CD48 (Lin et al. 1999, Munoz et al. 2003). RBL-2H3 is a rodent mast cell line that has been extensively used to study MC biology in general and MC-pathogen interactions in particular (de Bernard et al. 2005, Mollerherm, von Kockritz-Blickwede and Branitzki-Heinemann 2016, Munoz et al. 2003, Sakurai, Yamaguchi and Sonoyama 2012, Wesolowski, Caldwell and Paumet 2012)

17. To visualize these intracellular pathogens they were stained with CFSE. More than 90% of cells were stained with CFSE and labeling was stable till 24 h in case of BCG and *Leishmania.*

18. We found that uptake of BCG by RBL MCs was also further inhibited with MβCD, Nyastatin and Filipin which disrupts raft.

19. There are two routes of killing of pathogen by RBL Mast Cells such intracellular killing by phagocytic route as well as extracellular killing by degranulation or by Etosis

20. Live BCG were sonicated and were taken up by RBL Mast Cells. This may mimic phenomenon of macropinocytosis.

21. When *L. tropica* and *L. donovani* were cocultured with RBL Mast Cells, only *L. tropica* were taken up by RBL Mast Cells which was confirmed both by microscopy and flowcytometry.

22. We found that yeast form of *C. albicans* were not phagocytosed by RBL Mast Cells whereas hyphal form invades RBL Mast Cells which is similar to earlier reports (Lopes et al. 2015).

23. *C. albicans* study with Mast Cells and Macrophages have shown that different mutants of GPI - GnT pathway affect the efficacy of phagocytosis, invasion of RBL Mast Cells. This also gives an indication of virulence of different mutants of GPI–GnT pathway.

24. When we co-cultured BCG with RBL-2H3 for 24 h we found reduction in CFU compared to control as well as in the transwell system which clearly depicted that direct contact is required for killing of BCG. Co-culture of BCG with RBL cells lead to degranulation leading to release of β-hexosaminidase and histamine accompanied by releasing of LDH at higher time points. The secretion was further confirmed by Transmission Electron Microscopy showing empty granules after co-culture with BCG.

25. When we co-cultured *L. tropica* and *L. donovani* with PMCs of BALB/c mice, which is a strain susceptible to leishmaniasis (Sacks and Noben-Trauth 2002) , we found reduction in their viabilities. Significant decrease was seen in viability of both strains more specifically *L. tropica,* on co-culture with MCs. By using transwell during co-culture studies, we found significant increase in viability of *L. tropica*, as well as *L. donovani.* This indicated that direct cell-cell contact between MCs, and promastigotes of both *Leishmania* species was required for the observed killing of *Leishmania*

promastigotes. Compared to *L. tropica, L. donovani* is more viable on co-culture with MCs in 24 h.

26. Killing of *Leishmania* promastigotes by MCs can either be by phagocytosis or by extracellular means by mediator release or Etosis and Mast cell extracellular traps.

27. We did observe significant ROS production in co-cultured MCs, and also apoptosis of extracellular *Leishmania* parasites, confirmed by Annexin V binding assay. One recent study states that ROS from macrophages is required for killing of promastigotes of *L. amazonensis* (Roma et al. 2016)

28. One interesting observation of our study is that MCs are unable to phagocytose promastigotes of *L. donovani*, but still co-culture with these promastigotes leads to ROS production in MCs.

29. We saw generation of significant amounts of ROS and release of β-hexosaminidase indicating degranulation. This can also be the reason of reduction in viability of *L. donovani*. We have observed release of β-hexosaminidase during initial hour of interaction with *L. tropica* and *L. donovani.*

30. Co-culture of different GPI-GnT mutants of *C. abicans* with Mast Cells and Macrophages showed difference in virulence. GPI2 is the least virulent mutant among all the GPI-GnT mutant of *C. albicans* as the CFU formed after co-culturing with RBL Mast Cells and MH-S Macrophages. Cell death of RBL Mast Cells and Macrophages by Cagpi2 is lesser than wild type. Cell death of RBL Mast Cells and Macrophages by Cagpi19 and Eri1 is more than wild type.

31. In our study, the interaction of MCs with both *Leishmania* species led to significant death of MCs and release of DNA as confirmed by fluorescence microscopy, electron microscopy, and Sytox green staining.

32. Promastigotes of these parasites by DNase treatment on co-culture with PMCs as well as RBL-2H3. This leads to the conclusion that promastigotes of both species are

susceptible to MCETs. Thus MCETs may not only physically restrict them, but also able to kill them to some extent. Thus, this is the first report showing direct interaction of MCs with either

33. MCs act as initiators of immune responses in most intracellular pathogens such as BCG, *Leishmania*, Candida. Not only do they affect other cells by their secretions but they themselves play an active direct role in pathogen clearance – phagocytosis, ROS production and MCET formation. They can be recruited also can proliferate unlike some other immune cells.

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Annexures

OADC (100ml)

Oleic Acid	60 ul
BSA	5 g
Glucose	2 g
Catalase	0.004 g
NaCl	0.85 g

Annexin Binding Buffer (1x)

HEPES	10 mM
NaCL	140 mM
CaCl2	2.5 mM

SOUTONS Medium (1 L, **pH - 7.3**)

L-Asparagine monohydrate	4 g
Citric Acid 1 Hydrate	2 g
KH2PO4	0.5 g
MgSO4	0.5 g
Ammonium Ferric Citrate	0.05 g
Glycerol	60 ml
Tween-80	500 ul

PBS (1 L, pH – **7.3-7.4**)

NaCl	8 g
Na2HPO4	1.44 g
KCL	0.2 g
Kh2PO4	0.2 g

RPMI Medium (1 L, **pH - 7**)

Glucose	1 g
HEPES	2.5 g
NaHCO3	2.6 g
L-Glutamine	0.3 g
RPMI	1 Bottle
Gentamicin	120 mg

RPMI PR¯Medium (1 L, **pH - 7**)

Glucose	1 g
HEPES	2.5 g
NaHCO3	2.6 g
L-Glutamine	0.3 g
RPMI Phenol red free	1 Bottle
Gentamicin	120 mg

RBL Media (1 L, **pH – 7.0-7.25**)

Iscoves medium	8.85 g
MEM medium	4.8 g
Glutamine	0.3 g
NaHCO3	2.6 g
HEPES	1.25 g
Gentamicin	120 mg

The Journal of Immunology

Mast cells' interaction with *Leishmania* leads to effector responses in reducing initial parasite load

ANGAMUTHU SELVAPANDIYAN, Nilofer Naqvi, Kavita Ahuja, Ranadhir Dey, Hira L Nakhasi and Niti Puri

J Immunol May 1, 2017, 198 (1 Supplement) 68.12;

Abstract

Mast cells (MCs) are involved in clearing invading pathogens at the skin surface and play an important role in innate and adaptive defenses. Hence they may have an important role in the control of Leishmaniasis due to the protozoa *Leishmania* that are inoculated by infected sandfly vector on the skin surface. It is still unclear if their contributions occur directly through active pathogen killing or antigen uptake. Our objectives were to use *Leishmania donovoni* the causative agent of fatal visceral Leishmaniasis and *L. tropica* the causative agent of cutaneous leishmaniasis for studying the interactions between the parasite and MCs to clarify if MCs are directly involved in active antigen uptake, and its clearance. We found that co-culture of *Leishmania* with MCs led to significant killing of *L. tropica* and to a lesser extent of *L. donovoni.* Also, while there was significant uptake of *L. tropica* by MCs, *L. donovoni* was not phagocytosed by MCs. Uptake increased with time of exposure in case of *L. tropica.* We also found significant generation of reactive oxygen species by MCs on co-culture with these species of *Leishmania,* which may contribute to their clearance. However interactions of MCs with *Leishmania* led to generation of MC extracellular traps comprising of DNA, histones and tryptase probably to ensnare these pathogens. These results clearly establish that MCs may contribute to host defences to *Leishmania* in a differential manner by actively taking up these pathogens and also by mounting effector responses for their direct clearance by extracellular and non-phagocytic means. This gives us an understanding that MCs can also be used as a target for therapeutic approaches to leishmaniasis.

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J Immunol May 1, 2017, 198 (1 Supplement) 68.12;

ARTICLE INFORMATION

vol. 198 no. 1 Supplement 68.12

Published By	The American Association of Immunologists, Inc.
Print ISSN	0022-1767
Online ISSN	1550-6606
History	Published online June 12, 2017.

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