IDENTIFICATION OF RECEPTORS IN MAST CELLS FOR POSSIBLE ROLE IN ERYTHROPHAGOCYTOSIS

M. Sc Thesis

2017

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

By

Sumeena Karki

Roll No: 219/071

TU Registration No: 5-2-37-299-2010

Supervisors

Supervisor

Prof. Rajani Malla, Ph.D

Central Department of Biotechnology

TU, Nepal

Research Supervisor

Assistant Prof. Dr. Niti Puri

School of Life Sciences

JNU, India

Acknowledgements

This work could not have been complete by the grace of God who has sent me so many people to get involved with me to provide positive energy to move forward. Next to God is my parents especially my mom and sanima who always believed in me.

I am really fortunate to get kind supervision not just one but two supervisors. I am very obliged to thank ***Prof. Dr. Rajani Malla****, Central Department of Biotechnology, Tribhuvan University for believing in me and proving me a chance to complete my dissertation in India's one of the most prestigious university.*

I would like to express my appreciation to my supervisor ***Dr. Niti Puri****, Assistant Professor, School of Life Sciences, Jawaharlal Nehru University for her guidance and suggestions in completion of this project in her lab.I would also like to thank* ***Dr. Krishna Das Manandhar****, Head of Department, Central Department of Biotechnology, Tribhuvan University for allowing me to do my thesis in JNU.*

During my dissertation at JNU, I was guided by ***Priyanka Sharma****. I feel very lucky to be able to work under her direction because she gave me her precious time to guide me so that I can bring out best of me.*

I am extremely thankful to ***Suchhanda Agasti, Sandeep Poudel, Rahul Srivastava, Rangati Verma*** *and* ***Sayad Hadi Toughyan*** *for their endless support. I am also thankful to* ***Pieu Naska, Gagandeep*** *and* ***Nilofer Naqvi*** *for their ideas. I would like to thank my special friend* ***Geetu Bisht*** *for her love and care. A big thanks to* ***Vijay Pal, Ajay Chandila*** *and* ***Anwar ji*** *for providing clean lab environment. Each day spent with you all is memorable.*

I owe a deep sense of gratitude to all my teachers, seniors, friends, juniors and staffs of Central Department of Biotechnology for all those days spent together. You are all wonderful people who kept loving me and guiding me.

The name that I really can't miss is of my best friend ***Apshara Parajuli.*** *Thank you for supporting me always.*

LIST OF ABBREVIATIONS

APC	Allophycocyanin
ACK	Ammonium Chloride Potassium
BSA	Bovine Serum Albumin
CFSE	5-(and-6)-,carboxyfluorescein diacetate,succinimidyl ester
c-kit (CD-117)	Receptor of stem cell factor
DMEM	Dulbecco's Modified Eagle's medium
DNP-BSA	Dinitrophenyl-Bovine Serum Albumin
EDTA	Ethylene diamine tetra acetic acid
FACS	Flurorescence activated cell sorter
FBS	Fetal Bovine Serum
Fc	Fragment,crystallizable
FcεRI	High affinity IgE receptor
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter
HEPES	N-[2-100 hydroxyethyl] piperazine-NO-[2-ethanesulfonic acid]
Hb	Hemoglobin
IFNγ	Interferon γ
i.p	Intraperitoneal
MC	Mast Cell
MFI	Mean Fluorescence Intensity
MOPS	3-(N-morpholino)propanesulfonic acid
PFA	Paraformaldehyde
PBS	Phosphate Buffered Saline
PS	Phosphatidylserine
RBC	Red Blood Cell
RBL-2H3	Rat Basophil Leukemia cell line
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
SEM	Standard Errors of the Means
SSC	Side Scatter
t-BHP	tert-butyl hydroperoxide
VEGF	Vascular Endothelial Growth Factor

CONTENTS

Chapters Page No.

ABSTRACT

Mast cells are specialized secretory cells that are major participants in allergic inflammation. Release of different mediators like histamines and cytokines by mast cell causes activation of professional phagocytes causing inflammation and clearance of pathogens or apoptotic cells via phagocytosis. A number of investigation is done to study the interaction between macrophages and mast cells during inflammatory process that are modulated by number of receptors both activating and inhibitory receptors present on the surface of the mast cell.

Here we review the current state of knowledge about the mast cell response to normal as well as oxidatively damaged erythrocytes and identify the receptors that play key role in erythrophagocytosis.

For observing eyrthrophagocytosis P815 mastocytoma cell line was used and analysed by fluorescence microscopy and confirmed by flow cytometer. Rat basophilic cell line RBL-2H3 was used for the identification of receptors present in mast cell using RT-PCR technique.

Uptake of oxidatively damaged RBCs than normal RBCs by P815 mast cells was observed. From PCR result we found that same receptors are present in mast cells which were present on macrophages involved in erythrophagocytosis. So, out of 8 receptors, 5 receptors i.e., SIRPα, RAGE, VCAM-1, TIM3 and RC3a were present in spleen macrophages and 3 receptors i.e., RAGE, VCAM-1 and TIM3 were present in mast cell of which VCAM-1 and TIM3 expression being new finding in case of RBL-2H3.

The leading theory was proposed to know whether these interactions between mast cells and phagocytes like macrophages is just limited to inflammatory responses or it can be involved in other non-inflammatory responses as well like in case of erythrophagocytosis. From our study we conclude that the receptors are involved in uptake of oxidatively damaged erythrocytes which elicited role of mast cell as scavengers.

Keywords: RBL-2H3, RT-PCR, SIRPα, RAGE, VCAM-1, TIM3, RC3

CHAPTER 1
INTRODUCTION

1.1 Background

Erythrocytes are also called as Red Blood Cells (RBCs) which account 99% of total blood cells in mammals. RBCs are red in color due to presence of haemoglobin which is responsible for transport of oxygen from lungs to different organs. Cell metabolism releases carbon dioxide which is then carried back by RBCs to lungs. They also maintain blood pH as well as blood pressure level by balancing hydrogen and nitrogen level. Almost all vertebrates except crocodile icefish have RBCs. RBCs of mammals are biconcave in shape and have nucleus in their early stage of their life and nucleus is ejected before releasing into bloodstream whereas RBCs of the vertebrates with few exceptions are nucleated (Ruud JT, 1954). Life span of RBC in human is 120 days and their count normally ranges from 4.10-5.90 X 10^{12}/L (Vajpayee et al., 2011) and 40 days in mice (Wang et al., 2010).

RBC count is found to be elevated in conditions such as stress (high altitude and hypoxia), hemoconcentration and dehydration (for example, from severe diarrhea), cardiovascular disease , renal cell carcinoma and other erythropoietin-producing neoplasms, polycythemia vera, smokers whereas, decreased in conditions such as anemias, chronic renal failure, hemolysis, leukemia, malnutrition, hemorrhage and failure of marrow production (Ferri FF.,2011). Homeostasis condition is maintained by the cells of Reticulo-endothelial system (RES) which are mainly of two types named as professional and non-professional phagocytes. Professional phagocytes include Polymorphonuclear (PMN) granulocytes, Monocytes, Dendritic cells and Macrophages whereas Non-professional phagocytes include epithelial cells, fibroblasts and other cells. Macrophages are essential for RBC production, maintenance and clearance (de Back et al., 2014). Erythropoiesis condition is promoted not only under homeostasis but also under stress conditions by macrophages. Imbalance in homeostasis condition leads to membrane deformability which may be caused by any of the two ways: (1) mutations in various membrane/ skeletal protein or globin genes causing secondary effects on the membrane (e.g. sickle cell disease) and (2) Stressed condition (Osmolarity, Salinity, oxidants, temperature, senescence, etc). Removal of these stressed cells or senescent normal red cells is either by deformation-induced membrane fragmentation or by phagocytosis by macrophages in spleen (Mohandas and Gallaghar, 2008). Phagocytes maintain homeostasis with the help of scavaneger receptors by limiting inflammatory

response upon removal of pathogens as well as senescent cells from the host (Peiser and Gordon, 2001). Inflammatory response is initiated when macrophages detect pathogens or apoptotic signals. These signals also activate mast cells that mediate innate and modulate adaptive immune responses. Mast cells (MCs) releases cytokines which serves as chemoattractants for neutrophils, eosinophils and dendritic cells that clears pathogens and apoptotic cells by phagocytosis at the targeted site (Bulanova and Paus, 2010).

There are many studies that show mast cell involvement in pathogen clearance by exocytosis. Release of different mast cell mediators, proteases, and cytokines controls *Plasmodium* spp., *Leishmania* spp., *Trypanosoma* spp., and *T. gondi* infection (Lu and Huwang, 2017). Howerever, there are only few studies that have enlightened role of mast cells as phagocytes for uptake of pathogens by endocytosis. In 1979, Sher and his colleagues first observed involvement of mast cell as phagocytes mediated by complement system in case of *Salmonella*. Later in 1997, phagocytosis by mast cell in case of noninvasive strains of *E.coli, Enterobacter cloacae* and *K.pneumonia* has also been reported (Abraham and Malaviya, 1997). Macrophage inclusions have similarity to paranuclear inclusions in mast cells known to arise from ingestion of erythroblast nuclei. These ferritin containing granules exchange between macrophage and mast cell. Without degranulation the transfer of matrix content takes place from cytoplasmic granules to phagocytic vacuoles (Spicer et al., 1975). The study from above papers provides an overview that mast cells not being professional phagocyte can act as phagocyte under certain conditions like microbial infection, excessive hematopoesis of early erythrocytes and autoimmune diseases like anaemia. Till now prevalence of erythrophagocytosis by mast cells have been reported in case of rats (Spicer et al., 1975) and cats (Madewell et al., 1983) only.

Mature RBCs are particularly susceptible to oxidative damage. They have high oxygen-carrying capacity because they are rich in heme iron which generate H_2O_2 and lipid peroxides. Decreased ability to detoxify these reactive oxygen species (ROS) plays a causative role in tissue injury in many disease conditions, including cardiovascular diseases, neurological disorders, aging and even cancers. Clearance of these old or abnormal RBCs takes place in spleen by macrophages. This results in a decreased life span of RBCs leading to anemia (Wang et al., 2010). Studies till now are not sufficient to provide strong insight for mast cells direct involvement in erythrophagocytosis mechanism and their presence in humans and murines as well. So involvement of mast cells in this mechanism could provide insight in relation with several diseased or inflammatory condition as well as identification of erythrophagocytic receptors in mast

cell similar to macrophages will provide an approach in identifying molecular mechanisms involved in erythrophagocytosis by mast cells.

A body-wide system of phagocytic cells defined by anatomists is known as the '**reticuloendothelial system**' (RES) and descendents from these arise various types of macrophages, the major phagocytic tissue cell (Friedman, 2012). The cells of the mononuclear phagocytic system (MPS) have capability of both the 'recognition' and the 'mopping up' phase of the adaptive immune response. They are divided into two types: Professional and Non-professional phagocytes. Professional phagocytes include Ploymorphonuclear (PMN) granulocytes, Monocytes, Dendritic cells and Macrophages whereas Non-professional phagocytes include epithelial cells, fibroblasts and other cells which can take up particles (Kahn & Line, 2007). Through 'innate' receptors, macrophages and dendritic cells act as tissue sentinels responding to infection and tissue damage and signaling the alarm to adaptive immunity via both antigen presentation and the release of powerful cytokines. It is well recognized that the immune system and metabolism are highly integrated, and macrophages, in particular, have been identified as critical effector cells in the initiation of inflammation. Once an adaptive immune response is established the antibody promotes and amplifies phagocytosis, while T lymphocytes serve to activate macrophage microbicidal activity. Different types of phagocytic cell include:

- Macrophage: The resident and long-lived tissue phagocyte either free in the tissues, or 'fixed' in the walls of blood sinuses, where they monitor the blood for particles, effete red cells, keeps vital air sacs free of particles and microbes . Recognition is via antibody and/or complement bound to it, which greatly enhances phagocytosis.
- Osteoclast: A large multinucleate macrophage responsible for resorbing, shaping bone and cartilage. It is regulated by cytokines such as TNF-α and IL-1.
- Mesangium: Mesangial cells are specialized macrophages which phagocytose particularly complexes of antigen and antibody material found deposited in the kidney.
- Dendritic cells: These cells are weak phagocytic Langerhans cells('veiled' cells) of the epidermis that migrate through the lymphatic vessels or blood to lymph nodes and spleen leading T-cell stimulation which recognizes foreign antigens in association with cell-surface antigens coded for by the MHC, Different follicular dendritic cells presents antigen to B cells that specialize in trapping antigen–antibody complexes.

- Blood platelets: Although primarily involved in clotting, are able to phagocytose antigen–antibody complexes, and can also secrete some cytokines, such as transforming growth factor β (TGF-β).
- Kupffer cells: Specialized macrophages that make up a major fraction of the phagocytic cells in the body, found in the liver where they remove dying or damaged red blood cells and other material from the circulation.
- Polymorphonuclear leucocyte (PMN): Major phagocytic cell of the blood.
- Monocyte: Bone marrow derived macrophage which matures in tissue which patrols the surface of blood vessels to repair sites of damage or infection.
- Microglia: The phagocytic cells of the brain derived from a special precursor cell that enters the brain before birth and divides within the brain.
- Sinus: It is the tortuous channels present in spleen, liver, etc. through which blood passes to reach the veins, through which the lining macrophages removes damaged or antibody-coated cells and other particles (Rabinovitch, 1995).

They are highly elastic, highly responsible to fluid stresses and have strong structural resistance. In human the average life span of RBC is 120 days (De Flora & Magnani, 2013). The lipid membrane bilayer is composed of cholesterol and phospholipid in equal proportion (by weight). Outer membrane has Phosphatidylcholine and sphingomyelin, while inner layer constitute mostly phosphatidylethanolamine and phosphatidylserine (PS), and minor phosphoinositide. RBCs have different types of energy-dependent and energy-independent phospholipid transport proteins for maintaining phospholipid asymmetry. Specific membrane proteins of RBC (like flotillins, stomatin, G-proteins, and adrenegic receptors) are found to be associated with “lipid rafts” enriched in cholesterol and sphingolipids. 25 transmembrane proteins defining various blood group antigens exhibit diverse functional heterogeneity. They serve as transport proteins, as adhesion proteins (red cells with other blood cells and endothelial cells) and as signaling receptors. Membrane stability of RBC is maintained by family of Spectrin proteins which includes dystrophin, actinin and utrophin(Nikinmaa, 2012). Imbalance in homeostasis condition leads to membrane deformability which may be caused by any of the two ways: (1) mutations in various membrane/ skeletal protein or globin genes causing secondary effects on the membrane (e.g. sickle cell disease) and (2) Stressed condition (Osmolarity, Salinity, oxidants, temperature, senescence, etc). Depending upon the condition, there is structural changes in membrane done by membrane proteins like “Flippases” which move phospholipids from the outer to the inner monolayer and PS from inner to outer whereas in an energy dependent manner “floppases” do the opposite against a concentration gradient . “Scramblases” also work in an energy independent manner which moves phospholipids bi-directionally down their

concentration gradients. These structural changes (PS exposure at outer surface) are easily encountered by macrophages. Removal of these stressed cells or senescent normal red cells is either by deformation-induced membrane fragmentation or by phagocytosis by macrophages in spleen (Mohandas & Gallagher, 2008).

Table 1.1 List of adhesion molecules of erythrocytes

Adhesion Molecules of Erythrocytes	**Alternative Name**
CD36	Platelet Glycoprotein IV
VLA-4	α4β1 integrin
CD47	Integrin-associated protein
CD58	Lymphocyte-associated antigen-3
ICAM-4	-
B-CAM/LU	Laminin receptor
Band 3	Anion exchanger(AE-1)

The transfer of immune complexes and pathogens from RBC to macrophages is a tightly controlled process leading to clearance of red blood cells (RBC) known as erythrophagocytosis. Macrophages are essential for RBC production, maintenance and clearance. Erythropoiesis condition is promoted not only under homeostasis but also under stress conditions by macrophages. After 120 days in circulation, senescent or damaged RBC is cleared in spleen and liver(De Flora & Magnani, 2013).

Macrophages actively participate in erythroid development by providing iron for heme synthesis and by phagocytosing expelled nuclei during final erythroid differentiation (human bone marrow can contain 5–30 erythroblasts surrounding a central macrophage). Splenic red pulp macrophages returns iron to bone marrow by recycling senescent and damaged erythrocytes after catabolism of hemoglobin molecules. Erythroblastic island culture showed that ferritin produced by macrophages is released by exocytosis and engulfed by erythroblasts via endocytosis. Iron is released from ferritin upon acidification and proteolysis inside the erythroblast thus being

subsequently available for heme production in the erythroid precursor cell (de Back et al., 2014).

The direct association between the central macrophage and the erythroblasts is essential for erythroid maturation and prevention of cell death. The first molecule identified on the surface of both central macrophages and erythroblasts is Erythroblast macrophage protein (Emp), a protein that pro- motes binding between them (Bieker, 2008). Absence of Emp leads to aberrant erythropoiesis and increased levels of apoptosis in erythroblastic island cultures. During the final stage of terminal erythroid differentiation, the erythroblast expels its nucleus as part of its maturation into a reticulocyte. The macrophages phagocytose the expelled nucleus, aiding erythropoiesis (Figure 2.2). Not only this both the macrophage and the erythroblast/reticulocyte are equipped with adhesion molecules which promotes the retention of the nucleus on the surface of the macrophage before phagocytosis takes place. After expulsion, Emp and β1 integrin distributes on the nucleus maintaining the interaction between the nucleus and the macrophage. Studies performed with fetal liver erythroblasts demonstrate that expelled nuclei expose phosphatidylserine (PS) on their surface for 10min after expulsion .That means the time frame between nucleus expulsion and phagocytosis is 10min and PS might be assisting in the adhesion of the nucleus to the macrophage prior to phagocytosis. PS exposure on the cell surface is considered an apoptotic signal targeting cells undergoing cell death for clearance by phagocytes expressing PS receptors and also signifies ATP depletion(Van Lancker, 2006). PS is a membrane component normally situated on the inner leaflet of the cell membrane. An ATP-dependent amino phospholipid translocase enzyme maintains the lipid asymmetry by keeping PS on the inside of the plasma membrane (Kidd,P.M., 1998) . Furthermore, the PS-binding protein lactadherin (also known as MFGE8) is crucial for phagocytosis of extruded erythroblast nucleus which normally serves as a bridging molecule between an apoptotic cell and a phagocyte. At last an macrophage protein enzyme DNase II degrades nuclear DNA after phagocytosis.

Macrophages take up immune complexes and pathogens bound to complement receptor1 (CR1) on the RBC and can clear intracellular pathogens from the RBC leaving the RBC intact and return them into circulation. In the open circulation, RBC passes through endothelial slits of sinus where they are checked for their loss of deformability and removal of variety of intracellular inclusions such as Howell-jolly bodies(inclusion of nuclear chromatin remnants) (Al, 2000), Heinz bodies (inclusions of denatured hemoglobin caused by oxidative damaged), siderocytes (RBC containing granules of iron that are not part of cell's hemoglobin) (Lappin, 2001) and Pappenheimer bodies (inclusion bodies formed by phagosomes engulfing excessive amounts of iron) are

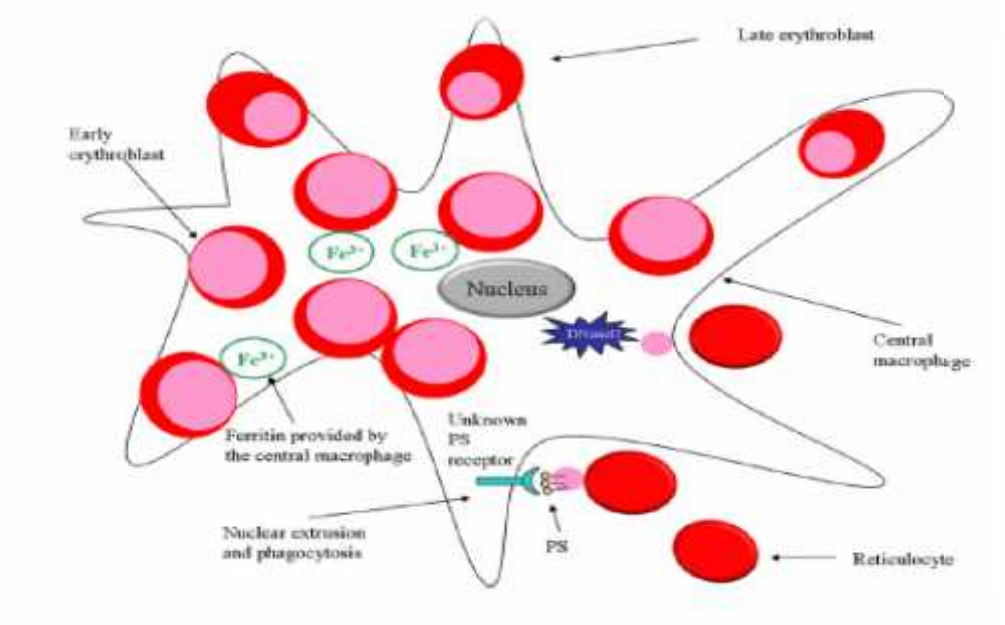


Figure 1.1: Interaction between early and late erythroblast with macrophage. Expelled nuclei from erythroblast is degraded by DNaseII. Ref: (de Back et al., 2014).

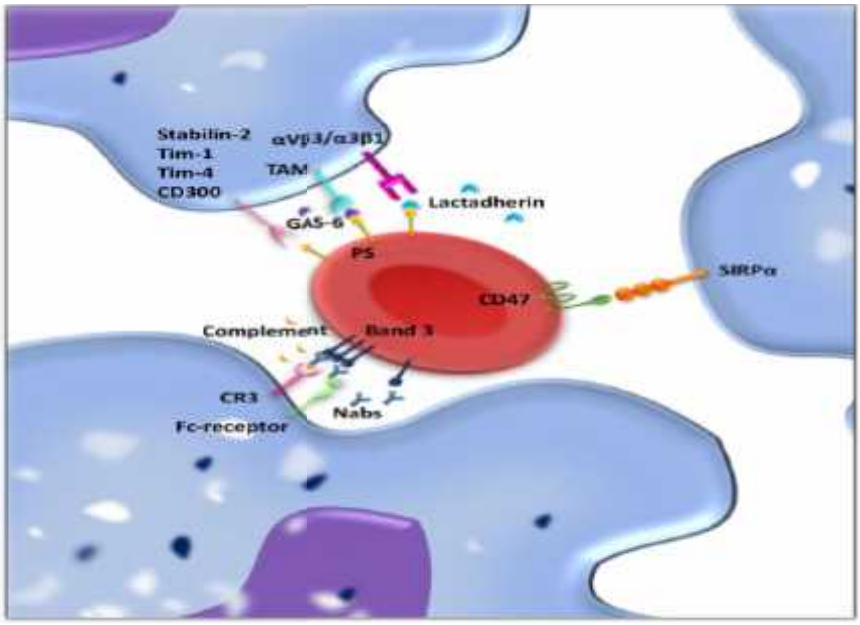


Figure1.2: Interaction between macrophages and mature RBCs regulating clearance. Aged RBC express PS, CR1 and CD47 on their surface that binds to Stabilin-2 or TIM1/3 or CD300, C3b and SIRPα respectively.Ref: (Klei et al., 2017)

Table 1.2 Receptors present in macrophages involved in erythrophagocytosis

S.No	Receptor	Ligands	Reference
PS RECEPTORS			
1.	BAI-1	PS	(Sierra et al.,2013)
2.	MER	PS[Gas6,ProteinS]	(Sierra et al.,2013)
3.	TAM	PS[Gas6]	(Klei et al.,2017)
4.	Stabilin-2	PS	(Sierra et al.,2013)
5.	TIM-1	PS	(Sierra et al.,2013)
6.	TIM-3	PS	(Bevers & Williamson, 2016)
7.	TIM-4	PS	(Sierra et al.,2013)
INTEGRIN RECEPTORS			
8	αvβ5	PS,vitronectin [MFGE8,thrombospondin]	(Sierra et al.,2013)
9	αvβ3/α3β1	PS [Lactadherin]	(Klei et al,.2017)
10	CR1	MBL,C1q,C4b,C3b,C3bi	(Sierra et al.,2013)
11	CR3	C3 and C1q[DAP12]	(Sierra et al.,2013)
12	CR4	iC3b	(Sierra et al.,2013)
Ig SUPERFAMILY RECEPTORS			
13	FCγRIIa	IgG3 ≥ IgG1 = IgG2	(Sierra et al.,2013)
14	RAGE	Aβ,AGEs,PS and HMGB1,C1q	(Sierra et al.,2013)
15	SIRPα	Myelin[SP-A,D;CD47]	(Sierra et al.,2013)
16	SIRPβ	Unknown ligand[DAP12]	(Sierra et al.,2013)
SCAVANGER AND RELATED RECEPTORS			
17	CD36	Ox-LDL,Ox PS[thrombospondin]	(Sierra et al.,2013)
18	CD68	Ox-LDL	(Sierra et al.,2013)
19	CD163	[Erythroid Precursors]	(VanGorp et al.,2010)
20	CD300	PS	(Klei et al.,2017)
OTHER RECEPTORS			
21	Ephrin-2	HTK	(de Back et al.,2014)

removed by residential macrophages of spleen. The molecular mechanism for clearance of inclusion bodies is largely unknown. However, removal of Heinz bodies takes place via vesiculation where red blood cells (RBCs) loses aggregated hemoglobin, becomes small and more dense finally phagocytosed by macrophages (older cell vesciculate more than younger ones) (de Back et al., 2014).

1.2 Rationale of Study

In general it is believed that as erythrocyte age in circulation, many changes occur in these cells that are recognized by the reticulo-endothelial system (RES) resulting in phagocytosis of the senescent/damaged erythrocytes by professional phagocytes like macrophages. Recent studies have shown that mast cell acts as endocytes under parasitic infection and in many cases variable number of mast cell has shown to be correlated with anemia. Endocytosis of erythrocytes by mast cell has been observed in rats. But it is still not known if mast cells have any role in the clearance of RBCs for homeostasis under normal conditions or during oxidative stress, inflammation and other disease condition. Thus, present study aims to know possible role of mast cells in erythrophagocytosis under normal and oxidative condition.

1.3 Objectives

General Objectives

Identification of the receptors involved in erythrophagocytosis of mast cells.

Specific Objectives

1. Studying erythrophagocytosis by mast cells under normal or during oxidative stress condition.

2. Comparing the receptors involved in erythrophagocytosis between macrophages and mast cells.

3. Designing primers for the receptors involved in erythrophagocytosis.

4. Analysis of expression of these receptors on mast cells from *in vitro* studies.

1.4 Research Hypothesis

Null hypothesis:

$H_0$1: There is no difference in uptake of normal and oxidatively damaged erythrocytes by mast cells.

H_o2: Receptors present in mast cell and macrophages are same involved in erythrophagocytosis.

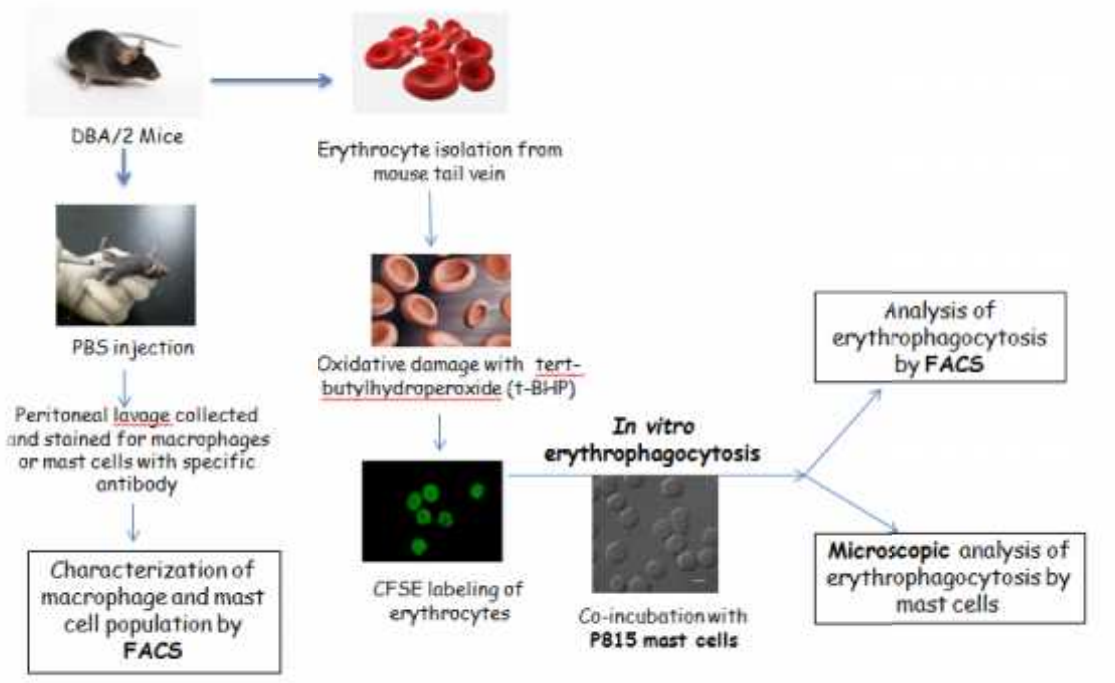
Alternative hypothesis:

$H_1$1: There is difference in uptake of normal and oxidatively damaged erythrocytes by mast cells.

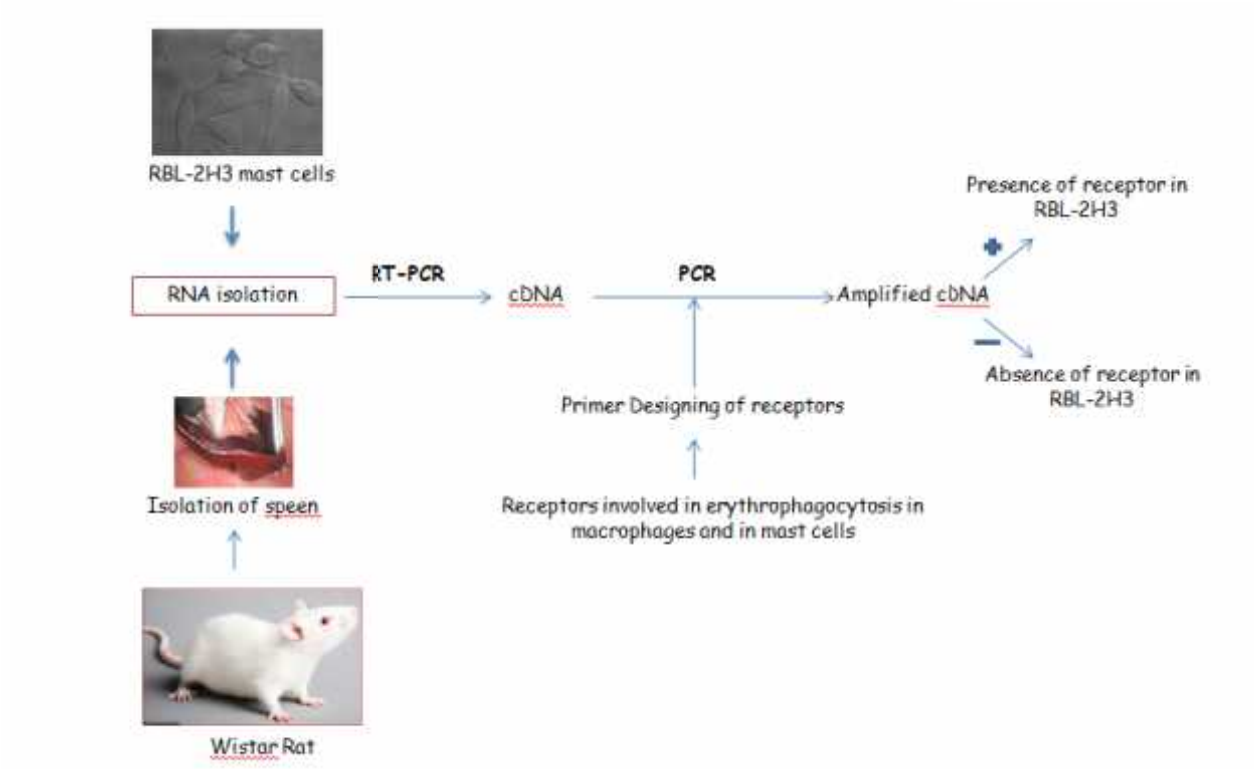
$H_1$2: Different receptors are involved in erythrophagocytosis present in mast cell and macrophage.

1.5 Work Plan

1.5.1 Work plan for observing erythrophagocytosis



1.5.2 Work Plan for identification of receptors on mast cell



CHAPTER 2
LITERATURE REVIEW

The term “Endocytosis” was coined by Christian deDuve in 1963 which is a Greek derived word in which “endon” means within, “kytos” means cell and “osis” means process (Rieger et al., 2012). So the process in which any kind of substance is brought inside the cell via in folding of plasma membrane and vesicle formation from the external milieu by utilizing energy is called endocytosis. This process is common in eukaryotes, amoeba and some bacteria like *Gemmata obscuriglobu* but not in archea*(Acton, 2012).* It is mainly of three types: Phagocytosis (cell eating), Pinocytosis (cell drinking) and Receptor mediated endocytosis (Morvillo & Schmidt, 2015). Phagocytosis plays specialized role depending upon cell type. For example in amoeba intake of food (bacteria and protozoa) takes place by phagocytosis whereas in case of multicellular animals it eliminates aged or damaged cells as well as invading microorganisms from the body serving as defense system. Pinocytosis takes place almost in every cell which uptake small amount of fluid into small vesicles which then fuses with lysosome following degradation. In endocytosis the best characterized pathway is Receptor-mediated endocytosis in which the macromolecules first bind to specific cell surface receptors which are present in specialized regions of the plasma membrane, called clathrin-coated pits. Small clathrin-coated vesicles are formed from plasma membrane as a bud containing the receptors and their bound macromolecules (ligands) which fuses with early endosomes and then their contents are sorted and decided whether to recycle or transport to lysosomes. Main importance of endocytosis is, it allows the entry of large polar molecules directly into the cells without contacting cell membrane that are not able to get into the hydrophobic plasma membrane and also plays a key role in regulation of intracellular signaling (GM, 2000)**.**

2.1 Phagocytosis

Phagocytosis of invading microorganisms by immune cells was first discovered by the father of cellular immunology Ilya Metchnikoff in 1882, for which he was awarded the Nobel Prize. A Greek-derived term “phagocytosis” means the cellular process of eating which includes recognition, engulfment, and degradation of large (>0.5 μm), particulated organisms or structures (Mukherjee et al., 2002). Most cell types, including unicellular as well as multicellular organisms have the capacity to phagocytose. In multicellular organisms phagocytosis is mostly performed by specialized, professional phagocytes:

macrophages, dendritic cells (DCs), and neutrophils. By the innate immune system together with inflammation, phagocytosis composes the first line of defense against multicellular organisms especially in jawed vertebrates, including mammals, phagocytosis also helps to initiate the more specific adaptive immune response through antigen-presentation to T lymphocytes (Litman et al., 2005). Cell biology of phagocytosis has been mainly established on bone marrow-derived tissue macrophages. Macrophage phagocytosis has two main targets: dead resident cells (apoptotic or necrotic) and live invading microorganisms. The level of expression of the receptors involved in phagocytosis may change under different stimuli such as inflammation (Poon, Hulett, & Parish, 2010). However, phagocytosis can also activate the respiratory burst, which produces toxic reactive oxygen species (ROS). Phagocytosis has been traditionally extensively studied in pathological conditions in which molecular pathways are involved in the recognition, engulfment, and degradation of the targets. Based on these steps three-step model of phagocytosis has been developed: Chemotaxis, Adherence and Engulfment.

2.1.1 Chemotaxis

In this process the damaged or apoptotic cells releases chemo attractant molecules (known as Find me signals) such as fractalkine/CX3CL1 and extracellular nucleotides (ATP,UDP) (Hemmi et al., 2009), C5a molecules after complement activation, lysophosphatidylcholine (LPC) and so on which stimulate chemotaxis of macrophages, neutrophils and monocytes to the target cell.

2.1.2 Adherence

Phagocytes recognize cells via signals present on cell surface. Normal cell posses “don’t-eat-me” signals (also known as self-associated molecular patterns (SAMPs)) which enables phagocyte to recognize their targets through “eat-me” signals. Different cell express different “Eat-me signals” e.g. Alarmins (e.g. uric acid/monosodium urate (MSU) crystals and high-mobility group box 1 (HMGB1) molecules) in case of necrotic cells, Pathogen Associated Molecular Patterns(PAMPs) in case of pathogens, Apoptotic-cell-associated molecular patterns (ACAMPS) in case of early apoptotic cells and both Alarmins and ACAMPS in late apoptotic cells. It is found that clearance of target cell dependents on both the quantity and quality of positive (i.e., ‘eat-me’) and negative (i.e., don’t eat-me’) phagocytic signals. The receptors involved in recognition vary according to their targets like scavenger receptors (e.g., CD14/TLR4 complex, c-type lectins) in conjunction with Toll-like receptors (TLRs) for detection of PAMPs, receptors involved in recognition of exposure of phosphotidylserine (PS) like T-cell immunoglobulin mucin (Tim-1) and Tim-4, Stabilin-2, brain-specific angiogenesis

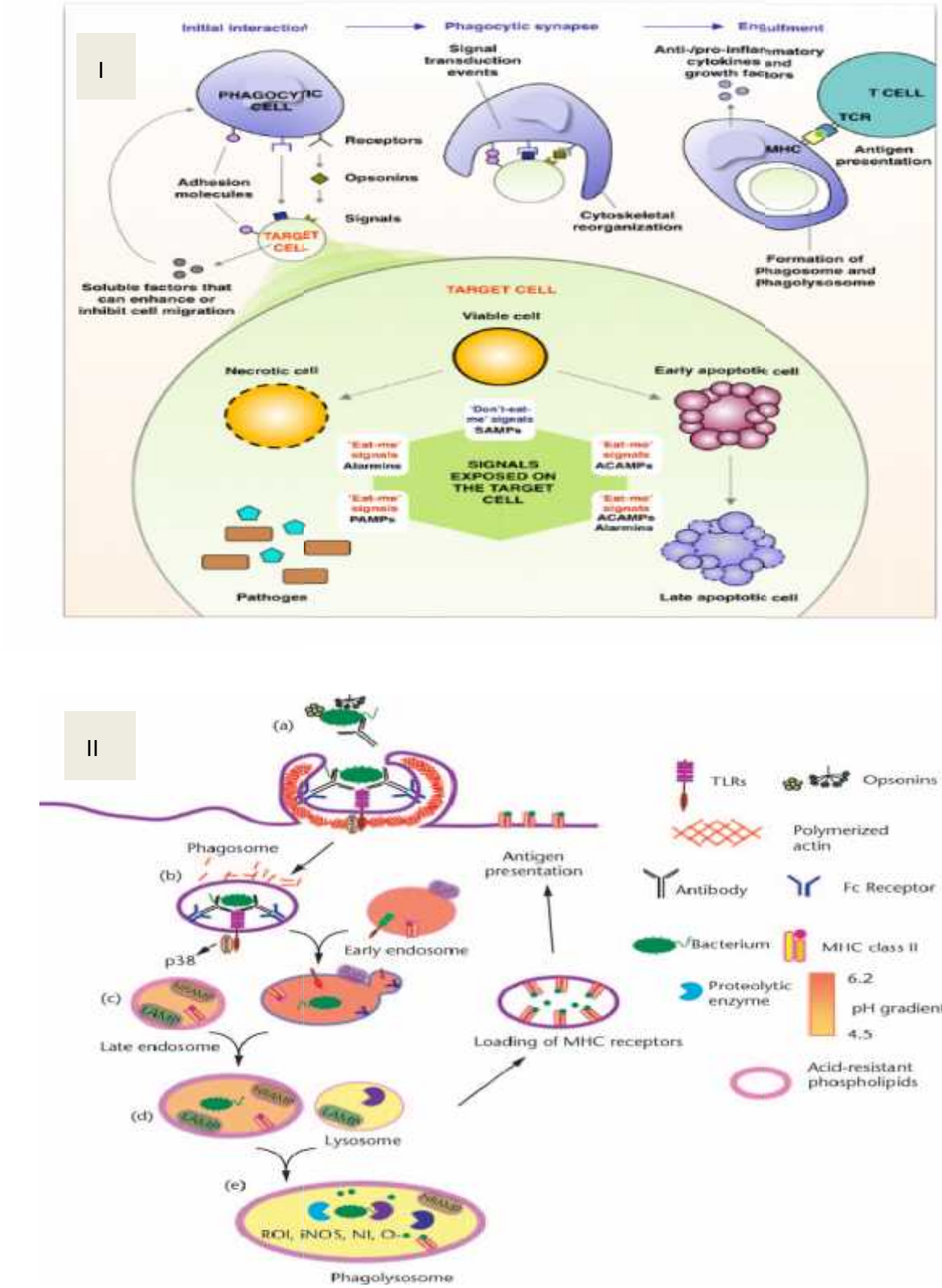


Figure 2.1: Process of Phagocytosis. (I)Cell uptake by phagocytes mediated by the type of 'eat-me' and 'don't-eat-me' signals exposed. Formation of 'Phagocytic synapse' inducing cytoskeletal reorganization.

II)Degradation of internalized cell takes place in phagolysosome finally presenting peptides to T cell through both MHC class I and II complex. Ref: (Poon et al., 2010)

inhibitor 1 (BAI1) and scavenger receptors, as well as the opsonins b2-glycoprotein I (b2GPI), milk fat globular-EGF factor 8 protein (MFG-E8) and Protein S. Several other candidates include bridging molecules, anionic oligosaccharides such as bacterial lipopolysaccharides, heat shock protein 60, soluble opsonins, antibodies such as IgG and proteins of the complement system such as C3b that bind to phagocyte Fc receptors and complement receptor 3 (CR3) mediating phagocytosis (Underhill & Goodridge, 2012). Adhesion of a target cell and phagocyte forms 'phagocytic synapse'.

2.1.3 Engulfment

Engulfment of cell takes place in a phagosome through formation of engulfment synapase via receptors and their ligands. This phagocytic or engulfment synapse initiates various signaling events triggering cytoskeleton reorganization through an actin-based mechanism (Figure 2.1a). The target cell is then engulfed and delivered to phagosome (Figure 2.1b). Phagosome and lysosome then fuse forming phagolysosome which again fuses with other cell organelles like endosomes (Figure 2.1 c and e). Maturation takes place through series of fusion and fission events leading to degradation of damaged or apoptotic cell in phagolysosome in less than 2 hr. After degradation especially DCs and macrophages present antigens to the adaptive immune system derived from the target cell via both major histocompatibility complex (MHC) class I and II molecules (Poon et al., 2010).

Overall, phagocytosis is considered as beneficial phenomenon essential for maintaining tissue homeostasis in health as in disease since it acts as an effector mechanism to clear infectious agents as well as dying cells (Sierra et al., 2013). Uptake of apoptotic cells by macrophages releases vascular endothelial growth factor (VEGF) and transforming growth factor-b (TGF-b), which promotes cell growth, wound healing as well as has a vital role in maintaining immunological tolerance against cell-associated antigens. Also at the site of tissue injury it facilitates process of also has a fundamental role in regeneration and angiogenesis. The type of immunological (anti- or a pro-inflammatory) response initiated from the site of phagocytosis depends upon the signals possessed by the cell type. For example display of certain PAMPs on bacteria such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), flagellin and peptidoglycans as well as virus-derived-RNA trigger the release of pro-inflammatory cytokines alerting host to invading pathogens which then activates TLRs. In contrast anti-inflammatory cytokines are produced during clearance of dying cells in nonphlogistic way. Hence the interaction between the phagocyte and the target cell plays a critical role in determining whether

the internalized material will raise an immunogenic or a tolerogenic adaptive immune response (Poon et al., 2010).

2.2 Homeostasis

Macrophages are relatively long-lived, biosynthetically active cells that express diverse surface receptors and secretory products in order to maintain homeostatic condition by clearing up apoptotic cells and cellular debris and also iron recycling (Kahn & Line, 2007). But upon imbalance in homeostasis initiate chronic inflammatory process which contributes to persistent tissue as well as organ damage. For maintaining homeostasis, Scavenger receptor (SR) present on professional phagocytes plays a vital role in innate immunity and tissue homeostasis. SRs are surface glycoprotein able to bind modified lipoproteins that controls inflammation. They are divided into 5 families namely Class A, B, C, E and F. SR-A, SR-B and CLA-1 can recognize apoptotic cells. Phagocytosis by SR is promoted by accumulation of the receptor in clathrin-coated pits and also by ITAM domain of FcγR receptors of endocytic SR. SR-A is expressed higher in lamina propria macrophages of gut, alveolar macrophages in the lung and Kupffer cells of the liver and also in some dendritic cell whereas, MARCO (a type of SR-A) is restricted to subpopulations of macrophages of lymph nodes and spleen. On the other hand SR-B (SR-BI and CD36) has role in adhesion by its ligand-binding properties with apoptotic cells. Besides macrophages, SR present on dendritic as well as B cells also presents modification-specific antigens to T cells via MHC-I. The type of inflammatory mediators depends on the type of phagocytes involved. For example, SR-A functions as an anti-inflammatory molecule in case of macrophage and as pro-inflammatory in dendritic cells (Peiser & Gordon, 2001). Hence phagocytes maintain homeostasis with the help of SR by limiting inflammatory response upon removal of pathogens as well as senescent cells from the host.

In macrophages iron trafficking is current focus of study. They maintain iron homeostasis because among all body tissues, especially red blood cells are the one that needs frequent repairing.

2.3 Mechanisms involved in erythrophagocytosis

Several mechanisms have been proposed for clearance of senescent RBC via macrophages. This is because RBC does not undergo classical apoptosis since they lack nucleus, mitochondria and other cell organelles and exhibit similarities with programmed cell death, a non-inflammatory process termed as “eryptosis”(Lang & FÃ¶ller, 2012). Proportion of RBC that is daily cleared is a mere 0.8% per day. Some of the mechanisms involved are discussed below:

2.3.1 Clearance based on Phosphotidyl Serine (PS) exposure

Eryptosis process relatively short i.e., 1-48hr and is characterized by efflux of K+ ions, cell shrinkage, membrane blebbing with shedding of micro- or nanovesicles, increased influx of Ca2+ and activation of lipid scrambling with subsequent PS exposure. PS exposure is early and general feature of apoptosis best characterized by “eat-me” signal exposure of PS in the outer leaflet of apoptotic cell plasma membrane that leads to recognition and phagocytic engulfment by professional phagocytes or non-professional neighbor cells (Nagata & Nakano, 2017). Phagocytes have two classes of membrane protein receptors that recognize exposed PS. The first class bind directly to PS and the second class mediates interaction without binding to PS directly via soluble proteins (or opsonins) which has affinity for both PS and receptor protein. The direct membrane receptors functions in binding PS-exposing apoptotic targets and then indirect receptors activate engulfment process. The first class of proteins include TIM4, BAI1, Stabilin-2 and RAGE which activates (except TIM4) Rac1 pathway for phagocytosis (Figure 2.2A). CD300 is immunoreceptor tyrosine based inhibitory motif(ITIM) containing myeloid receptor that bind to PS via Ca2+ ions and through association with adaptor molecules convey signal for engulfment via PI3K/Akt pathway. The second class of recognition involves binding of soluble proteins like Gas 6 /ProteinS to a TAM receptor (Tyro3, Axl and Mer) of phagocytic cell which causes dimerization and autophosphorylation causes docking of intracellular signaling molecules conveying signal(Figure 2.4B). Rac1 pathway gets activated when MFG-E8 binds with PS with its NH2-terminal C1 and C2 domain via RGD motif to its receptor integrin $\alpha v\beta 3/5$ on phagocytic cell. C1q globular region binds with PS and its tail with C1q receptor of macrophage then finally in masking of recognition motif for LRP causes binding of β2-GP1 with PS exposing cells triggering engulfment process (Bevers & Williamson, 2016).

In case of erythrocytes, recognition and engulfment takes place via PS exposure by apoptotic T lymphocytes by macrophages in a non-inflammatory manner. In normal RBC, PS is found on the inner leaflet of membrane (Wang, 2014). From an in-vivo experiment it is observed that with RBC age, there is increase in PS exposure as well as its clearance.

The receptors like Axl, Tim4, Tim3 and Stabilin-2 are expressed in red pulp macrophages that means PS-exposing RBC are cleared up by one or more PS/ligand receptor pairs in spleen. Other trigger for phagocytosis includes loss of phospholipid asymmetry or stress induced PS exposure (de Back et al.,2014).

2.3.2 Band -3 based mechanism

Senescent RBC is detected by their increased amount of denatured globin and decreased kinase activity. Their surface bears increased amount of adsorbed IgG and alteration of large band-3 associated polysaccharide causes reduced association with cytoskeleton. Band3 is a transmembrane protein which constitutes 25% of total RBC membrane protein has two domains. First one is the membrane spanning domain which is recognized by natural occurring antibodies (Nabs) that catalyses anion exchange, and second one is a cytoplasmic domain that helps RBC to regulate its structure and function by binding to different proteins(Bamberg & Passow, 2012). Different mechanisms has been hypothesized clearance by band-3.One being oxidative damage of hemoglobin produces hemi chromes which causes clustering of Band-3 and Nabs binds to the cluster. Another one is recognition of neoepitope of band 3 or “senescent cell antigen” present in aged RBC by Nabs which generate C3b2-IgG complexes in presence of active complement. Once opsonized with C3b, it will form complex with Nabs. Now recognition and phagocytosis of complement opsonized RBC takes place via expression of CR1 (C3b-receptor, CD35) and CR (iC3b-receptor, CD11b/CD18) present in red pulp macrophages. But phagocytosis through this mechanism results in secretion of pro-inflammatory cytokines and also senescent RBC adhere to vascular endothelial cells same as normal RBC suggesting that clearance may depend on macrophages either by immunoglobulin bound to RBC surface or by other adhesion mechanism but not by adhesion to splenic and other reticuloendothelial system endothelial cells (de Back et al., 2014; Telen, 2000).

2.3.3 RBC Complement Receptor 1

Complement Receptor 1(CR1) is involved in providing protection against blood-borne pathogens in humans and other higher primates via immune-adherence clearance (IAC) in which CR1 on RBC binds with complement opsonized particles bearing C3b/C4b in circulation (Tsokos, 2004). Immune complex removal efficacy increases upon increase in immune complexes bound to RBC CR1 compared to unbound opsonized immune complexes. Alteration of RBC membrane deformability caused by downstream signaling of CR1 after particle binding results in clustering of CR1 on RBC surface which increases binding of opsonized particle. This increases ATP secretion and hence particle uptake by phagocytes (de Back et al., 2014).

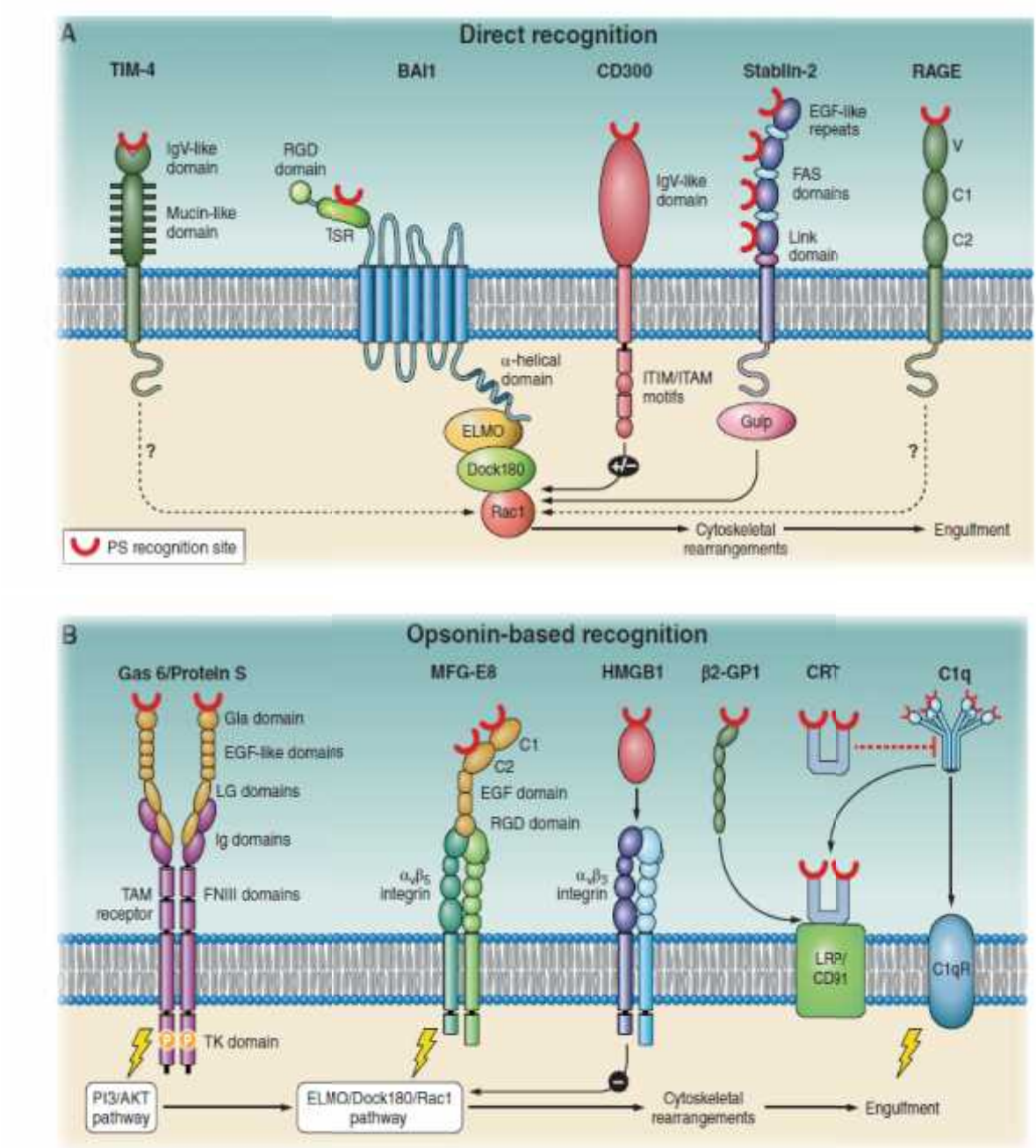


Figure 2.2: Recognition of both Phosphatidylserine and receptor protein exposing apoptotic cells either directly (A) or via opsonin proteins (B). First group of proteins like BAI1, CD300, RAGE and Stabilin-2 except for TIM-4 activates Rac1 pathway for phagocytosis. On the other hand, soluble proteins like Gas6 orProtein S and MFG-E8 binds to specific receptor activating phagocytic pathways. Calrecticulin (CRT) interacts with Lipoprotein like receptor (LRP, CD91) modulating clearance of apoptotic cells via C1q pathway.

Ref: (Bevers & Williamson, 2016)

2.3.4 CD-47 mediated RBC clearance

CD-47 can act as both “don’t eat-me” signal as well as “eat- me” signal but still there is debate .The CD47-SIRPα interaction function as marker of “self” on RBC providing strong negative signal for phagocytosis in spleen. CD-47 is a ubiquitously expressed protein that binds with inhibitory immunoreceptor signal regulatory protein alpha (SIRPα) which is expressed by macrophages as well as other myeloid cells. But conformational changes in CD-47 can switch it into activating from inhibitory one (Weiskopf et al., 2016). Aged RBC generally have tendency to bind the CD47-binding partner thrombospondin-1(TSP-1) which enables their phagocytosis via red pulp macrophages. It is also seen that there is 20% reduction in CD47 expression in old RBC than younger RBC which may be reason behind the clearance of senescent RBC resulted from total signals generated through macrophage inhibitory and pro-phagocytic receptors (de Back et al., 2014). The complexity of RBC clearance by CD47-SIRPα is due to their dual role in RBC uptake.

Not only this multifactor’s are involved in RBC clearance such as, infection inflammation, chemical substances, oxidative stress, or osmotic imbalance So, whether an RBC will be cleared from the circulation or not depends upon the balance between “eat me” and “don’t eat me” signals (Klei et al., 2017).

2.4 Inflammation and phagocytosis

Macrophages being the major phagocytic cell which provides first line of defense of our immune system but over-activation of macrophages leads production of high levels of reactive oxygen intermediates and the inflammatory cytokine TNF-α which leads to wide variety of chronic inflammatory conditions, including diseases such as Rheumatoid arthritis, Psoriasis, Alzheimer’s disease, Atherosclerosis ,Sickle cell disease and even cancer. Once a macrophage detects foreign materials an inflammatory response is initiated that may induce the production of pro-inflammatory cytokines, chemokines or activation and maturation of the macrophage (Lee, 2013). But apart from macrophages there is involvement of another cell during inflammation known as “mast cells”.

2.5 Mast cell

Mast cells are generally involved in allergic and anaphylactic reactions which get activated by aggregation of their IgE receptors (FcεRI)(Saito & Okayama, 2005). In case of inflammatory disease, mast cell activation is triggered by anaphylotoxins, immunoglobulin-free light chains, superantigens, neuropeptides and cytokines which leads to release of mediators without degranulation. In general mast cell activation takes place through cross-linking of their surface receptors for IgE causing degranulation releasing pro-inflammatory, vasoactive and nociceptive mediators like histamines, IL-6, IL-8, PGD2, tryptase and Vascular Endothelial growth factor (VEGF) that establishes mast cells peculiar role in innate or adaptive immunity (Holgate, 2012). Mast cells are precursors of bone marrow cells, matures in local tissue producing chemo attractants like stem cell factor (SCF), nerve growth factor(NGF),RANTES and monocyte chemo attractor protein 1(MCP-1). Mast cell regulates immune response process termed as "activation" or "piecemeal" degranulation in which without degranulation indication of secretion takes place through ultra structural changes of their electron dense granular core (Theoharides & Kalogeromitros, 2006). ATP and other nucleotides are present at a high concentration within the cytoplasm of cells. The concentration of extracellular nucleotides is low in healthy tissues(5–10 mM) may rise(up to 100 mmol/l) in damaged organs due to(i) the sheer stress, osmotic swelling, ischemia, inflammation, apoptosis,or necrosis leading to the passive leakage of ATP from the damaged cell; (2) vesicular release; and (3) channel mediated release and can be sensed by surrounding mast cells expressing varieties of purinoceptors which triggers degranulation, cytokine secretion, chemotaxis, and apoptotic cell death. ATP which is stored in their secretory granules and released upon stimulation. ATP is acknowledged as a "signal of danger" or damage associated molecular pattern triggering innate immune system in inflammatory settings together with pathogen-associated molecular patterns(PAMPs). Increase in ATP causes elevation in the intracellular Ca2+ concentration leading Ca2+ influx. Membrane permeabilization and nonselective cationic pore formation takes place within milliseconds with increased permeability to Ca2+ and equilibration of Na+ and K+ gradients.

Then degranulation takes place causing histamine release. Phosphorylation of intracellular proteins takes place then Cytokine/chemokine is expressed and released upon triggering of intracellular signaling cascades such as MAP kinases or Jaks/STATs). Increase in extracellular nucleotides serves as powerful chemoattractants for neutrophils, eosinophils, and dendritic cells. Increased ATP induces apoptosis with typical features phosphatidylserine externalization and membrane blebbing, cytoplasm condensation, and DNA fragmentation. Purinergic receptor-mediated blebbing,

membrane permeabilization and phosphatidylserine flip are reversible when the receptor activation is relatively brief (<10–15 min). All this causes inflammation and then clearance of apoptotic cells via phagocytosis mainly by macrophages, dendritic cells and thymocytes (Bulanova & Bulfone-Paus, 2010).

Mast cells are c-kit^{hi}, FcεRI+ granular cells of the innate immune system that range from 500 to 4,000 per mm^3 in the lungs, 7,000 to 12,000 per mm^3 in skin, and 20,000 per mm^3 in the gastrointestinal tract. Mast cells amplify autoimmune responses through multiple mechanisms. As demonstrated by several in vio and in vitro studies, mast cells are involved in clearance of pathogens as well as foreign particles (Acton, 2013). Previously it was thought that clearance was by exocytosis mechanism but there are very few papers that have talked about endocytosis mechanism involved but extensive study about its significance and implications of this property is yet to be examined. Involvement of mast cell as phagocytes was first observed by Sher and colleagues in case of Salmonella and their interaction was mediated by complement. Then after phagocytosis by mast cell in case of noninvasive strains of *E.coli, Enterobacter cloacae* and *K.pneumoniae* has been reported. Adherence of bacteria by mannose-lectin FimH to mast cell caused 40% reduction of bacteria. Macrophages and neutrophils involves combination of nonoxidative system in which acidification of phagocytic vacuoles and lysosomal granules takes place , and oxidative killing mechanism involves production of superoxide anions, singlet oxygen, hydroxyl radicals and hydrogen peroxides. Whereas in case of mast cell, oxidative brust is generated as well as other mechanisms include inhibition by secreting granule chymase or protease II or by triggering of high-affinity FcεR1 receptor (Abraham & Malaviya, 1997).

When mast cells were ultrastructurally examined then evidence of their endocytic activity were found. Ultrastructure study of mast cell via electron microscopy shows the presence of granules which are very dense, roughly spherical and crowded together in cytoplasm(Ross & Pawlina, 2006). Whereas mast cell structure differs from cell to cell in granule shape, size and density. Mast cell also contained different vacuoles believed to be counterpart of large metachromatic and ortgochromatic inclusions measuring 2-6µ in diameter (Sokolov, 1982). They are mainly classified into 4 types: 1)Large heterophagic (endocytic) vacuoles measuring upto 5µ, 2)small and multiple heterophagic vacuoles measuring upto 2.5 µ, 3)Communal vacuoles with coalesced granules and, 4)Intermediate vacuoles. Large and small heterophagic vacuoles contains endocytosed materials like erythrocytes or reticulocytes, remnants of unidentified homogenous materials like neutrophils as well as leukocyte. Macrophage inclusions have similarity to paranuclear inclusions in mast cells known to arise from ingestion of erythroblast nuclei.

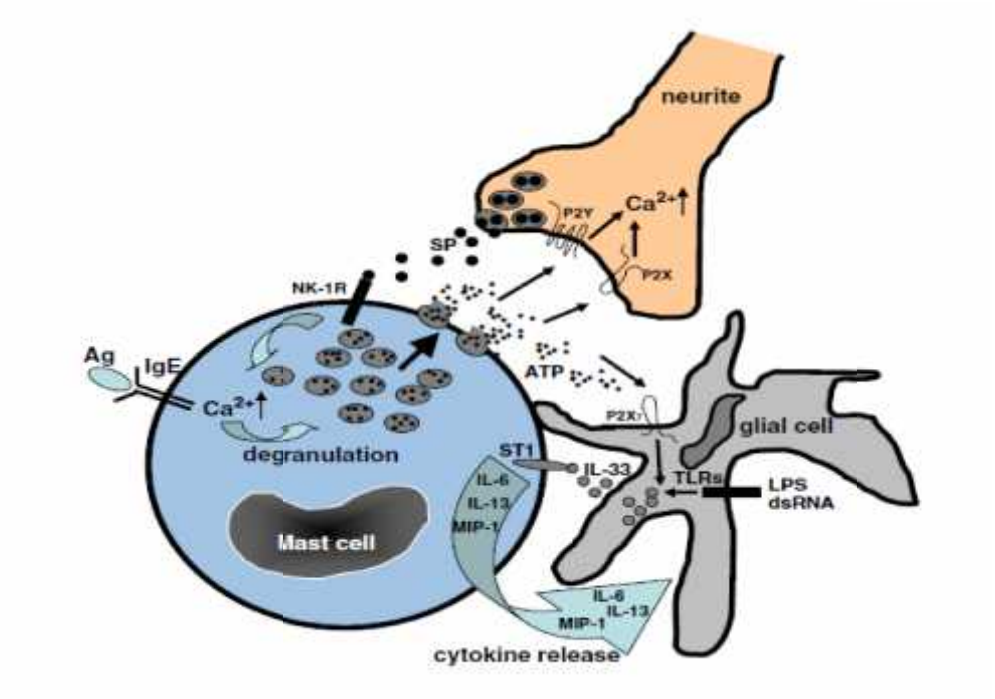


Figure2.3: Ag exposure leads to IgE Fc-receptor mediated mast cell activation following degranulation involving intracellular communication to dendritic and glial cells. Ref:(Bulanova & Bulfone-Paus, 2010)

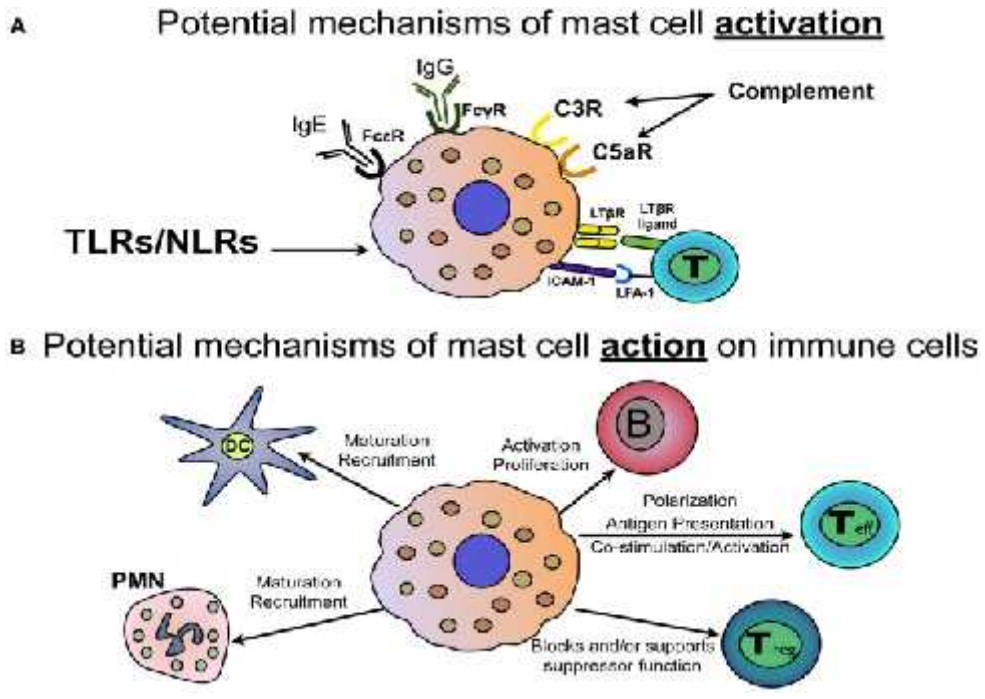


Figure 2.4: Mast cell activation and response in autoimmunity.(A) Mast cell activation by different receptors leading to activation, maturation and polarization of immune cells. Ref:(Brown & Hatfield, 2012)

Mast cells are associated with lipid or hemosiderin known as siderophage-associated mast cells have low content of mucosubstance and strong basic protein. These ferritin containing granules bodering siderophages exchanges between macrophage and mast cell. Without degranulation the transfer of matrix content takes place from cytoplasmic granules to phagocytic vacuoles. Hypersensitive state is acquired via loss of granule without secretion or exocytosis of granules. Large inclusions contains mucosubstances acquired from heparin –rich granules and altered granules are converted to heterophagosome rich in hydrolases. These release of lysosomal hydrolases into extracellular space leads fragmentation of RBC which are then ingested by macrophages.

The study from above papers only correlates phagocytic mechanism of mast cell with respect to bacteria or young RBC rather than senescent ones and studies have limited results due to low prevalence of erythrophagocytosis by mast cells encountered in animals like cats (Spicer et al., 1975). These papers discusses about the possibility and lack evidences. However we can get view that mast cells not being professional phagocyte can act as phagocyte under certain conditions like microbial infection, excessive hematopoiesis leads to endocytosis of early erythrocytes and autoimmune diseases.

2.5.1 Receptors present in mast cell that are similar to macrophage erythrophagocytic receptors

2.5.1.1 RAGE

RAGE is known as Receptor for Advanced Glycation end products (AGE), a member of immunoglobulin super family of cell surface receptors an IgSF containing one V-type, two C2-type domains and single transmembrane domain .AGE formation is considered to be one of the major etiologic factors in aging- and diabetes-related chronic inflammatory diseases through activation of Ras-MAPK pathway promoting cellular dysfunction including altered superoxide production($O_2^{\circ -}$), defective chemotaxis and phagocytosis ,and breaking immune tolerance. Glycated albumin (GA), an AGE can induce mast cell death not stimulated by high-affinity IgE receptor(FcεRI) but via GA-induced mitochondrial death pathway undergoing aoptosis. RAGE induces elevated $O_2^{\circ -}$ generation in mitochondria since GA but not FcεRI stimulation causes membrane potential collapse due to overload of Ca^{2+} resulting mitochondrial disintegrity, cytochrome c release and caspase-3/7 activation contributing to immunocompromised and inflammatory conditions. Diabetic patients have abnormal RBC membranes caused

Table 2.1 Receptors present in mast cell that are similar to macrophage erythrophagocytic receptors

S.no	Receptors	Mast cell	RBL-2H3	References
1.	CR3	+	C3aR,C5aR	(Klei et al., 2017; Péterfy, 2008)
2.	SIRP	SIRPα	SIRPβ	(Klei et al., 2017; Karra & Levi-Schaffer, 2011)
3.	CD300	CD300a	CD300b,c,d	(Martínez-Barriocanal et al.,2010)
4.	TIM1,TIM3 AND TIM4	+	Unknown	(Klei et al., 2017; Phong, 2016)
5.	αVβ3, αV	+	+	(Klei et al., 2017)
6.	VCAM-1	+	Unknown	(de Back et al., 2014; Hamawy, 2013)
7.	Ephrin-B2	+	Unknown	(Salvucci & Tosato, 2012; Yoneda et al., 2006)
8.	RAGE	+	+	(Bevers & Williamson,2016; Yoshimaru et al.,2008)

by oxidative damage to membrane proteins and lipids deficient in reduced glutathione leading production of AGEs. In high glucose concentration aldimines are formed then rearrange to form ketoamines leads to decrease in membrane fluidity and increased lipid peroxidation hence shortening RBC life span (Yoshimaru et al., 2008; Telen,2000).

2.5.1.2 CD300

It is leukocyte surface molecule encoded by cluster of genes present on 17q25.1 human chromosome. CD300 family has six molecules that when present in complexes can modify signaling properties of individual receptors to both agonist and/or antagonist at the same time. CD300a (IRp60 or CRMF-35) and CD300f (IREM-1) acts as inhibitory signals whereas CD300b and CD300e (IREM-2) triggers signals but CD300c and CD300d function is still uncertain (Hattori & Seifert, 2017). CD300a is expressed on NK cells, monocytes, MCs, T-cell subsets, granulocytes and dendritic cells. CD300 family constitute new mechanism which precisely control immune responses including cell growth, survival and differentiation, adhesion, migration, phagocytosis, cytokine production and/or cytotoxicity. CD300 complexes are formed by intergration of more than two receptors. Intergral membrane proteins undergoes secretory pathway starting in rough ER proceeding through golgi apparatus compartments. Fusion of vesicles to plasma membrane, post translational modification including N- and O-glycosylation and pre-assembly of activated immune complexes with transmembrane adaptor modules takes place in ER. Pre-synthesized granules containing CD300 complexes are exported to plasma membrane during up-regulation which in normal condition stored in cell cytoplasm. CD300 family members are stimulated by different process like CD300c is increased following FcεRI cross linking in mast cells (Martinez-Barriocanal et al., 2010).

2.5.1.3 TIM (T-cell immunoglobulin- and mucin-domain-containing molecule)

It is type-I transmembrane protein with IgV-like and mucin domains, a transmembrane region and a cytoplasmic region. In mouse there are 8 TIM genes in which Tim-1, 2, 3, and 4 encode functional proteins whereas Tims 5-8 appear to be pseudo-genes. In human there are 3 TIM genes. From the sequence analysis it is found that human TIM-1shows homology with murine Tim-1 and Tim-2 and, murine Tim-3 and Tim-4 to human TIM-3 and TIM-4. Also mouse Tim-1 is homologous to rat kidney injury molecule 1(*Kim1)* (78% identity) and with human and monkey hepatitis A virus cellular receptor (hHAVcr1) with 42% identity. Tim-1 is expressed on activated and Th2-polarized T cells, highly

expressed on the surface and intracellular of Th1 and Th17 and also expressed by DCs, germinal and regulatory B cells, natural killer T (NKT) cells and mast cells. Tim-1 interacts with PS which facilitates cell death recognition and clearance in the injured kidney. The Tim-2 shows 85% nucleotide sequence identity with Tim-1. Tim-2 is mainly expressed on B cells and dendritic cells that can enhance T cell proliferation and cytokine production (IL-2, IL-4 and IL-10) as well as on hepatocytes and non-immune cells of the bile duct epithelial in the liver.Tim-3 is expressed by Th17, regulatory T cells, CD8+, natural killer (NK) cells, peritoneal macrophages, monocytes, DCs and mast cells. Tim-3 recognizes apoptotic cells by binding to PS mediating phagocytosis which then triggers a pro-apoptotic signal on T cells. Tim4 is expressed on macrophages and mature lymphoid DCs. Tim-4 is also a receptor for PS which facilitate uptake of apoptotic thymocytes as well as plays active role in development of food allergy. It is generally found that different types of macrophages use different Tim molecules for phagocytosis (Binh Le Phong, thesis, 2006).

2.5.1.4 SIRP (Signal Regulatory Protein)

SIRP is a family of surface receptors which consists of SIRPα, SIRPβ and SIRPγ in which SIRPα and SIRPβ has opposite roles. SIRPβ positively regulate neutrophil migration and macrophage phagocytosis (Zhang et al., 2015). SIRP-α belongs to the Ig-superfamily having three Ig-like domains in the extracellular portion and contains four tyrosine residues in their intracellular domain which form two ITIMs, thus negatively regulating signal transduction pathways. SIRP-α is expressed on human basophils and CBMC as well as on HMC-1 cells. Expression of SIRP- α on mast cells is still not evident in murine besides having their homologue. When SIRP-α is coligated with FcεRI, phosphorylation of FcεRI ITAMs is decreased leading to inhibition of mast cell degranulation. Also reduction of intracellular Ca^{2+}mobilization, influx of extracellular Ca^{2+} and the activation of the MAP kinases Erk1 and Erk2 caused inhibition of IgE-induced mast cell mediator release. CD47 is identified as the ligand SIRP-α which is an integrin-associated transmembrane protein which is expressed on many cell populations. The interaction between ligand and its receptor also inhibits FcγR dependent/independent phagocytosis by macrophages. Moreover, SIRP-α inhibits the development of Th2 cytokines that drive allergic responses by inhibiting production of IFN-γ by mature dendritic cells (Karra & Levi-Schaffer, 2011).

2.5.1.5 Complement Receptor (CR)

Anaphylatoxins C3a and C5a are generated by activation of the complement cascade. These fragments specifically bind to their subsequent membrane receptors named as C3aR and C5aR (CD88). These receptors are coupled to a Gi protein and belong to the seven-transmembrane receptor superfamily. They are expressed in myeloid cells as well as in non-myeloid cells and tissues which functions as vasodilation, mast cell degranulation, smooth muscle contraction, and recruitment of immune cells to the site of inflammation. C3aR and C5aR is constitutively expressed by glial cells in the central nervous system and their expression is increased in inflammatory conditions (Benard et al., 2004).

2.5.1.6 VLA-4 (Very late Antigen-4) and VCAM-1 (Vascular cell adhesion molecule-1)

VLA-4 is an integrin (α4β1) expressed on Hematopoetic stem cells (HSCs). VLA-4 ligates with VCAM-1 present on stromal and endothelial cells, which makes important adhesion pair involved in leukocyte attachment to the endothelium at inflammatory sites and also in lymphocyte differentiation. VLA-4 is involved in adhesion of B cell precursors and the ligation (VLA-4/VCAM-1) mediates T-cell proliferation. The molecular interaction between VLA-4 on erythroblasts and VCAM-1 on central macrophages is found to have significance in stress erythropoesis and also maintaining erythroblastic island integrity (de Back et al., 2014).

2.5.1.7 Ephrin (Eph)

In mammals, Eph family receptor is the largest family of tyrosine kinase receptors. There are Ephrin (Eph receptor interacting) ligands which binds to Eph receptors which promotes high order clustering and promotes bidirectional signaling(Eph receptor in forward signaling and Ephrin ligands in reverse signaling). Both Ephs and ephrins are divided according to their sequence similarity and binding specificities into into two groups as A and B. There are nine EphA (EphA1-8 and EphA10) and five EphB (1-4 and 6). The Eph receptors are expressed in all embryonic germ layers and functions in homeostasis and cell signaling. This signaling induces cell repulsion, directs cell positioning and migration, tissue morphogenesis, axon guidance and development of the vascular system. Central macrophage and erythroblast also interact via the ligand-receptor pair EphB4 (expressed on the surface of erythroblasts) and Ephrin-2 that are found on macrophages. Knockout mice lacking EphrinB2 or EphB4 expression targeted

to the endothelial cells display a severely compromised vascular system and die at their midgestation period (Salvucci & Tosato, 2012).

Mast cells originate from the bone marrow and develop into c-kit + FcεRI+ cells. They provide first line of defense providing both innate and adaptive immunity. However complications may arise upon mast cell dysfunction which may be due to excessive proliferation of mast cells also known as mastocytosis or due abnormal reactivity of mast cells known as Mast cell activation syndrome (MCAS). Both affect functions in potentially every organ system, particularly the skin, the gastrointestinal tract and the cardiovascular and nervous systems. Mastocytosis can be broadly divided into two groups: Localised mastocytosis and Systemic mastocytosis. Localised mastocytosis is localised to a single tissue whereas systemic mastocytosis involves one or more tissues. Almost 45% of patients show Anemia as the manifestations of systemic mastocytosis. Also in MCAS patients, anemia is the most common issue affecting red blood cells (Krishnan and Jaishankhar, 2017). In cats, erythrophagocytosis by neoplastic mast cells contributed to a part of anemia (Madewell et al., 1983). So arise in anemic condition with increase of mast cell lead to the dissussion point whether increased hemolysis was due to involvement of professional phagocytes whose activation was lead by mast cell via exocytosis or involvement of mast cells functioning as phagocytes.

CHAPTER 3
MATERIALS AND METHODOLOGY

3.1 Reagents and supplies

The following reagents including Cloroform,Isopropanlol,ethanol,Ethylene Diamine Tetra Aceric Acid (EDTA), Trizol (TRI reagent),Diethyl pyrocarbonate (DEPC), agarose and primers were purchased from Sigma Aldrich(India).Reagents such as RNase inhibitor ,Oligo(dt)18,dNTPs mix,M-MuLV reverse transcriptase enzyme and its buffer and Nuclease free water (GeNei) were purchased from NEB (UK). 100bp DNA ladder was purchased from NEB (UK) and GeneDireX (Taiwan). All solutions were prepared in MQ. Roswell Park Memorial Institute(RPMI-1640) Medium, Dulbecco's Modified Eagle's medium(DMEM), tert-butyl hydroperoxide (t-BHP), HEPES (N-[2-100 hydroxyethyl]piperazine-NO-[2-ethanesulfonic acid]) and Bovine Serum Albumin(BSA) were purchased from Sigma Aldrich (MO, USA). Allophycocyanin (APC) Hamster anti-mouse CD80 and isotype control Armenian Hamster IgG2 were purchased from BD Pharmingen (USA). Costar(NY,USA) was source of all disposable plastic culture wares. All the syringes and needles were purchased from Becton Dikinson(Singapore), Allophcocyanin (APC) anti-mouse CD117 from Bio-legends (San Diego, CA), anti-mouse F4/80 from E-biosciences (India), Paraformaldehyde(PFA), 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE), and flourmount from Thermofisher (India). Fetal Bovine Serum(FBS) , Minimum Essential Medium Eagle with Earle’s salts and Iscove’s Modified Dubecco’s Medium from Gibco, Life Technologies(Grand Island, NY, USA).

3.2 Animals

Inbred DBA/2 mice (8-12 weeks old, 24-28g body weight) and Wistar rats (2 months old, 250-300 g body weight) were used throughout this study. Animals were bred and maintained in microbe free environment in the animal house facility at Jawaharlal Nehru University (JNU), New Delhi. The animals were housed in positive-pressure air conditioned units (25˚C, 50% relative humidity) and kept on a 12h light/dark cycle. Water and mouse chow were provided *ad libitum.* All the experimental protocols were conducted strictly in compliance with the Standard Operating Procedures (SOP) for Institutional Animal Ethics Committee (IAEC)of the CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals), Government of India. This study was duly approved by Institutional Animal Ethics Committee (IAEC) of Jawaharlal Nehru

University. All the mice were randomly assigned to experimental groups .Experiments were designed so as to use the minimum number of mice and rats.

3.3 Maintenance of cell lines

The Rat Basophilic Leukaemia (RBL-2H3) mast cell line was a kind of 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 and Iscove's Modified Dulbecco's Medium and supplemented with 25mM HEPES, 50µg/ml gentamicin sulfate, and 20% heat-inactivated Fetal Bovine Serum (FBS) in a humified atmosphere containing 5% CO2 at 37^{o}C. This cell line was maintained as adherent and subcultured by trypsinization.

P815 mastocytoma cell line was obtained from National Centre for Cell Sciences, Pune, India. They were maintained in RPMI 1640 medium with 2mM Glutamine and 10% heat-inactivated FBS in a humified atmosphere containing 5% CO2 at 37^{o}C. This cell line was maintained as non-adherent and subcultured by replenishing media.

Viability of cells were counted using trypan blue exclusion method using formula, Percent (%) Viability = (Live cells/Total number of cells) X 100.

3.4 DNP-BSA induced activation of RBL-2H3

1 X 10^{6} cells/ml were seeded in a 6 well plate overnight. TIB-142 was used as a source of IgE. RBL-2H3 was treated with IgE at 1:100 concentrations for 18h. Cells were crosslinked with 200ng of Dinitrophenyl-Bovine Serum Albumin (DNP-BSA) added and incubated for 15 mins and 45 mins at 37^{o}C, 5% CO_2 incubator. Cells were harvested by trypsinization, washed three times with Phosphate Buffer Saline (PBS).

3.5 Isolation of blood and spleen cells

For the analysis of blood erythrocytes, 40-50µl blood sample was drawn from tail–vein of DBA/2 mouse.

Wistar rat was euthanized by CO2 asphyxiation with the help of guideline provided IAEC and spleen was collected after dissection of rat. In brief, ventral surface skin was wiped with dettol and with 75% ethanol and a small incision was made in the skin over the caudal half of the abdomen with a scissor. Spleen was removed from left side of the rat and placed in a sterile petriplate having 1X PBS made in DEPC treated water. Spleen was

placed in wire guage and minced with the help of syringe plunger .Single cell suspension was prepared and filtered through cheese cloth in a 15ml falcon tube and centrifuged at 1500rpm for 5 mins at 4^{o}C. RBC was lysed by adding 5 ml of 1 X ACK lysis buffer to the spleen cell pellet followed by incubation on ice for 5 mins. Then equal amount of PBS was added and centrifuged at 1500rpm for 5 mins at 4^{o}C. The cell pellet was washed twice with PBS containing 2% FBS and counted under hemocytometer.

3.6 Processing of macrophage and mast cell population in mouse peritoneal cells

Mice were euthanized by CO2 asphyxiation before dissection. With the help of forcep, skin was lifted just below abdominal region and a small cut was made with the help of a scissor.From the small cut, a straight cut towards the mouth was made and then towards the limbs. Skin was made apart and secured with pins at the side of their abdominal region.With a 5ml syringe ,PBS (containing 2% FBS) was injected in the peritoneal cavity. We should be careful not to puncture any of the organs. The peritoneal cavity was then massaged for about one minute so that all the cells stuck to peritoneum gets detached. With the help of same syringe peritoneal lavage was collected in 15 ml falcon tube kept on ice. The above step to collect peritoneal lavage was repeated for 2 more times and cells were pelleted down at 1400rpm for 5mins at 4^{o}C. Cell pellet was then resuspended in 1 ml PBS (containing 2% FBS) and cells were counted under hemocytometer. 0.2 million cells were stained for anti mouse F4/80 (murine macrophage marker) and anti mouse CD117 (c-kit) (murine mast cell specific marker) respectively. In brief, Fc receptor was blocked by anti mouse CD16/32 antibody by incubating on ice for 20 minutes. APC conjugated anti-mouse F4/80 Anitibody, its Isotype control and APC conjugated anti-mouse CD117 (anti-mouse c Kit) antibody and its isotype control was added to respective tubes, and incubated for 20mins in ice. Cells were washed twice with PBS (containing 2% FBS) at 1400 rpm, 4^{o}C for 5mins. Cells were fixed with 2% PFA (paraformaldehyde) and analyzed on BD FACS calibur (BD Biosciences) flow cytometer within 48hrs.

3.7 Growth Curve of P815 cell line

2 X 10^5 P815 cells were plated per well in a 48 well plate and incubated at 37^{o}C incubator supplied with 5% CO_2. Cells were harvested after 12, 24, 36, 48, 60 and 72 hrs and counted under hemocytometer. Handling should be very careful with repeated flushing so that not a single cell was left on the plate. Doubling time was calculated using

the formula from obtained data. Cells in their log phase were used to perform the experiment based on the obtained curve.

$$DoublingTime = \frac{duration * \log(2)}{\log(FinalConcentration) - log(InitalConcentration)}.$$

3.8 CFSE labeling of normal erythrocytes

Blood was collected from DBA/2 mice and then washed twice with PBS containing 2% FBS at 1400rpm for 5 mins at 4°C.To find out the efficient concentration of CFSE to be used further for the experiment RBCs were checked with different concentration 1,5,10 and 15µM of CFSE. RBCs with CFSE was kept for 10mins at 37°C. To stop the reaction ,complete medium containing 10% serum was added to the cells and washed thrice with PBS containing 2% FBS to remove out unlabelled CFSE. Unstained RBCs as well as labeled RBCs were fixed with 2% PFA and then acquired and analyzed on BD FACS calibur using Cell quest pro software within 48hrs.

3.9 Induction of oxidative damage to erythrocytes and CFSE labeling

Blood was collected from DBA/2 mice and was washed thrice with PBS at 1400rpm for 5 mins at 4°C.Oxidative damage of RBC was induced by t-BHP(3mM) for 1 hr at 37°C.Cells were then washed thrice with PBS. Oxidatively damaged RBCs were labeled with CFSE(according to the experiment) at 37°C for 10mins.Complete media containing 10% FBS was added to the labeled cells and kept on ice for 5 mins followed by three times washing with PBS.

3.10 Erythrophagocytosis by P815 cells of t-BHP treated RBC in-vitro

0.1 million P815 mast cells were seeded in 48 well plate overnight and co-incubated with normal as well as t-BHP treated oxidatively damaged erythrocytes in 1: 250 (mast cell to RBC ratio) at 37°C for 1 hr. Cells were then harvested by flushing the wells and pelleted at 1200 RPM for 5mins at RT. Unphagocytosed RBCs were then lysed with ACK lysis buffer for 5mins on ice. Cells were washed twice with PBS and fixed with 2% PFA. Mast

cells alone, co-incubated with normal RBC or oxidatively damaged RBC were acquired and analyzed on BD FACS calibur using Cell quest pro software within 48hrs.

3.10.1 Fluorescence Microscopy

For visualization of uptake of t-BHP treated RBCs, P815 mast cells were co-incubated with CFSE labeled damaged RBCs for one hour at 37^{o}C in ratio 1:250(mast cell to RBC). Cells were then harvested at 1200 rpm at RT for 5mins.To remove unphagocytosed RBCs ACK lysis buffer was added and kept on ice for 5mins.Cells were then washed twice with PBS and fixed with 2% PFA. Fixed cells were adhered on poly l-lysine(0.01%) coated coverslips for 1hr and then washed with PBS. After drying the coverslips with cells was mounted on a glass slide with flourmount. Cells were then examined under Nikon Ti-E fluorescence microscope at 100X magnification.

3.11 Isolation of RNA

RNA was isolated from RBL-2H3 and rat splenocytes using TRIZOL (TRI) reagent (1ml TRI for 5-10million cells).To create RNase free condition, all the equipments and working area was treated with DEPC and even the solutions were prepared in nuclease free water. 200µl chloroform per ml TRI was added, mixed by inverting and incubated at RT for 15mins.After incubation, it was then centrifuged at 12000g for 15mins at 4^{o}C. Three layers were obtained. Upper aqueous layer, middle and lower phenol-chloroform layer containing RNA, DNA and protein respectively. The upper aqueous phase was transferred to a fresh tube and 0.5 ml of isopropanol was added to precipitate RNA and incubated at RT for 15mins and then centrifuged at 12000g for 15mins at 4^{o}C. The white pellet of RNA was obtained which was further washed with 75% ethanol at 7500g for 5mins at 4^{o}C for at least two times to remove protein contamination. Remaining ethanol was removed by air drying and the pellet was resuspended in Tris/borate/EDTA (TBE) buffer. For proper dissolution of pellet in buffer, tubes were incubated at 70^{o}C for 10mins.Concentration and purification of RNA was checked by Nanodrop and RNase inhibitor (40U/µl) was added to the suspension and stored at -80^{o}C until use for RT-PCR.

3.11.1 Determination of yield and quality of RNA

By using Nanodrop ND2000 spectrophotometer according to the baseline set by a buffer the amount of RNA (yield) and quality (integrity) of RNA was determined. Readings were based upon absorbance that is all absorbed by ds or ss nucleotides i.e, at 260nm. The

TRIzol reagent being phenolic solution absorbs the UV both at 230 nm and ~270 nm.A260/280 ratio of ~2.0 accepted as pure for RNA and value below may be resulted due to phenol, protein or other contaminants. A260/230 ratio in range of 2.0-2.2 considered as pure for nucleotides. Presence of contaminants have lower A260/230 ratio.

3.11.2 Formaldehyde Agarose gel electrophoresis

RNA can exist in both secondary and tertiary structures so forms smearing upon electrophoresis.In order to get distinct bands RNA has to be denatured before electrophoresis.Heating is insufficient to denature RNA so denaturing chemical agents like formaldehyde,ethidium bromide to the agarose gel prevents from reforming of secondary structure.Formaldehyde also serves as RNase inhibitory agent maintaining RNA intergrity during separation whereas 3-(N-morpholino)propanesulfonic acid (MOPS) and sodium acetate serves as conductive medium.1.2% Formaldehyde Agarose gel was prepared in 1X formaldehyde buffer by adding 1.2% agarose.Before casting the gel EtBr was added and sample was loaded (4 volume of RNA +1 volume 5X RNA loading dye) in the gel tank having 1X formaldehyde buffer.Electrophoresis was conducted at 60V for 2-3 hrs and visualized under BIO-RAD Gel documentation system.

3.12 Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse Transcription Polymerase Chain Reaction (RT-PCR) is a method to detect gene expression of mRNA in the form of cDNA. It is a qualitative reverse transcription method in which mRNA is converted to cDNA by Avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (M-MLV or MuLV) reverse transcriptases hence named RT-PCR and then cDNA is further amplified by normal PCR using specific primers. The steps in PCR are: template denaturation, primer annealing and primer extension which needs template DNA, two oligonucleotide primers, a thermostable DNA polymerase, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium. Denaturation process takes place at higher temperature in which two interwined strands of DNA separates forming single-stranded for replication by the thermostable DNA polymerase. In the next step, temperature is reduced so that the oligonucleotides can form stable associations with the denatured target DNA and serve as primers for the DNA polymerase and at optimum temperature for DNA polymerase synthesis of new DNA begins. The last step is the extension step. This completes one cycle of amplification and

next cycle begins with a return to denaturation step. Theoretically each PCR cycle doubles the amount of targeted sequence or amplicon in the reaction.

Reverse transcription was performed using M-MuLV Reverse Transcriptase and an oligo(dT) primer in a total reaction mixture of 20µl (Table3.1). The amount of RNA template used was determined on basis of different standardization conditions.

For the synthesis of cDNA from RNA template, the reaction mixture was started by heating to 37^{o}C for 60mins followed by heating at 70^{o}C for 10mins in order to terminate the reaction. The reaction product was finally held at 4^{o}C till further analysis.

3.12.1 Agarose gel electrophoresis for cDNA

1.8% of agarose gel was prepared in 1X running buffer (5X TBE buffer+ Milli Q water)by heating and after cooling to 60-70^{o}C ,2µl of EtBr was added and casted on tray.After gel was set it was transferred to gel tank containing 1X running buffer and cDNA samples(1 volume of 6X DNA loading dye+5 volume of cDNA) were loaded along with DNA ladder.The gel electrophoresis was carried out at 90V ,for 1-2 hrs and visualized under BIO-RAD gel documentation system.

Table3.1: Reaction mixture for RT-PCR

Components	**20 µl Reaction**
Nuclease Free water	Calculated accordingly(to 20 µl)
RNA template	Variable
Oligo(dT)-18	1.0 µl
Heated for 5mins at 65^{o}C and kept at room temperature for 2mins and then on ice	
5X M-MuLV RT buffer	4.0 µl
dNTPs mix	2.0 µl
M-MuLV RT enzyme200µ/µl	2.0 µl
Total	20.0 µl

3.12.2 Primer designing

Literature review was done to search the receptors involved in erythrophagocytosis mechanism present in mast cells and macrophages. It was found that most of the receptors present in both macrophages and mast cells are similar and few of them were present in macrophages and their expression needed to check in mast cells also. Here we have used RBL-2H3 as a mast cell model in which erythrophagocytosis of oxidatively damaged erythrocytes have been observed and confirmed *in vitro* (Sharma P and Puri N, 2017 (manuscript under preparation). Primers were designed by using IDT (Integrated DNA technology) and Primer-BLAST. PCR product size was chosen between 200 to 850bp, primer melting temperature(Tm) was calculated according to nucleotide content of the primers.Primers were 20-24 bases long with GC content 45-55%. Bioinformatics tools such as "Oligo Calc" was used to calculate oligonucleotide properties. It detects possible formation of hairpin loop within primers and also self complementarity of primers.

Table 3.2: Bioinformatics tools

Bioinformatics Tools	URL Link
Oligo Calc	http://biotools.nubic.northwestern.edu/OligoCalc.html
Primer-BLAST	https://www.ncbi.nlm.nih.gov/tools/primer-blast/
MultAlin	https://multialin.toulouse.infra.fr/multialin/
Sequence editor/Sequence Massager	https://www.fr33.net/seqedit.php http://www.attotron.com/cybertory/analysis/seqMassager.html

A NCBI based tool "Primer-BLAST" is used to design primers specific to PCR checking against a selected database from Consensus coding sequences (CCDS) of molecules."MultAlign"(Multiple sequence alignment by Florence Corp) is a tool to check alignment of two sequences ,so the alignment of primers and their position was determined by using this tool.The blast results are itself enough for analysis(all combinations including forward-forward, forward-reverse as well as reverse-reverse,GC content, self complementarity and so on). "Sequence editor" or "Sequence Massager" both can edit DNA and RNA sequences like converting sequences, removing tags, generating antiparallel, inverse or complement sequences.

3.12.3 Primer designing for the receptors involved in erythrophagocytosis

Mast cells and macrophages being the important cells of immune system which are responsible for inflammatory responses. Receptors involved to regulate these inflammatory responses in macrophages and mast cells are found to be similar. The aim of this study is find out the receptors involved in erythrophagocytosis in macrophages and mast cells and similarity between the pathways involved. For this study we used splenocytes of rat as a positive control for the receptors involved in erythrophagocytosis since spleen has highest population of macrophages and RBL-2H3(Rat Basophilic Leukemia 2H3) mast cell line was used to check whether these receptors present in macrophages are also present in mast cells or not. RBL-2H3 shows similarity with basophils as well as mucosal mast cells making it an important in vitro tool that shows same phenomenon of degranulation in response to immunological stimuli to that of in vivo(Frankish, 2009).

3.12.4 Amplification of cDNA (semi-quantitative PCR)

Amplification of cDNA was performed using different primers for different molecules in total reaction mixture of 25µl.The amplification started with heating the sample to 95^{o}C for 5mins, followed by number of cycles (30 and 40) consisting of denaturation for 1min at 95^{o}C, annealing at 60/65^{o}C for 1min and extension for 72^{o}C for 1 min. The last cycle was followed by an additional extension for 5mins at 72^{o}C and final hold at 4^{o}C.

Table 3.3: Reaction mixture for semi-quantitative PCR

Components	**25 µl Reaction**	**Final Concentration**
Nuclease Free Water	Calculated accordingly (to 25µl)	-
Taq Buffer (20mM $MgCl_2$)	4.0 µl	1.6X
dNTPs Mix	3.0 µl	1200µM
Forward Primer	2.0 µl	0.8µM
Reverse Primer	2.0 µl	0.8µM
Template(cDNA)	4.0 µl	-
Taq.polymerase	0.125 µl	U/ml

3.12.5 Agarose gel electrophoresis of amplified DNA

1.8% agarose gel was prepared with 2µl EtBr in 1X TBE buffer. To the PCR product (25µl), 5µl of loading dye was mixed and loaded to the respective well in the gel along with DNA ladder (100bp,100µg/ml) and run for 2 hrs at 90V. The gel was visualized under gel documentation system.

3.12.6 Quantitation of band intensity

QUANTITY ONE software (BIO-RAD) was used for measuring band intensity of PCR products since band intensity is directly proportional to amount of DNA present in the gel.

CHAPTER 4
RESULTS

4.1 Erythrophagocytosis of t-BHP treated oxidatively damaged RBCs by P815 mast cells

Phagocytic mechanism involves professional phagocytes. The aim of the present study was to find out whether mast cells are also involved in erythrophagocytosis mechanism in normal or oxidative stress conditions and if yes then, was there any similarity between mast cells and macrophages in the uptake process.

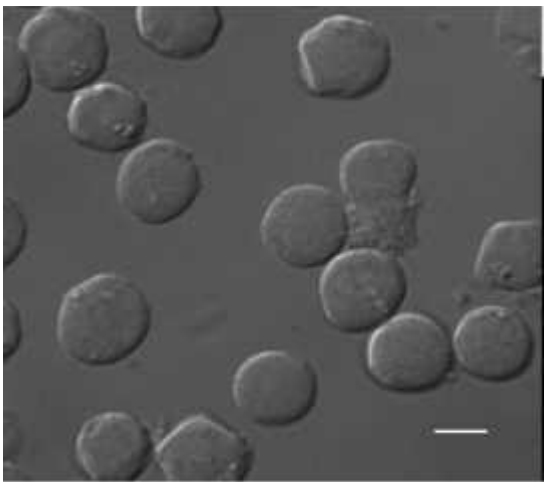
4.1.1 P815 cells and their growth kinetics

P815 is mastocytoma suspension cells some being adherent derived from DBA/2 (Gajewski et al., 2001). P815 cells were round in shape. Most of the cells were segregated and only fewer portions attached with other adjacent cells (Figure 4.1A). Growth curve was plotted based on viable cell count. As shown in figure 4.1B P815 cells have lag phase of less than 24hr, exponential phase after 36 hrs and stationary phase after 60hrs. From this growth curve the doubling time was found to be 13hrs as calculated according to the formula mentioned in materials and methods.

4.1.2 CFSE labeling of normal erythrocytes

At 1µM, 5µM, 10µM and 15µM concentration, 98.15%±1.20, 99.93%±0.01, 99.99%±0.0 and 100%±0.0 cells were found to be CFSE positive respectively. With the increase in concentration of CFSE, the Mean fluorescent intensity (MFI) value was also found to be increased. The MFI values of 1µM, 5µM, 10µM and 15µM were 28.05±1.06, 66.21±1.12, 263.36±22.65 and 397.74±10.87 respectively (figure 4.2A). Graph was plotted showing percent of positive cells with increasing concentration of CFSE (Figure 4.2B) and also with their MFI values (Figure 4.2C).

A



B

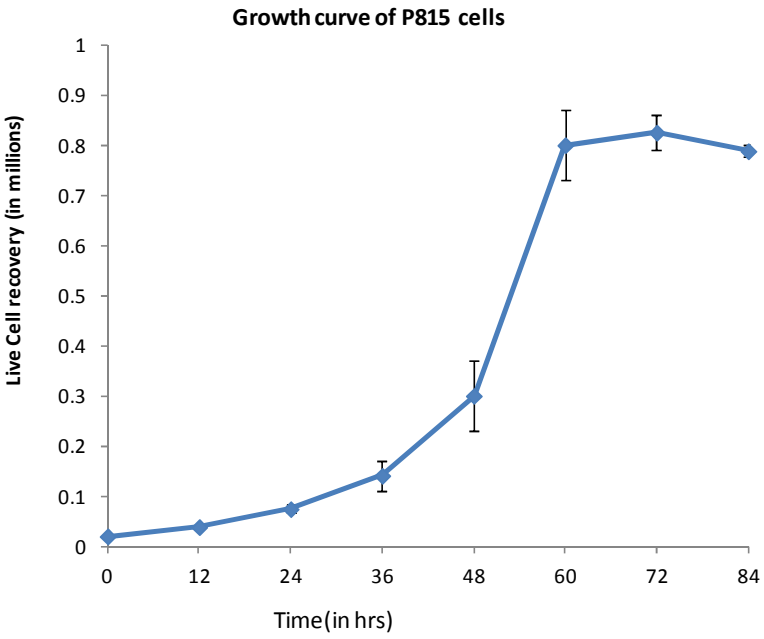


Figure 4.1.Growth kinetics of P815 cells: (A)0.1 X 10^6 P815 cells were adhered on poly l-lysine coated coverslips and after 1hr coverslip was mounted on glass slide with flourmount.(B)represents recoveries of viable cell number in which each time points represents mean±SEM of values obtained from duplicate assays(Magnification 100X,Scale bar 8µM**).**

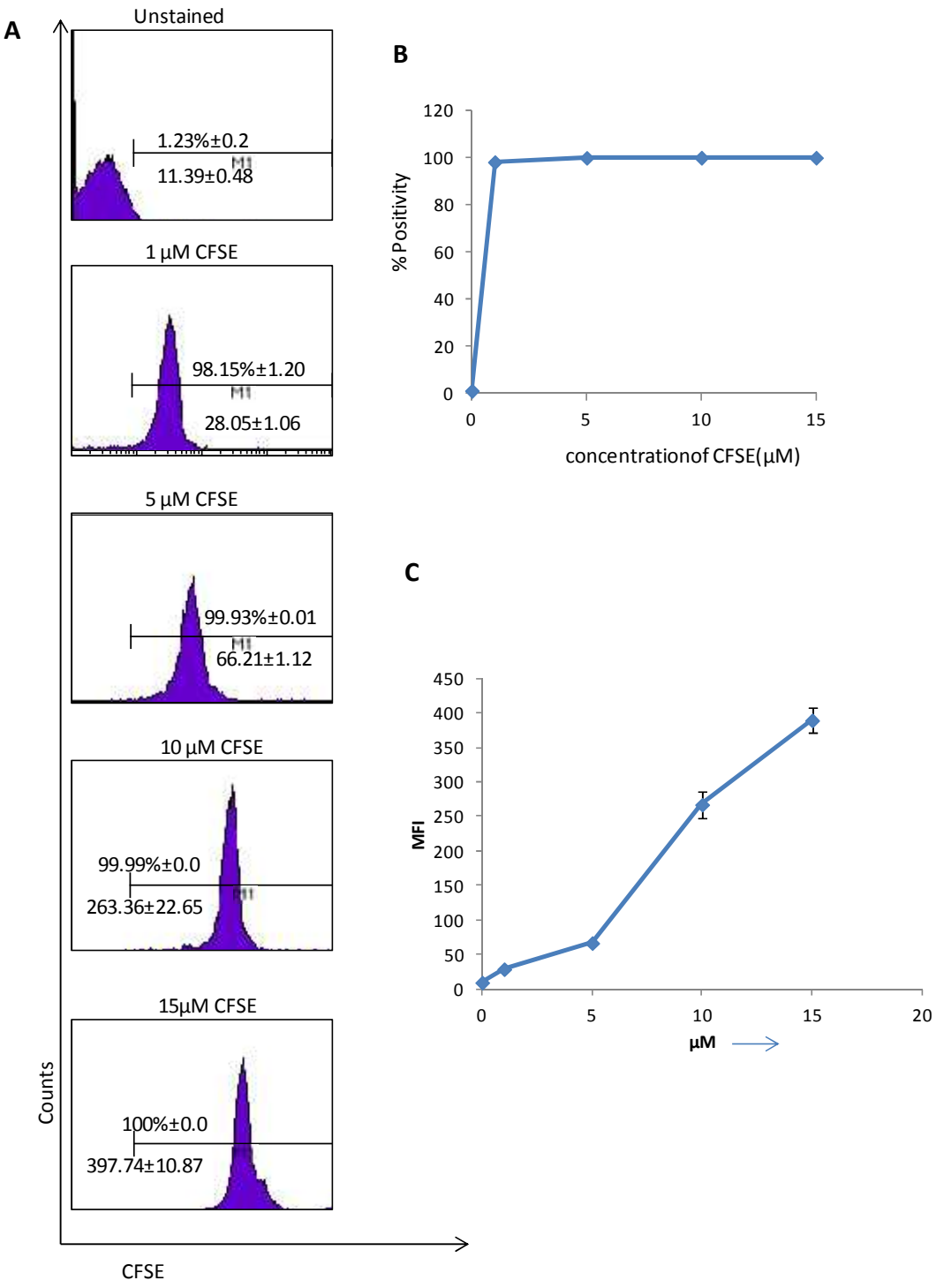


Figure 4.2.CFSE labeling of normal erythrocytes: 1X10^6 RBCs were stained with different concentration of CFSE and (A) analysis was done using FACS Calibur. (B)Graph showing percent of total cells that retained CFSE dye at different concentration and (C) shows mean fluorescence Intensity (MFI) of labeled cells at different concentration of CFSE.

4.1.3 Erythrophagocytosis by P815 cells of normal and t-BHP treated erythrocytes in-vitro

The uptake of normal as well as damaged erythrocytes by P815 mast cells was checked. From the FACs data, the MFI values of P815 cells alone, P815 cells co-incubated with CFSE labeled normal erythrocytes (P815+NRBC) and P815 cells with CFSE labeled t-BHP treated oxidatively damaged erythrocytes (P815+DRBC) were 13.10, 20.13±0.26 and 47.56±1.14 respectively as shown in figure 4.3A. From the data observed uptake of damaged RBCs by P815 mast cells was found to be higher (16.85%±2.16) than that of normal RBCs (2.55%±0.06) as shown in the graph (Figure 4.3B).

For the confirmation of erythrophagocytosis by P815 mast cells, fluorescence microscopy was performed. From FACs data we knew that there was no significant uptake of normal erythrocytes by P815 cells so there was no point in capturing image of P815 cells with normal RBCs. Images of P815 mast cells alone (Figure 4.4A) and P815 mast cells with damaged RBCs were only taken. Erythrophagocytosis was clearly observed in case damaged RBCs (Figure 4.4B).

4.2 Processing of total macrophage and mast cell population in mouse peritoneal lavage

Erythrophagocytosis by mast cells was examined in-vitro by using P815 mast cells. But the real challenge is to get similar result in-vivo also. So at first characterization of macrophage and mast cell in-vivo was done. Peritoneal cells were harvested as described above in materials and methods section and macrophage and mast cell population was distinguished by using specific antibody. From the dot plot analysis by using FACS Calibur, total macrophage and mast cell population was found. Macrophage population was found to be 47.75% determined by detecting F4/80 surface marker by using specific F4/80 antibody as shown in figure 4.5A. Mast cell population was found to be 2.32% by the help of c-kit antibody for detecting c-kit surface marker as shown in figure 4.5B.

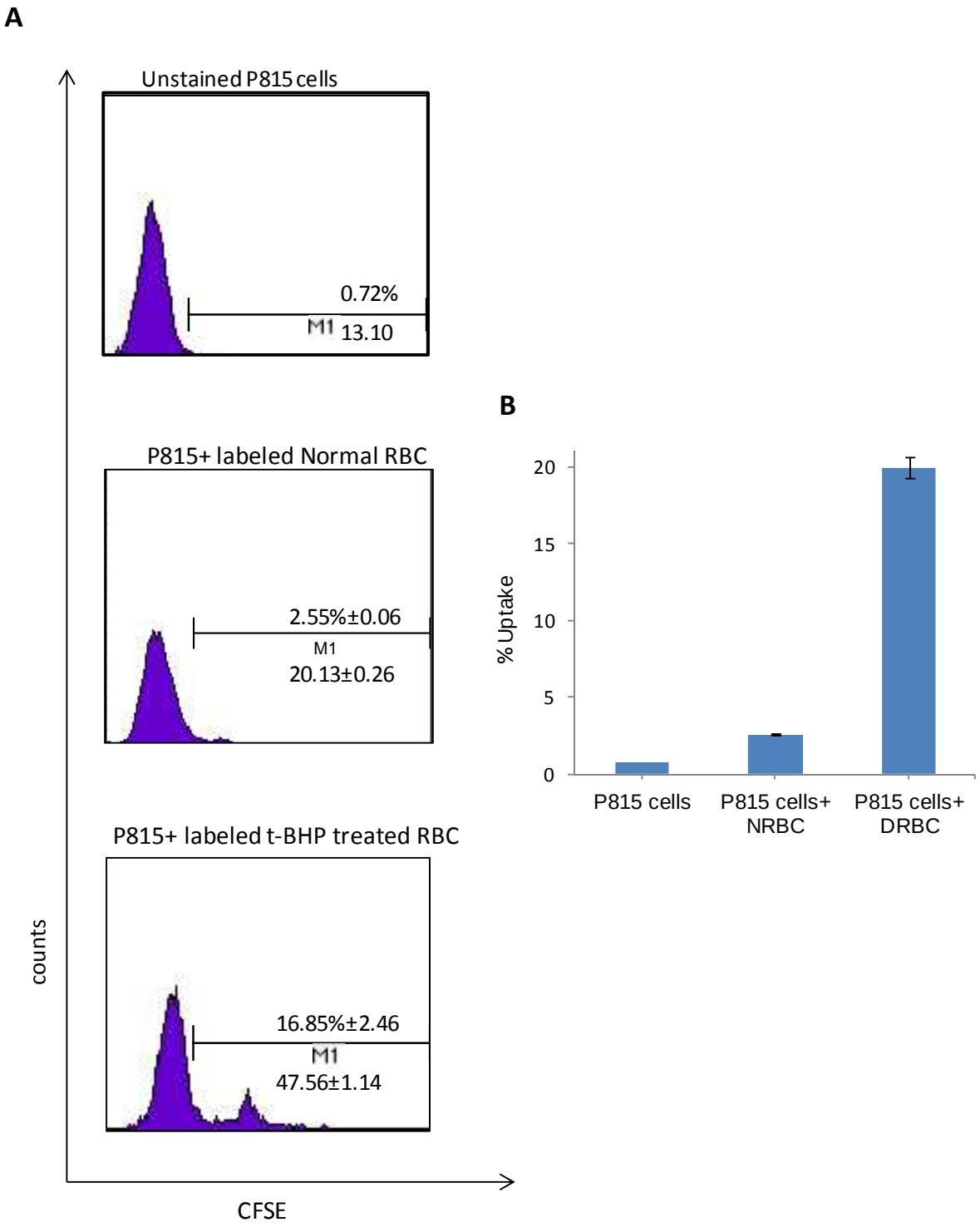


Figure 4.3.Uptake of t-BHP treated erythrocytes by P815 mast cells: (A) P815 cells alone (upper panel), P815 cells with CFSE labeled normal erythrocytes (middle panel), P815 cells co-incubated with CFSE labeled t-BHP treated oxidatively damaged erythrocytes (lower panel) analyzed using FACS Calibur. (B)represents bar graph for the FACs histogram shown in figure A.

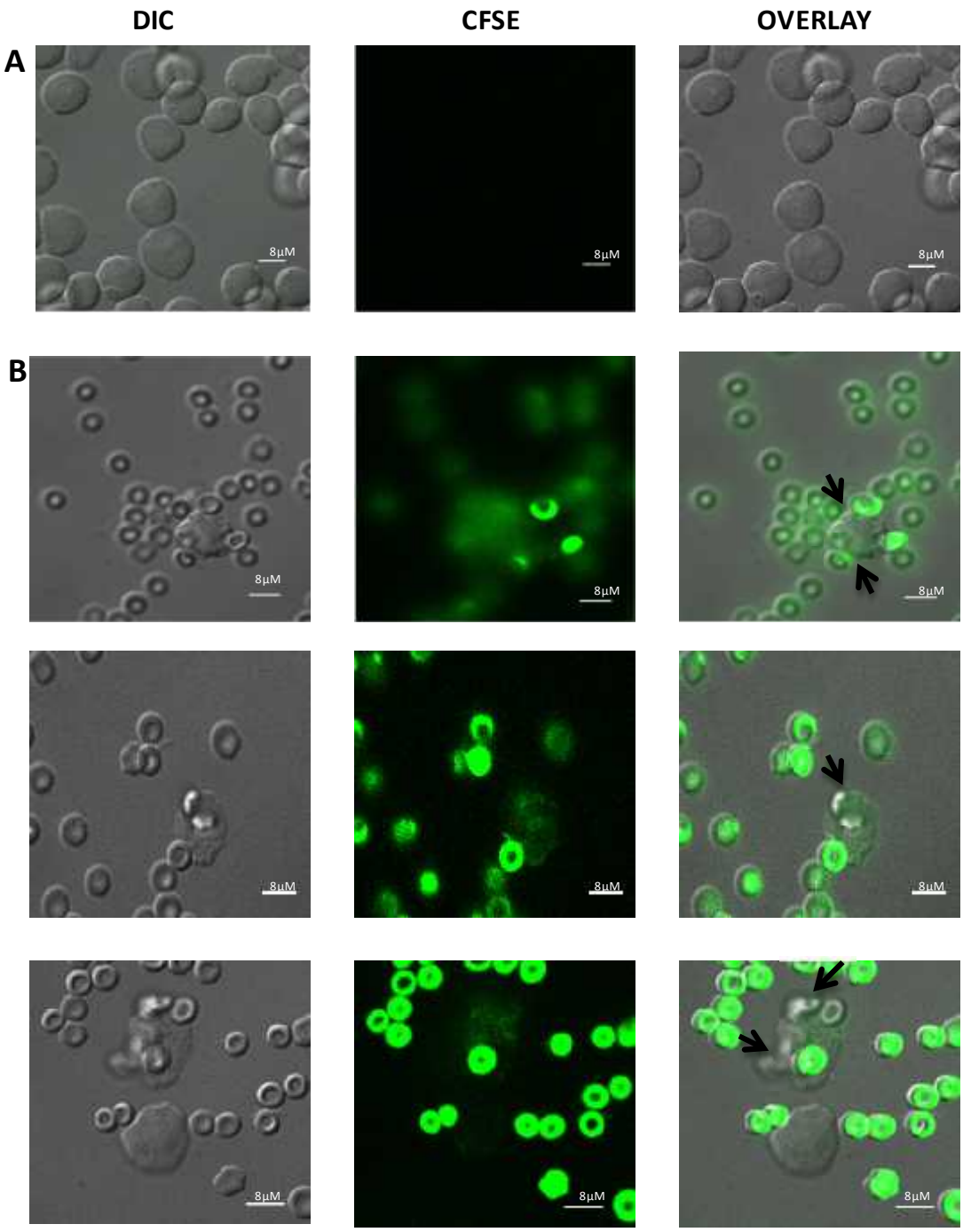


Figure4.4: Fluorescence microscopy image of P815 cells (A) and t-BHP treated oxidatively damaged erythrocytes by P815 mast cells(B).Images were captured from Nikon Ti-E(Magnification100X,Scale bar 8µM).

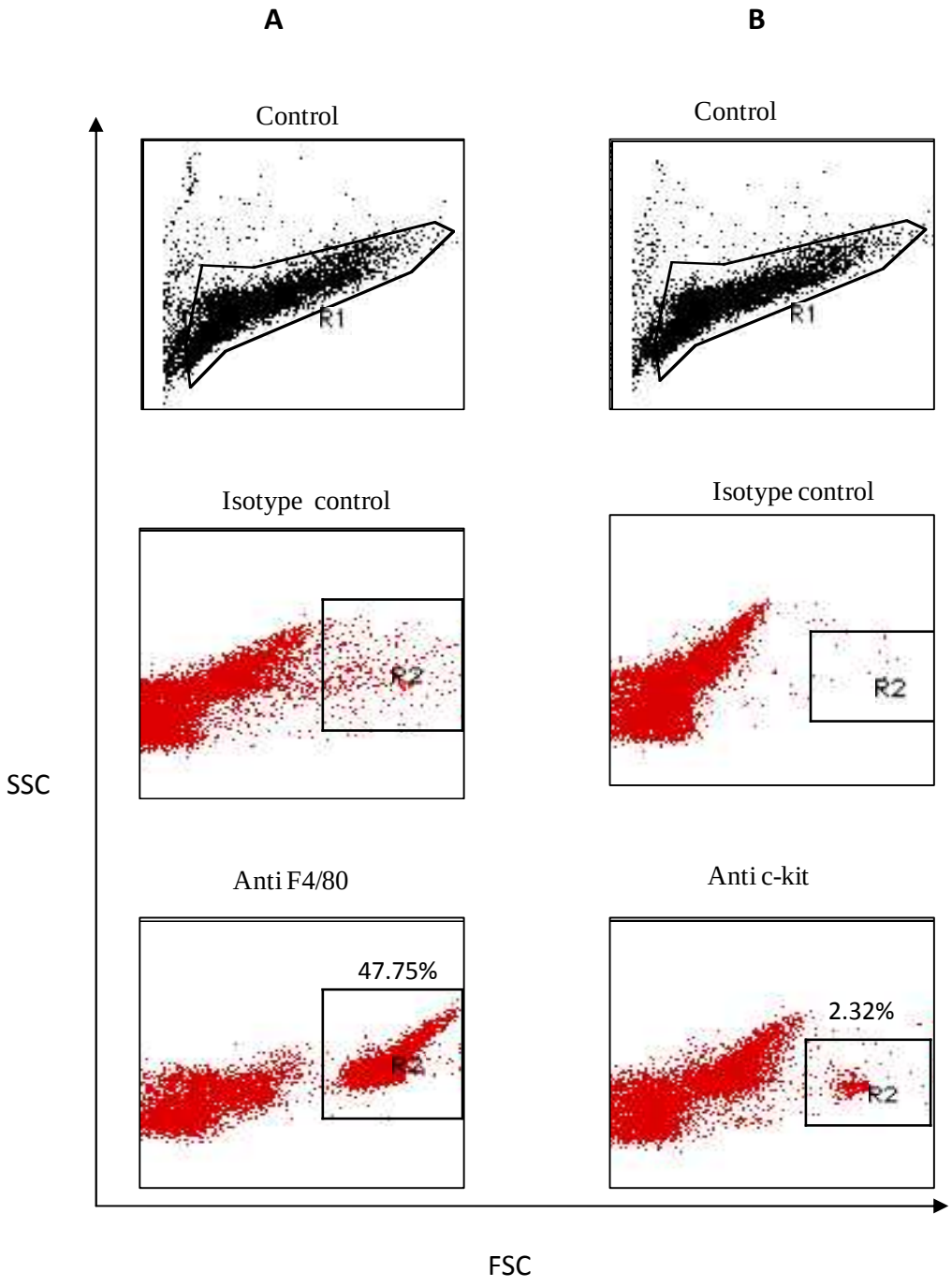


Figure4.5: (A) Characterization of total macrophage population with the help of F4/80 marker and **(B)** mast cell population with the help of Anti c-kit of total peritoneal cells from DBA/2 mice. Acqusition and analysis was done by flow cytometer. Upper panel shows Forward Scatter and Side Scatter obtained from the dot plot of total peritoneal cells. Middle panel explain respective Isotype control and lower panel determines macrophage population and mast cell population.

4.3 Receptors involved in erythrophagocytosis

4.3.1 Isolation of splenocytes

Spleen was isolated from Wistar rat and its single cell suspension was made. Erythrocytes were removed by treating it with ACK lysis buffer and after centrifugation white pellet was obtained (Figure 4.6). Splenocytes were recovered and total cell recovery was calculated (Table 4.1).

4.3.2 Isolation of RNA from splenocytes and RBL-2H3

RNA was isolated from rat splenocytes using Trizol reagent which permits complete dissociation of nucleoprotein complex. The amount and integrity of RNA were determined by measuring absorbance at 260nm using Nanodrop spectrometer. The purity ratio with respect to phenol and organic contaminants were found in splenocytes (Table 4.1) and RBL-2H3 (Table 4.2).

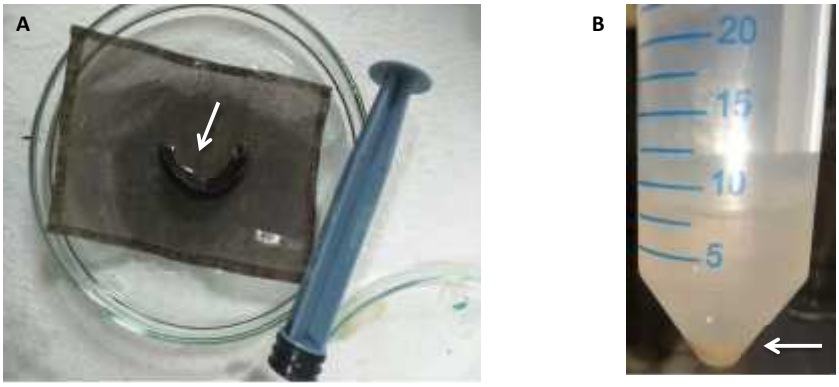


Figure 4.6: Isolated rat spleen kept on wire gauge for preparing single cell suspension (A) and (B) white pellet obtained after ACK treatment.

Table 4.1: Cell recovery. Cells were recovered from spleen from Wistar rat. The cells recovery was determined using hemocytometer by trypan blue staining as shown in table 1.

Rat strain	RNA sample(Splenocytes)	Viable cells counted per rat
Wistar	In vivo(Naïve)	368 X 10^6
		376 X 10^6

Table 4.2: Quantitation of RNA. Total RNA was isolated from rat splenocytes using Tri-zol reagents. Yield and integrity of RNA were determined by measuring absorbance at 260nm using Nanodrop (ND-2000) spectrophotometer as shown in table2.

Rat Strain	**RNA sample (Splenocytes)**	**Amount of RNA per million cells(µg)**	**Purity ratio(260/280)**	**Purity ratio(260/230)**
Wistar	***In vivo*(Naïve)**	0.032	2.07	0.87
		0.029	2.06	0.83
		0.024	2.10	0.31
		0.033	2.08	1.35
		0.030	2.08	1.03

Table 4.3: Quantitation of RNA. Total RNA was isolated from RBL-2H3 using Tri-zol reagents. Yield and integrity of RNA were determined by measuring absorbance at 260nm using Nanodrop (ND-2000) spectrophotometer as shown in table 4.3.

Cell line	**RNA sample**	**Amount of RNA per million cells(µg)**	**Purity ratio (260/280)**	**Purity ratio (260/230)**
RBL-2H3	***In vitro*** (Cultured)	0.384	2.10	2.12
		0.270	2.09	2.04
		0.504	2.07	1.46
		0.533	2.09	1.45
		0.344	2.08	1.72

4.3.3 RNA and cDNA gel of splenocytes and RBL-2H3

RNA was isolated from rat splenocytes and RBL-2H3 at resting and activated (IgE receptor crosslinked) state and were run on 1.2% formaldehyde Agarose gel. Figure 4.7A shows RNA gel of rat splenocytes with presence of 3 distinct bands of 28S, 18S and 5S rRNA, Figure 4.8A of RBL-2H3 at resting state and figure 4.9A of activated RBL-2H3 at two time points 15 and 30mins, which confirm the integrity of RNA. Using this total RNA, cDNA was synthesized and the product was run on 1.8% Agarose gel against 100bp DNA ladder. Figure 4.7B shows cDNA smear of rat splenocytes, figure 4.8B of rest RBL-2H3 and figure 4.9B of activated RBL-2H3. Disappearance of RNA band or presence of long smear confirms that RNA was reverse transcribed to cDNA.

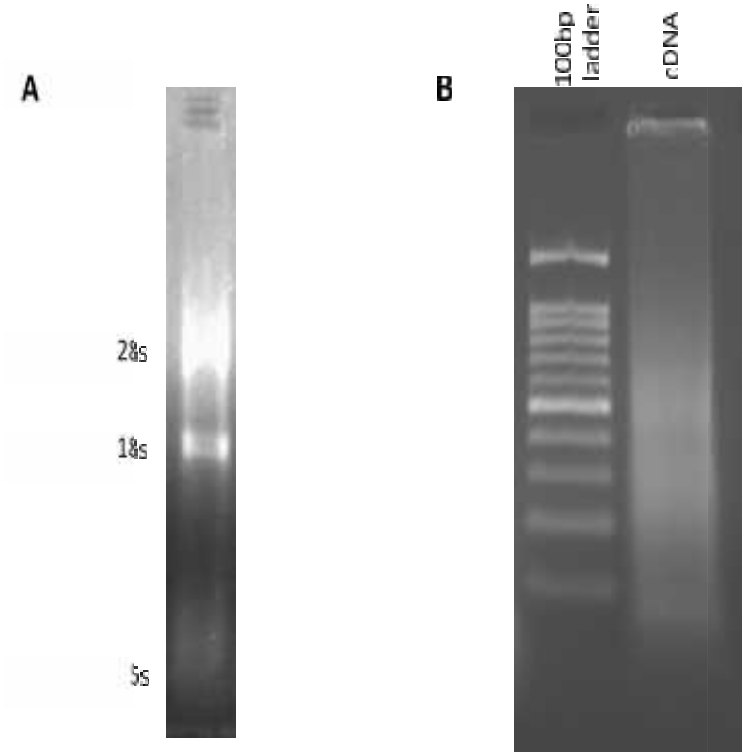


Figure 4.7: Agarose gel electrophoresis(with EtBr staining) of total RNA isolated from rat splenocytes(A) and cDNA obtained by reverse transcription of same RNA using oligo dT primerlong with 100bp ladder(B).

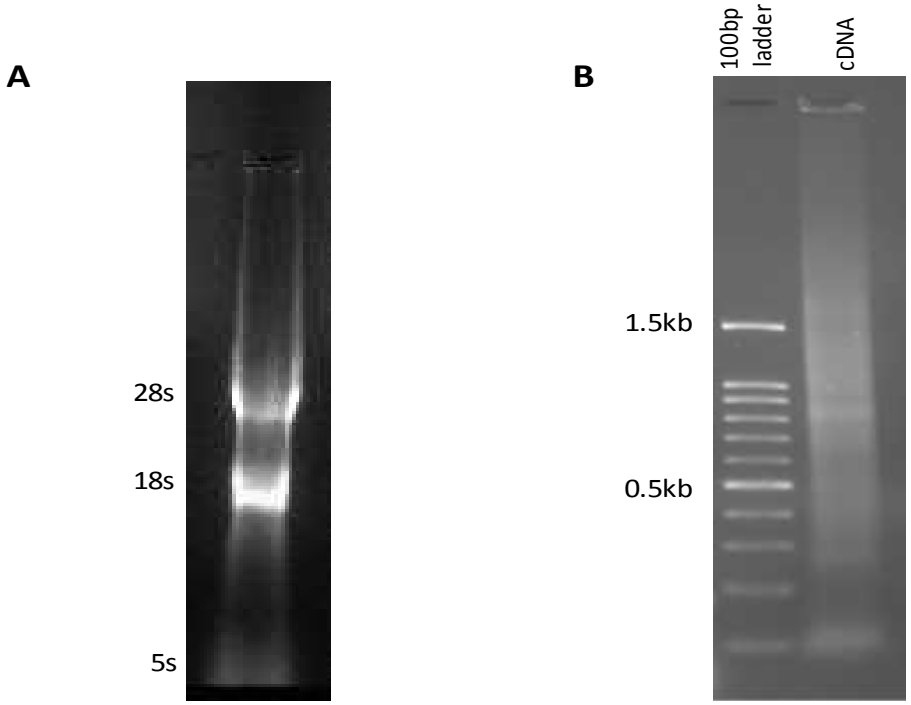


Figure 4.8: Agarose gel electrophoresis(with EtBr staining) of total RNA isolated from RBL-2H3(A) and cDNA obtained by reverse transcription of same RNA using oligo dT primer along with 100bp ladder(B).

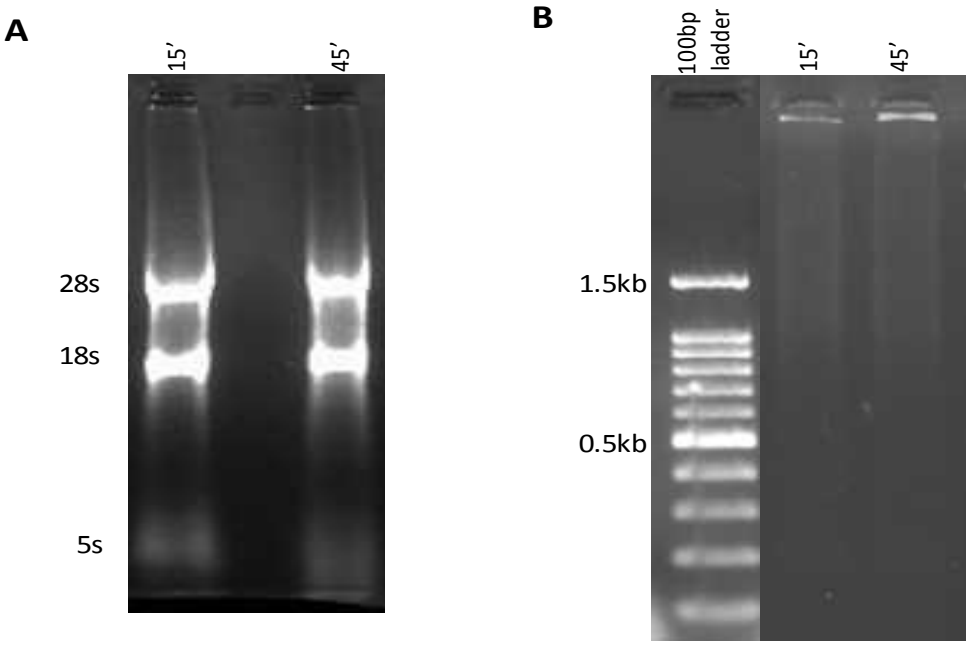


Figure 4.9: Agarose gel electrophoresis(with EtBr staining) of total RNA isolated from activated RBL-2H3 at time points15mins and 45mins(A) and cDNA obtained by reverse transcription of same RNA using oligo dT primer along with 100bp ladder(B).

4.3.4 Design of RT-PCR primers

Using NCBI gene/nucleotide sequence, *Rattus norvegicus* consensus coding sequences (CCDS) for the genes coding for such receptors were retrieved and used to design primers using NCBI primer-BLAST, Oligo Calc, MultAlign and Sequence massager tools. The list of primers with their primer length, melting temperature, GC content along with their product length is listed in Table 4.4.

Table 4.4: RT-PCR primers used for studying receptors involved in erythrophagocytosis

Name of the receptor	Primers Forward Primer(F) Reverse Primer(R)	Primer length (b)	Melting Tempe-rature (Tm^oC)	GC (%)	Product length (bp)
RAGE	F CTACCTATTCCTGCACTTC R CTGATGTTGACAGGAGGGCTTTCC	20 24	55.33 63.44	50 54.17	346
SIRPα	F GCTTAATGGGACCGCTAACT R CGTGAATACCACATCCTCTCTG	20 22	57.67 58.29	50 50	274
CD300b	F CGAACCACAGGATCAGAGAAA R CTGCTTATCCATCGGTCTAAGG	21 22	57.68 58.08	47.62 50	457
VCAM-1	F GACAGGAGACATGGTGCTAAA R CATCCCAATGGCGGGTATTA	21 20	57.67 57.42	47.62 50	226
RC3a	F AGCCCCATCCCAGATGTTTG R AGCCTCCCGCACAAACTAAA	20 20	60.03 59.89	55 50	214
EphrinB2	F AGGGACTCTGTGTGGAAGTA R TCTGTCGGCTTGCTCTTTATC	20 21	57.30 58.11	50 47.62	252
TIM3	F CGGCCAAGCACTCATGTTTT R TGGGATGACTTTGGCTGGTT	20 20	59.69 59.52	50 50	427
TIM1	F TGGATCTGTACCCAGTGCTTT R AGGAATCTCCACTCGGCAAC	21 20	59.01 59.75	47.62 55	815

4.3.5 Checking all the primers in rat splenocytes

Rat spleenocytes served as positive control for the above designed primers as they contain large number of macrophages. The PCR condition for RAGE was already mentioned by Yoshimaru et al., 2008 so we followed their protocol and performed RT-PCR and also standardized optimum PCR conditions for rest of the primers. At first RAGE, VCAM-1, SIPRα, CD300b primers were checked, GAPDH was taken as positive control (Figure 4.10). In this case out of 4 only VCAM-1 expression was observed by the presence of its band at 226bp. Correct band of GAPDH at 176bp represented correct PCR conditions. Under similar PCR conditions this experiment was repeated by replacing PCR buffer containing $Mg2^{+}$ (Figure 4.11). Now, clear and specific band of VCAM-1 at 226bp, SIRPα at 274bp (Figure 4.11A) and RC3a at 214bp (Figure 4.11B) was observed whereas non-specific bands of RAGE, TIM1 and TIM3 were found whereas bands for CD300b and EphrinB2 were not observed.

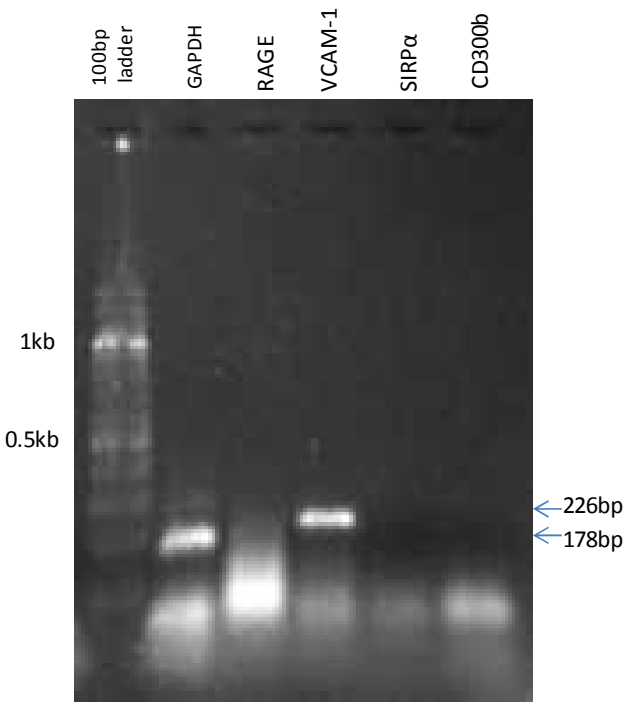


Figure 4.10: Agarose gel electrophoresis (with EtBr staining) of amplified cDNA using GAPDH, RAGE, VCAM-1, SIRPα and CD300b primers. PCR was performed by starting with 1µg RNA from rat splenocytes for 40 cycles at 60^{o}C annealing temperature.

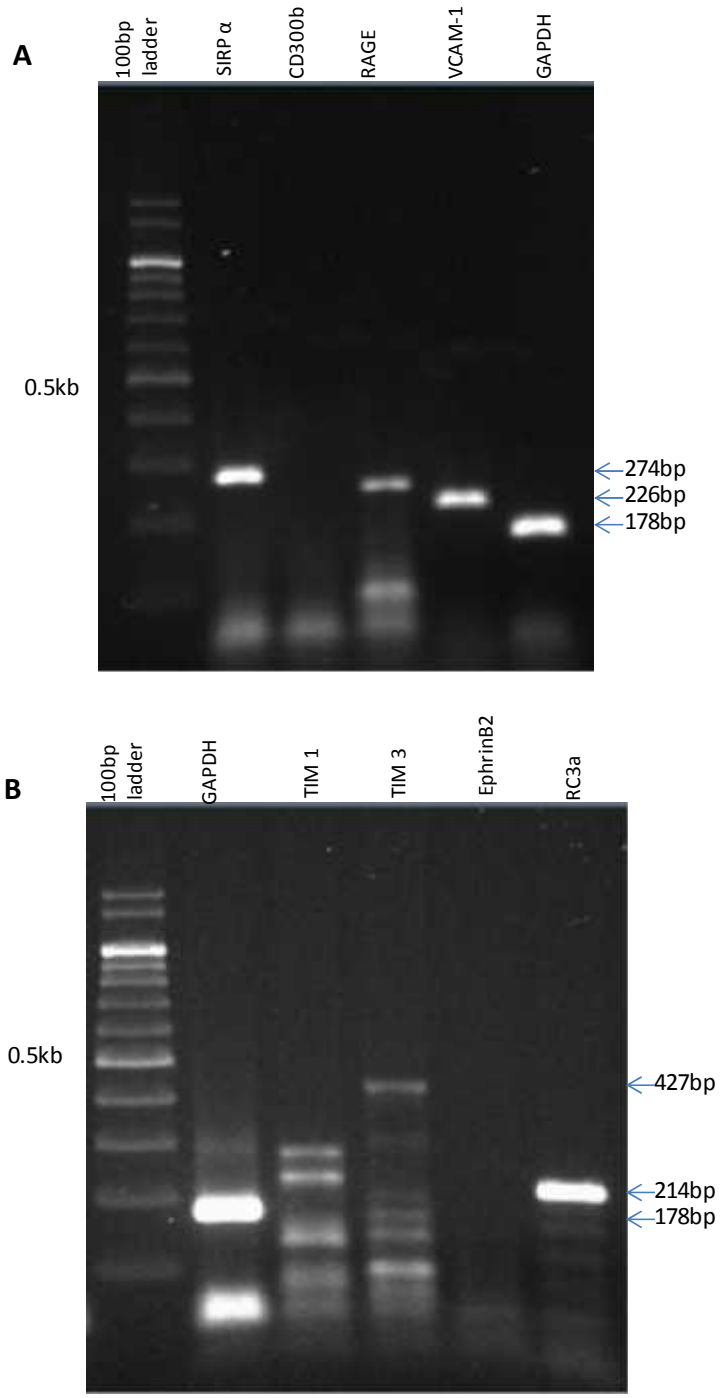


Figure 4.11: Agarose gel electrophoresis (with EtBr staining) of amplified cDNA using SIRPα, CD300b, RAGE, VCAM-1 and GAPDH primers (A), And GAPDH, TIM1, TIM3, EphrinB2 and RC3a primers (B). PCR was performed by starting with 1µg RNA from rat splenocytes for 40 cycles at 60^{o}C annealing temperature.

4.3.6 Standardization of RT-PCR conditions for RAGE

RT-PCR was performed for RAGE according to the conditions mentioned by Yoshimaru et al.,2008. Temperature gradient (50-65^{o}C) was done using 1µg of RNA isolated from splenocytes. The PCR products were run on 1.8% agarose gel and thus obtained bands were quantified using BIO-RAD quantity one software. After quantification, ideal conditions were found to be 65^{o}C as annealing temperature and 40 number of cycles as as shown in figure 4.12.

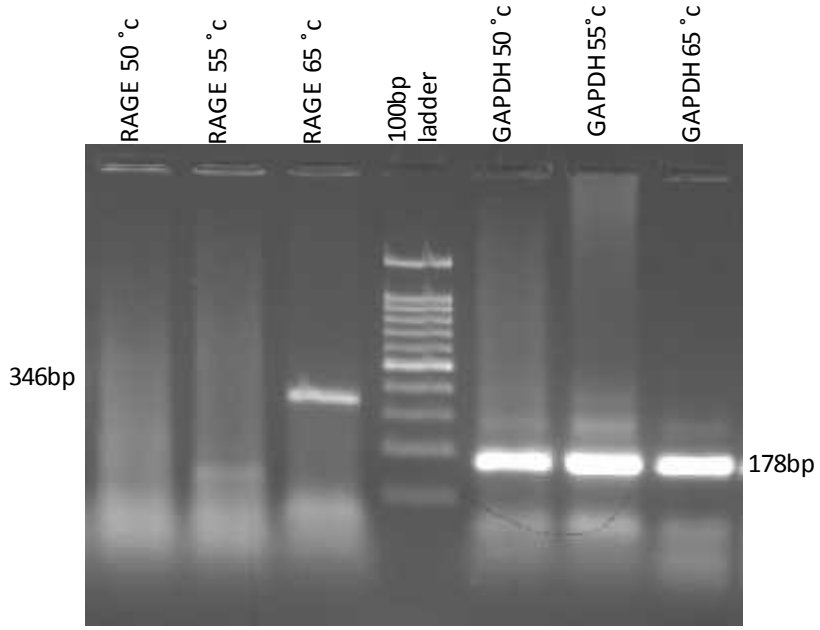
4.3.7 Standardization of RT-PCR conditions for TIM3

RT-PCR was performed for TIM3 for temperature gradient (50-65^{o}C) was done using 1µg of RNA isolated from splenocytes. The PCR products were run on 1.8% Agarose gel and thus obtained bands were quantified using BIO-RAD quantity one software. After quantification, ideal conditions were found to be 65^{o}C as annealing temperature and 40 number of cycles as shown in figure 4.13.

4.3.8 RT-PCR conditions for TIM 1

RT-PCR was performed for TIM1 for temperature gradient (50-65^{o}C) was done using 1µg of RNA isolated from rat splenocytes for 40cycles.The PCR products were run on 1.8% Agarose gel .Ideal conditions for TIM1 was not found due to formation multiple non specific bands as shown in figure 4.14.

A



B

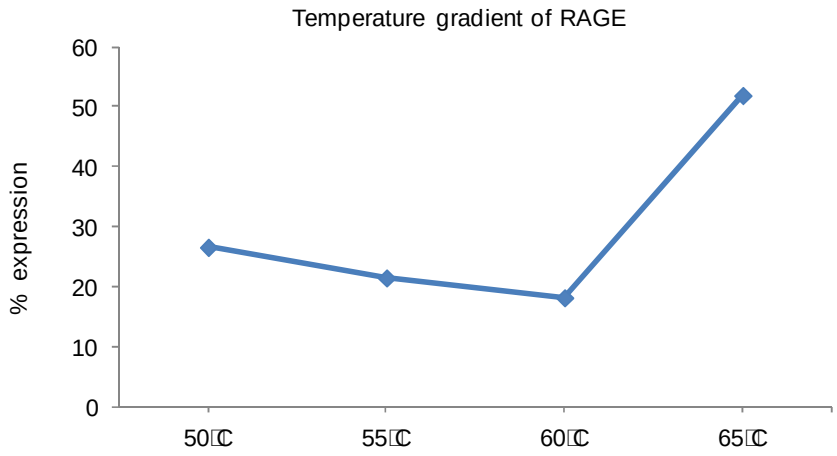


Figure 4.12: Standardization of annealing temperature for RAGE.PCR reaction was carried out with 1µg RNA from rat splenocytes for 40 cycles at various temperatures. Temperature gradient for GAPDH was also carried out to check for correct temperature condition.The PCR product were run on 1.8% agarose gel and visualized under gel documentation(A). Thus obtained band intensity of RAGE was quantified using BIO-RAD quantity one software (B).

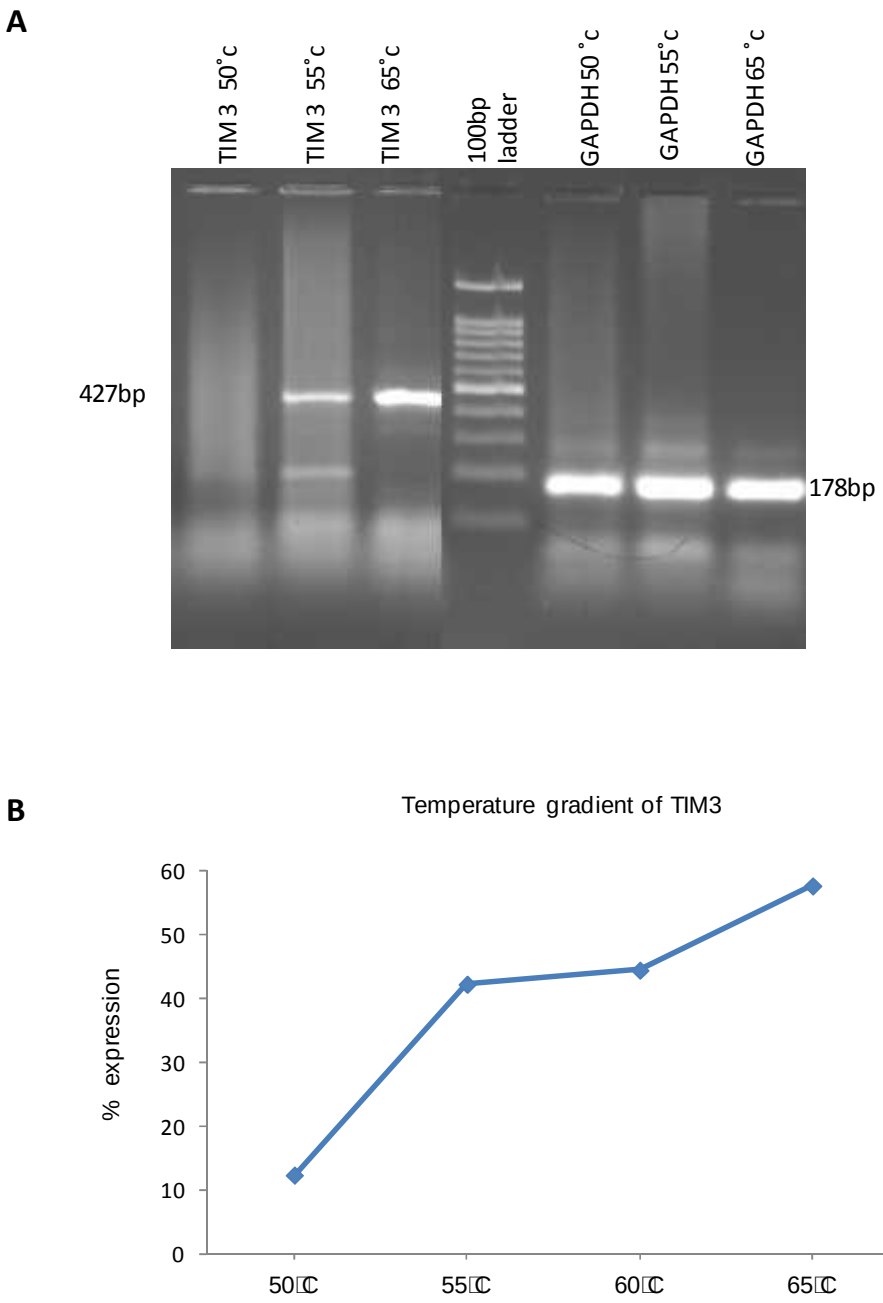


Figure 4.13: Standardization of annealing temperature for TIM 3.PCR reaction was carried out with1µg RNA from splenocytes for 40 cycles at various temperatures. Temperature gradient for GAPDH was also carried out to check for correct temperature condition.The PCR product were run on 1.8% agarose gel and visualized under gel documentation (A). Thus obtained band intensity of TIM 3was quantified using BIO-RAD quantity one software (B).

A

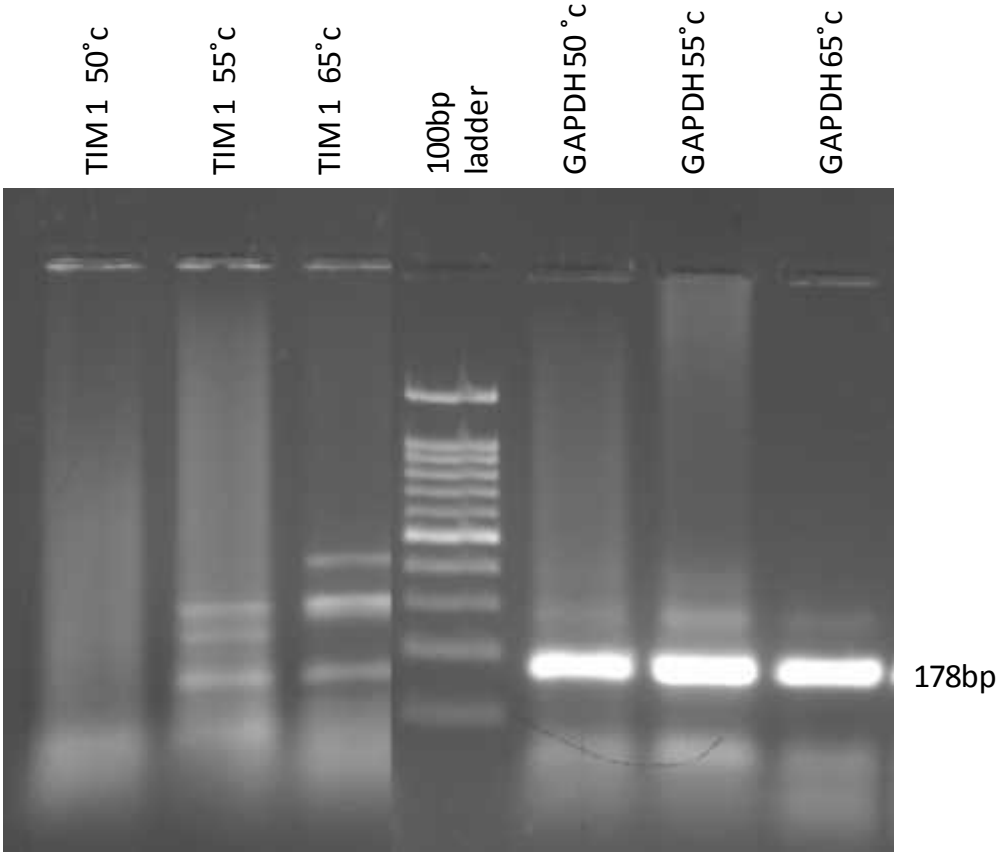


Figure 4.14: Standardization of annealing temperature for TIM1. PCR reaction was carried out with 1µg RNA from splenocytes for 40 cycles at various temperatures. Temperature gradient for GAPDH was also carried out to check for correct temperature condition.The PCR product were run on 1.8% agarose gel and visualized under gel documentation.

4.3.9 RT-PCR conditions for GAPDH using RBL-2H3 mast cells

RT-PCR was performed for GAPDH using RNA isolated from RBL-**2**H3 (resting and activated) at 60^{o}C annealing temperature for 30 cycles. RBL-2H3 was activated at two time points 15 and 45mins. The PCR products were run on 1.8% agarose gel and thus obtained bands were quantified using BIO-RAD quantity one software. According to the graph, activated RBL-2H3 for 15mins expression was greater (26%) than rest RBL-2H3 but not much difference was there in case of activated RBL-2H3 for 45mins (23%) as shown in figure 4.15.

4.3.10 RT-PCR conditions for primers (RAGE, SIRPα, TIM3, RC3a, EphrinB2 and TIM1) in RBL-2H3 mast cells

The receptors that showed positive result in case of rat splenocytes were checked for its presence in RBL-2H3 by using same PCR conditions i.e., at first 60^{o}C (Figure 4.16A) and at 65^{o}C annealing temperature (Figure 4.16B). RAGE and VCAM-1 expression was seen at 60^{o}C but not of SIRPα and TIM1 (Figure 4.16A) whereas TIM3 and RAGE band were seen at 65^{o}C (Figure 4.16B). Temperature gradient was performed in case of TIM1 and SIRPα (50-65^{o}C) but no any bands were observed in both the cases whereas GAPDH band was clearly observed taken as positive control (Figure 4.18). For RAGE 60^{o}C was taken as optimum temperature although it was expressed at both the temperatures i.e., 60^{o}C and 65^{o}C. A faint non-specific band was observed below specific band of RAGE at 65^{o}C.

4.3.11 Standardization of RT-PCR conditions for VCAM-1in RBL-2H3 mast cells

RT-PCR was performed for VCAM-1 for temperature gradient (50-65^{o}C) using RNA isolated from RBL-2H3. The PCR products were run on 1.8% agarose gel and thus obtained bands were quantified using BIO-RAD quantity one software. After quantification, ideal conditions were found to be 60^{o}C as annealing temperature, 40 number of cycles and 1µg of RNA as shown in figure 4.17.

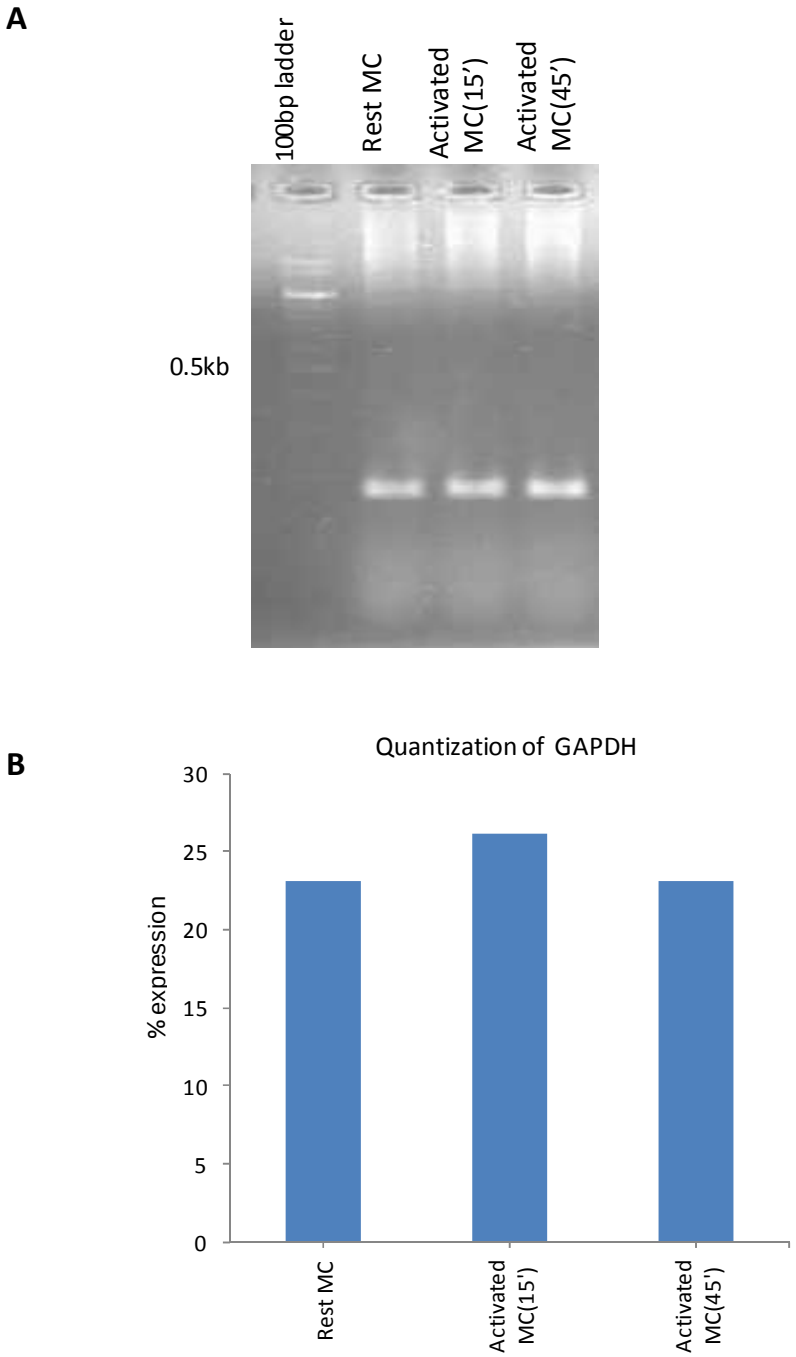


Figure 4.15: Agarose gel electrophoresis (with EtBr staining) of amplified cDNA from rest and activated (for 15' and 45') RBL-2H3 using GAPDH primer for 30 cycles at 60°C. The PCR product were run on 1.8% agarose gel and visualized under gel documentation as shown in the figure A and Thus obtained band intensity was quantified using BIO-RAD quantity one software (B).

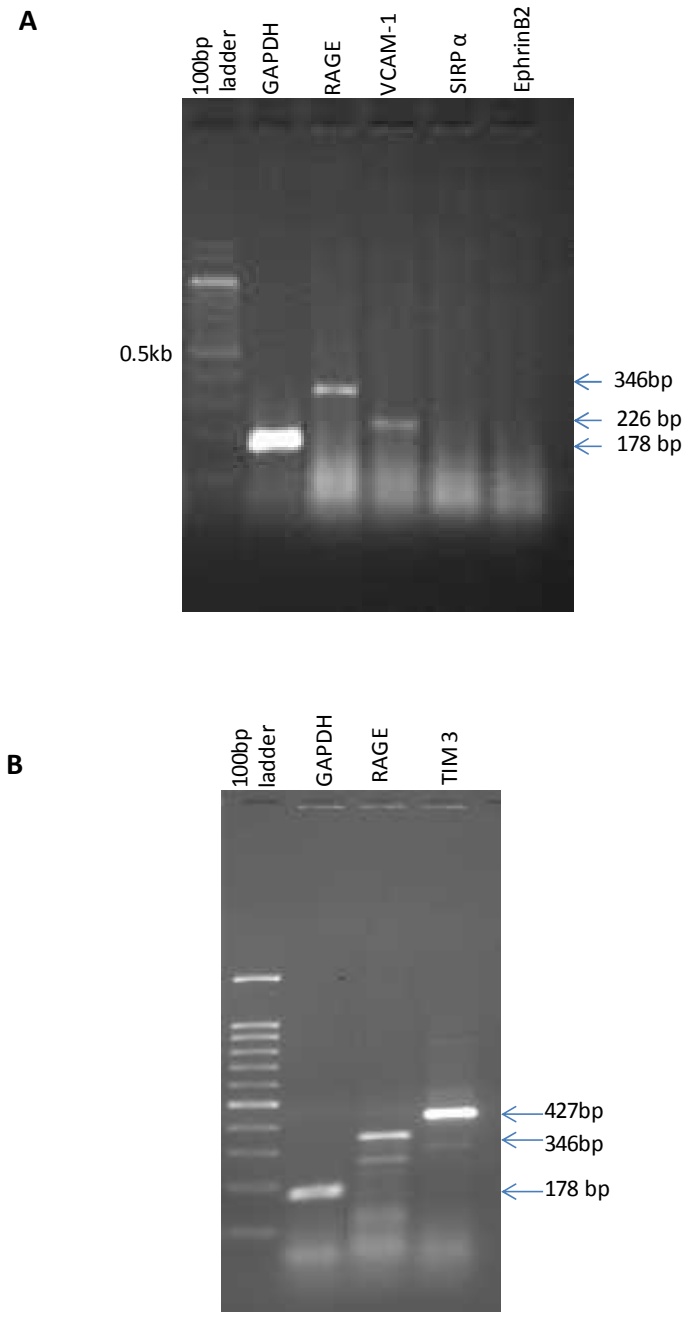


Figure 4.16: Agarose gel electrophoresis(with EtBr staining) of amplified cDNA from RBL-2H3 using GAPDH, RAGE, VCAM-1, SIRPα and EphrinB2 primers at annealing temperature 60^{o}C(A) and using GAPDH,RAGE and TIM3 at 65^{o}C(B).PCR was performed by starting with 1µg RNA from RBL-2H3 for 40 cycles.

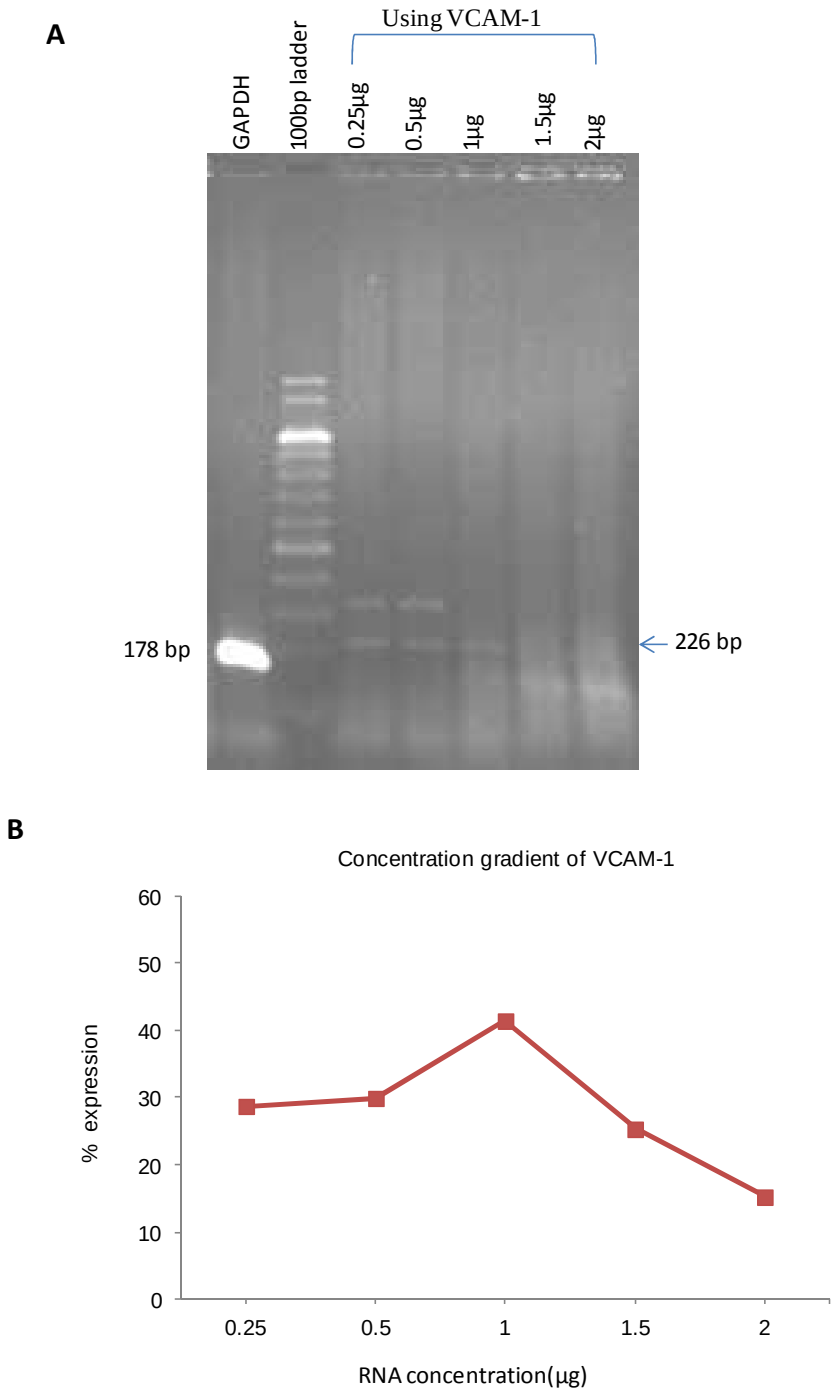


Figure 4.17: Standardization of amount of RNA template for VCAM-1.PCR reaction was performed using different amount of RNA from RBL-2H3 for 40 cycles at 60^{o}C. GAPDH was used as positive control. The PCR product were run on 1.8% agarose gel and visualized under gel documentation as shown in the figure A and thus obtained band intensity was quantified using BIO-RAD quantity one software (B).

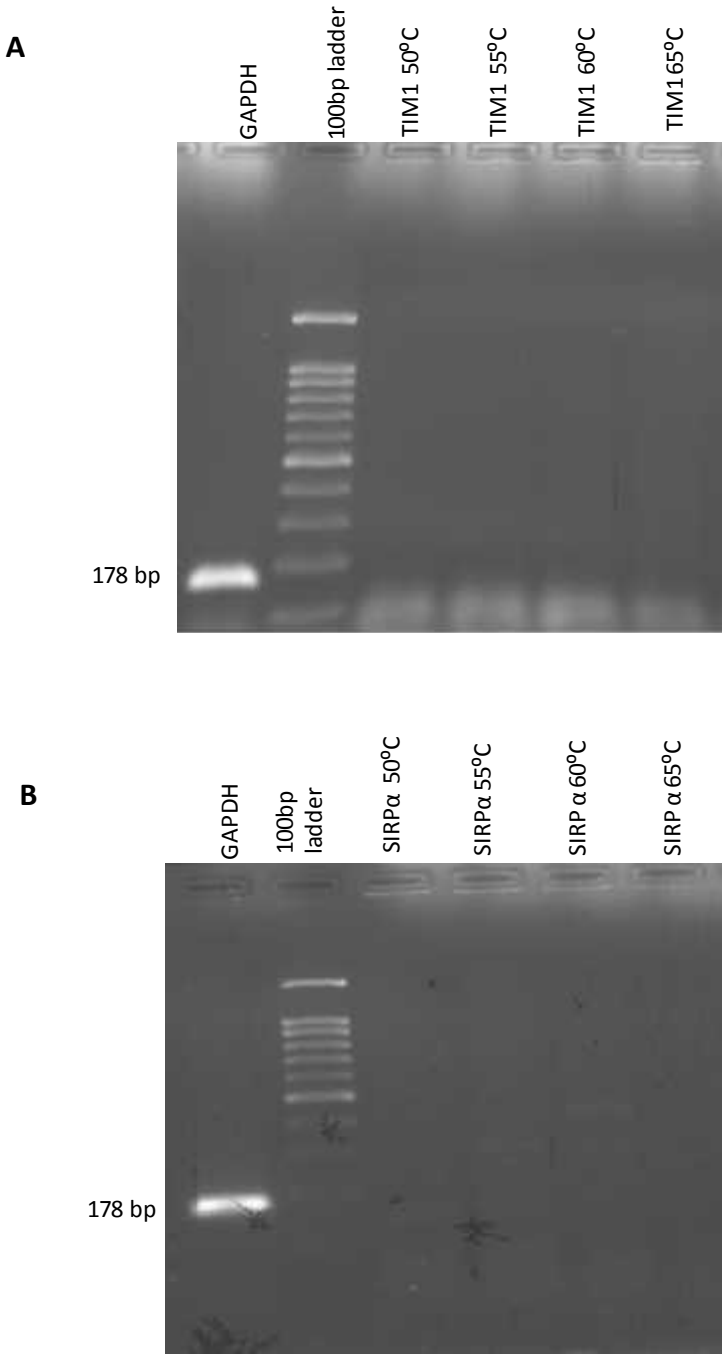


Figure 4.18: Standardization of annealing temperature for TIM1 (A) and for SIRPα (B). PCR reaction was carried out with 1µg RNA from RBL-2H3 for 40 cycles at various temperatures. GAPDH was used as positive control. The PCR product were run on 1.8% agarose gel and visualized under gel documentation.

CHAPTER 5
DISCUSSION

Phagocytosis mechanism involves professional phagocytes like macrophages, dendritic cells and neutrophils. Macrophages are found to be involved in erythropoesis as well as erythrophagocytosis mechanism. Erythrophagocytosis by mast cells increases when there is damage in RBCs either due to increased oxidative stress or diseased condition. During inflammatory response, phagocytosis also increases due to activation of macrophages by various inflammatory mediators released by cells such as mast cells and other immune cells. From above studies we posed two questions. First, to find out whether mast cells were involved in erythrophagocytosis mechanism and under which condition the uptake is more, in normal or oxidative stress conditions? Second query was to find out if there is any similarity between the receptors involved during the uptake process of erythrocytes in mast cells and macrophages. For studying erythrophagocytosis mechanism we have chosen P815 mastocytoma cell line derived from DBA/2 mouse. It is factor-independent rodent MC line which confers spontaneous activation of Kit receptor. They express Fc receptors for IgG (Fc gamma RII) but not Fc epsilon RI, but contain some mRNA for gamma chains while lack mRNA for the alpha and beta subunits of the Fc epsilon RI receptor. P815 is commonly used as an experimental tumor model *in vivo* experimentation because it grows progressively in the majority of syngeneic DBA/2 mice when implanted either intraperitoneally or subcutaneously (Gajewski et al., 2001). Also P815 have been used to study MC biology *in vitro* like for cell degranulation studies which was evaluated by detecting the release of histamine, β-hexosaminidase and tryptase by colorimetric assays (Peng et al., 2011).

We used tert-butyl hydroperoxide (t-BHP), to induce oxidative stress at 3mmol/l concentration. tert-butyl hydroperoxide (t-BHP) is the membrane-permeant water soluble organic hydroperoxide which damages RBC by lipid peroxidation, ankyrin and spectrin degradation which binds globin to the membrane causing alteration in skeletal network at the horizontal junction sites by detaching actin from the spectrin network, thus modifying cytoskeleton assembly and responsible for a reduction in GSH level and increase in reactive oxygen species (ROS) concentration. So, depending on the concentration used (ranging from 0.1 mmol/l up to 3 mmol/l) t-BHP causes RBC fragmentation. As from the data obtained in the present study, there was a significant uptake of t-BHP treated oxidatively damaged RBCs as compared to normal RBCs by P815 cells. This result was further confirmed by fluorescence microscopy in which we can clearly see from the images the uptake of oxidatively damaged erythrocytes by P815 cells *in vitro*. Uptake of oxidatively damaged erythrocyte was evident from the

fluorescence shift as compared to normal erythrocytes by P815 cells from FACS. These results clearly suggest the involvement of murine mast cells in the uptake of oxidatively damaged erythrocytes.

For validation of any result experiment should be done *in vivo* also. Isolation of peritoneal cavity cells is an easy and important technique to study different immune cells without altering their physiological properties. Mouse specific antibody F4/80 is usually used for distinguishing macrophages since peritoneal macrophages are $F4/80^{high}$, $CD11b^{high}$, and $CD68^{+}$ (Zhang et al., 2010). Mast/stem cell growth factor receptor that is also known as proto-oncogene Kit (CD117) are used for verification of mast cells since α subunit of FcεRI, c-kit, CD34 and CD13 are expressed on surface of peritoneal mast cells (Dahlin et al., 2015). We have characterized mast cell and macrophages population from total peritoneal cells of DBA/2 mouse by isolating peritoneal lavage and staining it with anti c-kit and anti F4/80 antibodies respectively. The total macrophages were found to be 47.75% and mast cells were found to be 2.32% of the total peritoneal cells which was found within the range. From an unelicited peritoneal cavity we can expect to harvest 5-10 × 10^6 total peritoneal exudate cells that will typically contain ~30% macrophages (Ray & Dittel, 2010) whereas macrophages can range from 50% to 75% depending on the eliciting agent used (Zhang et al., 2008) and mast cells can range from 0.05% to 2% (Dahlin et al., 2015). The parallel analysis of both MC and macrophages will allow setting up studies in which the biological effects during various inflammatory settings can be comparatively analyzed.

We have already proved mast cells were involved in erythrophagocytosis by using P815 as a model of mast cells. We then focused on finding the mechanism involved, so we compared receptors involved in erythrophagocytosis in macrophages with that of MCs (List of receptors shown in review of literature). For checking presence of receptors in mast cell we used RBL-2H3 mast cell line because in our lab erythrophagocytosis by RBL-2H3 has been standardized. Also erythrophagocytosis of oxidatively damaged erythrocytes by RBL-2H3 was found to be significantly higher in activated state (Sharma and Puri, 2017, manuscript under preparation). RBL-2H3 cells is extensively used as a mast cell model in studying different aspects of mast cells like expression of rat mast cell protease II (RMCP-II),binding of IgE to FcεRI receptors, similar expression of c-kit receptor tyrosine kinase (in human HMC-1 and murine P-815 mast cells) and subsequent downstream events. RBL-2H3 shows similarity with basophils as well as mucosal mast cells making it an important *in vitro* tool that shows same phenomenon of degranulation in response to immunological stimuli to that of *in vivo* (Passanate & Frankish, 2009).

Before checking for the receptors in mast cells we need to check the primers we made by confirming the presence of these receptors in macrophages. We used rat spleen cells

as positive control. Spleen is largest secondary lymphoid organ which is able to initiate immune responses to blood-borne antigens. It acts as a blood filter comprising red pulp, white pulp and marginal zone. Spleen has highest population of macrophages comprising 20-30% of macrophages (Franken et al.,2015), lymphocytes(both T- and B-cells), plasma cells, dendritic cells, blood cells including erythrocytes, circulating mononuclear cells and granulocytes. Macrophages present in red pulp are involved in erythropoesis and are also actively phagocytic, removing old and damaged erythrocytes as well as blood-borne particulate matter. White pulp as well as red pulp macrophages have homosiderin and ferritin deposites in their cytoplasm which is the converted product of hemoglobin of phagocytosed erythrocytes (Cesta.M, 2006).We designed our primers according to the receptors present in rat that shared homology with humans. RNA was then isolated from rat splenocytes and with their cDNA, all the primers were checked. We have shortlisted 8 receptors that are involved in erythrophagocytosis from literature mining i.e., SIRPα, CD300b, RAGE, VCAM-1, TIM1, TIM3, EphrinB2 and RC3a which were predicted to be present in macrophages (Yoshimaru et al., 2008; Telen, 2000; Martinez-Barriocanal et al., 2010; Binh Le Phong, thesis, 2006; Karra & Levi-Schaffer, 2011;Benard et al.,2004; de Back et al., 2014; Salvucci & Tosato, 2012). In the present study, we found clear band for SIRPα, RAGE, VCAM-1, TIM3 and RC3a receptors in murine splenocytes which confirms the respective primer accuracy. SIRPα, VCAM-1 and RC3a were observed best at 60^{o}C annealing temperature while RAGE and TIM3 was observed at 65^{o}C annealing temperature.

Further, the presence of these positive receptors on mast cells was checked using RBL-2H3 mast cell line. Expression of RAGE and SIRPβ was already reported in RBL-2H3 (Karra & Levi-Schaffer, 2011; Yoshimaru et al., 2008) but not of VCAM-1, EphrinB2, TIM1 and TIM3. Out of these receptors RAGE, VCAM-1 and TIM3 expression was observed. Even on performing temperature gradient of SIRPα and TIM1, no any bands in case of SIRPα whereas multiple bands in case of TIM1 was observed. This may be because SIRPα expression is reported in case of human mast cells (Karra,L & levi-Schaffer, 2011) but its presence is contradictory in case of murine mast cells and recent finding reported that TIM1 had become a pseudogene and its expression in mRNA level is very rare (Acton,Q.A., 2013). So, this maybe the reason we did not get any bands in case of SIRPα and multiple bands in case of TIM1 in the present study. However, the expression of these receptors involved in erythrophagocytosis was only checked in case of RBL-2H3 mast cell in resting state. So, from our study out of 8 receptors, 5 receptors i.e., SIRPα, RAGE, VCAM-1, TIM3 and RC3a were present in spleen macrophages and 3 receptors i.e., RAGE, VCAM-1 and TIM3 in RBL-2H3 which were found to be involved in erythrophagocytosis as described above in review of literature section.

CHAPTER 6

SUMMARY

Depending upon type of organism phagocytosis has its own definition. In prokaryotes it serves as a way of survival whereas in eukaryotes especially in mammals, phagocytosis plays an important role in defense as well as in maintaining homeostasis condition. Professional phagocytes provide first line of defense in human immune system in which macrophage acts as major phagocytic cell. Once a macrophage detects foreign materials an inflammatory response is initiated. During inflammatory response, phagocytosis also increases due to activation of macrophages by various inflammatory mediators released by cells such as mast cells and other immune cells. Previously it was though that clearance of such foreign materials or apoptotic cells by mast cell was by exocytosis mechanism but there are very few papers that have discussed about its endocytosis mechanism. Ultrastructure study of mast cell showed presence of inclusions similar to macrophages and also different vacuoles which contained endocytosed materials like erythrocytes, neutrophils as well as leukocytes.

Our study aims to find out whether mast cells were involved in erythrophagocytosis mechanism in normal or oxidative stress conditions. In endocytosis the best characterized pathway is Receptor-mediated endocytosis so next aim was to find out the receptors involved during the uptake process of erythrocytes in mast cells and to check if there is any similarity with the receptors on macrophage. P815 cells were used as a model of mast cells to observe erythrophagocytosis of oxidatively damaged erythrocytes by fluorescence microscopy which was further confirmed by flow cytometry analysis. . As per the protocol P815 mast cells were co-incubated with normal as well as t-BHP treated (3mmol/l) oxidatively damaged erythrocytes for one hour at $37^{o}C$ in 5% CO_2 incubator. After harvesting the cells, unphagocytosed RBCs were lysed with ACK lysis buffer. After fixing the cells the data was acquired and analyzed by using flow cytometer. For checking presence of receptors in mast cell we used RBL-2H3 mast cell line because RBL-2H3 shows similarity with basophils as well as mucosal mast cells making it an important *in vitro* tool that shows same phenomenon of degranulation in response to immunological stimuli to that of *in vivo*.

We have studied growth kinetics of P815 cells, 2×10^4 cells were seeded in 48 well cell culture plates and at fixed time points cells were harvested by flushing and counted by trypan blue exclusion method using hemocytometer. The doubling time was found to be 13hrs of P815 mast cells. In order to track phagocytosed RBCs by P815 mast cells,

erythrocytes were labeled with CFSE fluorescence dye. To find out efficient concentration of CFSE to be used further for the experiment, normal erythrocytes were labeled with different concentration 1,5,10 and 15µM of CFSE. As compared with control or unstained erythrocytes, it was found that at all the concentrations of CFSE used, almost all the cells were stained but with increasing fluorescent intensities with increasing concentration of CFSE dye. So, for further experiment 10µM CFSE concentration was used for staining erythrocytes. From the FACs data observed uptake of damaged RBCs by P815 mast cells was found to be higher (16.85%±2.16) than that of normal RBCs (2.55%±0.06). Uptake of oxidatively damaged erythrocytes was further confirmed by fluorescence microscopy.

From the dot plot analysis by using FACS Calibur, total macrophage population was found to be 47.75% determined by detecting F4/80 surface marker by using specific F4/80 antibody and mast cell population was found to be 2.32% by the help of c-kit antibody for detecting c-kit surface marker.

RT-PCR technique was used for identification of the receptors. Primers were designed according to the receptors present in rat that shared homology with humans and also we were using rat cell line. Spleen was used as positive control since it consists of highest percentage of macrophages. Erythrocytes were damaged b treating it with ACK lysis buffer. RNA was extracted and cDNA was prepared from both spleen cells as well as RBL-2H3 cells. Presence of SIRPα, RAGE, VCAM-1, TIM3 and RC3a receptors in splenic macrophage and RAGE, VCAM-1 and TIM3 expression was observed in mast cells out of these 8 receptors i.e, SIRPα, CD300b, RAGE, VCAM-1, TIM1, TIM3, EphrinB2 and RC3a which were predicted to be present in macrophages involved in erythrophagcytosis.

Our study suggests that erythrophagocytosis by mast cells takes place in oxidative stress condition and same receptors are involved in erythrophagocytosis present in mast cell and macrophages of which VCAM-1 and TIM3 expression being new finding in case of RBL-2H3 mast cells.

CHAPTER 7
CONCLUSION

In our study we have discussed evidence for role of mast cells involved in erythrophagocytosis. Here we have shown that mast cells are capable to phagocytose oxidatively damaged erythrocytes by a process known as erythrophagocytosis commonly done by macrophages. We have also described about the common receptors present in macrophages and mast cells involved in erythrophagocytosis mechanism of which VCAM-1 and TIM3 expression being new finding in case of RBL-2H3 mast cells. However, the molecular pathways leading to phagocytic mechanism and how they integrate with their signaling pathways are yet to be understood. If we are able to find out under what conditions mast cells perform phagocytosis will itself be an important advance. Blocking these receptors and confirming their involvement in phagocytosis may open a wide range of therapeutic opportunities because the drugs given to treat allergic and autoimmune diseases only control symptoms and often cause serious side-effects. Upon exposure to an allergen or pathogens, mast cells get activated which in turn activates other immune cells like macrophages that leads to increased phagocytosis.

From our *in vitro* study we found that P815 cells show uptake of only oxidatively damaged erythrocytess but not normal erythrocytes similar to RBL-2H3 cells (observed previously in lab), which elicit the role of MCs as scavengers. This is further supported by presence of receptors on MC that are responsible for uptake of erythrocytes in macrophages. Erythrophagocytosis by MCs can be good or bad. It could be harmful if they damage normal erythrocytes during allergies or in case of inflammatory diseases. Hence it is an open question and needs to be confirmed by performing *in vivo* experiments. So, on the basis of our findings in the present study we can conclude that manipulating these receptors in mast cells could be a novel therapeutic target to control anemia during allergic, inflammatory and autoimmune diseases.

CHAPTER 8

REFERENCES

Abraham, S. N., & Malaviya, R. (1997). Mast cells in infection and immunity. *Infect Immun, 65*(9), 3501-3508.

Acton, Q. A. (2012). *Issues in General Science and Scientific Theory and Method: 2011 Edition*: ScholarlyEditions.

Acton, Q. A. (2013). *Endocytosis: New Insights for the Healthcare Professional: 2013 Edition*: ScholarlyEditions.

Al, O. E. (2000). *Medical Laboratory Science : Theory And Practice*: McGraw-Hill Education (India) Pvt Limited.

Bamberg, E., & Passow, H. (2012). *The Band 3 Proteins: Anion transporters, binding proteins and senescent antigens*: Elsevier Science.

Benard, M., Gonzalez, B. J., Schouft, M. T., Falluel-Morel, A., Vaudry, D., Chan, P., . . . Fontaine, M. (2004). Characterization of C3a and C5a receptors in rat cerebellar granule neurons during maturation. Neuroprotective effect of C5a against apoptotic cell death. *J Biol Chem, 279*(42), 43487-43496. doi: 10.1074/jbc.M404124200

Bevers, E. M., & Williamson, P. L. (2016). Getting to the Outer Leaflet: Physiology of Phosphatidylserine Exposure at the Plasma Membrane. *Physiol Rev, 96*(2), 605-645. doi: 10.1152/physrev.00020.2015

Bieker, J. J. (2008). *Red Cell Development*:Academic Press.ISBN:0123743664.Vol 82 Pg.37

Binh Le Phong (2008). Regulation of mast cell function by TIM-1 and TIM3 signaling. d-scholarship.pitt.edu/27700/1/BP-thesis_2016_041316.pdf

Brown, M. A., & Hatfield, J. K. (2012). Mast Cells are Important Modifiers of Autoimmune Disease: With so Much Evidence, Why is There Still Controversy? *Front Immunol, 3*, 147. doi: 10.3389/fimmu.2012.00147

Bulanova, E., & Bulfone-Paus, S. (2010). P2 receptor-mediated signaling in mast cell biology. *Purinergic Signal, 6*(1), 3-17. doi: 10.1007/s11302-009-9173-z

Dahlin, J. S., Ding, Z., & Hallgren, J. (2015). Distinguishing Mast Cell Progenitors from Mature Mast Cells in Mice. *Stem Cells Dev, 24*(14), 1703-1711. doi: 10.1089/scd.2014.0553

de Back, D. Z., Kostova, E. B., van Kraaij, M., van den Berg, T. K., & van Bruggen, R. (2014). Of macrophages and red blood cells; a complex love story. *Front Physiol, 5*, 9. doi: 10.3389/fphys.2014.00009

De Flora, A., & Magnani, M. (2013). *Red Blood Cell Aging*: Springer US.

Ferri, F. F. (2010). *Ferri's Clinical Advisor 2011: 5 Books in 1, Expert Consult*: Elsevier Health Sciences.

Frankish, E. P. a. N. (2009). The RBL-2H3 cell line: its provenance and suitability as a model. *Inflammation research*.

Friedman, H. (2012). *The Reticuloendothelial System: A Comprehensive Treatise Volume 5 Cancer*: Springer US.

Gajewski, T. F., Markiewicz, M. A., & Uyttenhove, C. (2001). The p815 mastocytoma tumor model. *Curr Protoc Immunol, Chapter 20*, Unit 20 24. doi: 10.1002/0471142735.im2004s43

GM, C. (2000). Endocytosis *Endocytosis*. Sunderland (MA): Sinauer Associates.

Hamawy, M. M. (2013). *IgE Receptor (FcεRI) Function in Mast Cells and Basophils*: Springer Berlin Heidelberg.

Hattori, Y., & Seifert, R. (2017). *Histamine and Histamine Receptors in Health and Disease*: Springer International Publishing.

Hemmi, H., Idoyaga, J., Suda, K., Suda, N., Kennedy, K., Noda, M., . . . Steinman, R. M. (2009). A new triggering receptor expressed on myeloid cells (Trem) family member, Trem-like 4, binds to dead cells and is a DNAX activation protein 12-linked marker for subsets of mouse macrophages and dendritic cells. *J Immunol, 182*(3), 1278-1286.

Holgate, S. T. (2012). *Mast Cells, Mediators and Disease*: Springer Netherlands.

Kahn, C. M., & Line, S. (2007). *The Merck/Merial Manual For Pet Health: The complete health resource for your dog, cat, horse or other pets - in everyday language*: Merck & Company, Incorporated.

Karra, L., & Levi-Schaffer, F. (2011). Down-regulation of mast cell responses through ITIM containing inhibitory receptors. *Adv Exp Med Biol, 716*, 143-159. doi: 10.1007/978-1-4419-9533-9_9

Kidd, P. M. *PS (PhosphatidylSerine) Nature's Brain Booster A Vital Lipid Nutrient For Memory Mood and Stress*: Parris M. Kidd, PhD.

Klei, T. R., Meinderts, S. M., van den Berg, T. K., & van Bruggen, R. (2017). From the Cradle to the Grave: The Role of Macrophages in Erythropoiesis and Erythrophagocytosis. *Front Immunol, 8*, 73. doi: 10.3389/fimmu.2017.00073

Krishnan Koyamangalath, D. J. (2017). *Medscape*. Retrieved from Medscape: http://emedicine.medscape.com/article/203948-overview

Lang, F., & FÃ¶ller, M. (2012). *Erythrocytes: Physiology and Pathophysiology*: World Scientific Publishing Company.

Lappin, M. R. (2001). *Feline Internal Medicine Secrets*: Hanley & Belfus.

Lee, K. M., Yin, Charles, Verschoor, Chris P, and Bowdish, Dawn ME. (2013). *Macrophage Function Disorders*. Chichester: eLS. John Wiley & Sons Ltd.

Litman G. W., Cannon J. P., Dishaw L. J. (2005). Reconstructing immune phylogeny: new perspectives. Nat. Rev. Immunol. 5, 866–87910.1038/nri1712

Lu, F., & Huang, S. (2017). The Roles of Mast Cells in Parasitic Protozoan Infections. *Front Immunol, 8*, 363. doi: 10.3389/fimmu.2017.00363

Madewell, B. R., Gunn, C., & Gribble, D. H. (1983). Mast cell phagocytosis of red blood cells in a cat. *Vet Pathol, 20*(5), 638-640. doi: 10.1177/030098588302000518

Martínez-Barriocanal A[1], Comas-Casellas E, Schwartz S Jr, Martín M, Sayós J(2010).CD300 heterocomplexes, a new and family-restricted mechanism for myeloid cell signaling regulation. *Journal of Biological Chemistry, 285(53*), 41781–41794. doi: 10.1074/jbc.M110.140889

Mohandas, N., & Gallagher, P. G. (2008). Red cell membrane: past, present, and future. *Blood, 112*(10), 3939-3948. doi: 10.1182/blood-2008-07-161166

Morvillo, N., & Schmidt, M. (2015). *The MCAT Biology Book*: Nova Press.

Mukherjee, K., Parashuraman, S., Krishnamurthy, G., Majumdar, J., Yadav, A., Kumar, R., . . . Mukhopadhyay, A. (2002). Diverting intracellular trafficking of Salmonella to the lysosome through activation of the late endocytic Rab7 by intracellular delivery of muramyl dipeptide. *Journal of Cell Science, 115*(18), 3693-3701. doi: 10.1242/jcs.00034

Nagata, S., & Nakano, H. (2017). *Apoptotic and Non-apoptotic Cell Death*: Springer International Publishing.

Nikinmaa, M. (2012). *Vertebrate Red Blood Cells: Adaptations of Function to Respiratory Requirements*: Springer Berlin Heidelberg.

Peiser, L., & Gordon, S. (2001). The function of scavenger receptors expressed by macrophages and their role in the regulation of inflammation. *Microbes Infect, 3*(2), 149-159.

Peng, B., He, R., Xu, Q.-H., Gao, J., Lu, Y.-L., & Li, J.-R. (2011). Comparison of RBL-2H3 and P815 Cell Lines for Establishing in vitro Model of Mast Cell Degranulation. *Chinese Journal of Natural Medicines, 9*(3), 227-231. doi: http://dx.doi.org/10.3724/SP.J.1009.2011.00227

Péterfy, H. (2008). *Regulation of the function of mast cells and basophil granulocytes by complement C3a-derived peptides;in vitro and in vivo studies.* (PhD), Eötvös Loránd University, Budapest, Hungary.

Phong, B. L. (2016). *REGULATION OF MAST CELL FUNCTION BY TIM-1 AND TIM-3 SIGNALING.* (Ph.D), University of Pittsburgh.

Poon, I. K., Hulett, M. D., & Parish, C. R. (2010). Molecular mechanisms of late apoptotic/necrotic cell clearance. *Cell Death Differ, 17*(3), 381-397. doi: 10.1038/cdd.2009.195

Rabinovitch, M. (1995). Professional and non-professional phagocytes: an introduction. *Trends Cell Biol, 5*(3), 85-87.

Ray, A., & Dittel, B. N. (2010). Isolation of mouse peritoneal cavity cells. *J Vis Exp*(35). doi: 10.3791/1488

Rieger, M. A., & Schroeder, T. (2012). Hematopoiesis. *Cold Spring Harbor Perspectives in Biology*, *4*(12), a008250. http://doi.org/10.1101/cshperspect.a008250

Ross, M. H., & Pawlina, W. (2006). *Histology*: Lippincott Williams & Wilkins.

Ruud, J. T. (1954). Vertebrates without Erythrocytes and Blood Pigment. *Nature, 173*, 848. doi: 10.1038/173848a0

Saito, H., & Okayama, Y. (2005). *Mast Cells in Allergic Diseases*: Karger.

Salvucci, O., & Tosato, G. (2012). Essential roles of EphB receptors and EphrinB ligands in endothelial cell function and angiogenesis. *Adv Cancer Res, 114*, 21-57. doi: 10.1016/B978-0-12-386503-8.00002-8

Sierra, A., Abiega, O., Shahraz, A., & Neumann, H. (2013). Janus-faced microglia: beneficial and detrimental consequences of microglial phagocytosis. *Front Cell Neurosci, 7*, 6. doi: 10.3389/fncel.2013.00006

Spicer, S. S., Simson, J. A., & Farrington, J. E. (1975). Mast cell phagocytosis of red blood cells. *Am J Pathol, 80*(3), 481-498.

Sokolov, V. E. (1982). *Mammal Skin*: University of California Press.

Telen, M. J. (2000). Red blood cell surface adhesion molecules: their possible roles in normal human physiology and disease. *Semin Hematol, 37*(2), 130-142.

Theoharides, T. C., & Kalogeromitros, D. (2006). The critical role of mast cells in allergy and inflammation. *Ann N Y Acad Sci, 1088*, 78-99. doi: 10.1196/annals.1366.025

Tsokos, G. C. (2004). *Complement in Autoimmunity*: Karger.

Underhill, D. M., & Goodridge, H. S. (2012). Information processing during phagocytosis. *Nat Rev Immunol, 12*(7), 492-502. doi: 10.1038/nri3244

Vajpayee, P., Khatoon, I., Bali Patel, C., Singh, G., Gupta, K., & Shanker, R. (2011). *Adverse Effects of Chromium Oxide Nano-Particles on Seed Germination and Growth in Triticum aestivum L* (Vol. 7).

Van Gorp, H., Delputte, P. L., & Nauwynck, H. J. (2010). Scavenger receptor CD163, a Jack-of-all-trades and potential target for cell-directed therapy. *Mol Immunol, 47*(7-8), 1650-1660. doi: 10.1016/j.molimm.2010.02.008

Van Lancker, J. L. (2006). *Apoptosis, Genomic Integrity and Cancer*: Jones & Bartlett Learning.

Wang, P. (2014). *MFG-E8 and Inflammation*: Springer Netherlands.

Wang, S., Dale, G. L., Song, P., Viollet, B., & Zou, M. H. (2010). AMPKalpha1 deletion shortens erythrocyte life span in mice: role of oxidative stress. *J Biol Chem, 285*(26), 19976-19985. doi: 10.1074/jbc.M110.102467

Weiskopf, K., Weissman, I. L., Garcia, K. C., Kohrt, H. E., Levy, R., Mitchell, B. S., & Program, S. U. C. B. (2016). *Targeting the CD47-SIRP Alpha Interaction to Stimulate Macrophage Destruction of Cancer*.

Yoshimaru T, Suzuki Y, Inoue T, Nishida S, Ra C.(2008). Extracellular superoxide released from mitochondria mediates mast cell death by advanced glycation end products.*Mol Cell Research, 1783(12)*, 2332-2343. doi: 10.1016/j.bbamcr.2008.08.010

Yoneda, M., Guillaume, V., Ikeda, F., Sakuma, Y., Sato, H., Wild, T. F., & Kai, C. (2006). Establishment of a Nipah virus rescue system. *Proc Natl Acad Sci U S A, 103*(44), 16508-16513. doi: 10.1073/pnas.0606972103

Zhang, H., Li, F., Yang, Y., Chen, J., & Hu, X. (2015). SIRP/CD47 signaling in neurological disorders. *Brain Res, 1623*, 74-80. doi: 10.1016/j.brainres.2015.03.012

Zhang, X., Goncalves, R., & Mosser, D. M. (2008). The isolation and characterization of murine macrophages. *Curr Protoc Immunol, Chapter 14*, Unit 14 11. doi: 10.1002/0471142735.im1401s83

Appendices

Appendix 1. 5X TBE Buffer

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

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

Appendix 2. 6x Gel loading dye

10mM Trix pH8

0.03% bromophenol blue

60% glycerol

60mM EDTA

Appendix 3. 5x RNA loading Buffer

Saturated aqueous bromophenol blue solution	16µl
500mM EDTA,pH 8	80µl
37% 12.3 M formaldehyde	720µl
Glycerol	12ml
Formamide	3084µl
10x formaldehyde agarose gel buffer	4ml

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

Appendix 4. TRIZOL Reagent

4M Guanidine thiocyanate Phenol

0.8 M Sodium citrate

0.5% N-laurosyl-5arcosine

0.1 M B-mercaptoethanol

Appendix 5. ACK lysis buffer (1 litre)

NH_4Cl (0.15M)	8.29g
$KHCO_3$ (10mM)	1.001 g
Na_2EDTA(0.1mM)	37.22mg

pH maintained at 7.2-7.4 by adding 1N HCl. Filter sterilize through 0.2µ filter.

Appendix 6. 10x Formaldehyde Agarose gel buffer

200mM MOPS	41.9g
50mM Sodium acetate	410g
10mM EDTA	3.722g

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

Appendix 7. 1x Formaldehyde Agarose gel buffer (1 litre)

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

Appendix 8. Phosphate Buffer Saline (1 litre)

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

pH was maintained to 7.3-7.4 with HCl.

Appendix 9. Trypan Blue (2% Stock solution)

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

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

Appendix 10. Trypan Blue (0.2% Working solution)

2% Stock solution	3ml
PBS	27ml

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

List of Tables

List of Figures

14	4.8	51	Agarose gel electrophoresis(with EtBr staing) of total RNA isolated from RBL-2H3 (A) and cDNA obtained by reverse transcription of same RNA using oligo dT primer long with 100bp ladder(B)
15	4.9	51	Agarose gel electrophoresis(with EtBr staing) of total RNA isolated from activated RBL-2H3 at time points 15 mins and 45 mins(A) and cDNA obtained by reverse transcription of same RNA using oligo dT primer long with 100bp ladder(B)
16	4.10	53	Agarose gel electrophoresis (with EtBr staing) of amplified cDNA using GAPDH, RAGE, VCAM-1, SIPRα and CD300b primers.
17	4.11	54	Agarose gel electrophoresis(with EtBr staing) of amplified cDNA using SIRPα, CD300b, RAGE, VCAM-1 and GAPDH primers (A) and GAPDH, TIM1, TIM3, EphrinB2 and RC3a primers(B).
18	4.12	56	Standardization of annealing temperature for RAGE.
19	4.13	57	Standardization of annealing temperature for TIM3.
20	4.14	58	Standardization of annealing temperature for TIM1.
21	4.15	60	Agarose gel electrophoresis of amplified cDNA from rest and activated (for 15’ and 45’) RBL-2H3 using GAPDH primer for 30cycles at 60^{o}C.
22	4.16	61	Agarose gel electrophoresis of amplified cDNA from RBL-2H3 using GAPDH, RAGE, VCAM-1, SIRPα and EphrinB2 primers at annealing temperature 60^{o}C (A) and using GAPDH, RAGE and TIM3 at 65^{o}C(B). PCR was performed by starting with 1µg RNA from RBL-2H3 for 40 cycles.
23	4.17	62	Standardization of amount of RNA template for VCAM-1.
24	4.18	63	Standardization of annealing temperature for TIM1 (A) and for SIRPα(B).