STUDY THE MODULATION OF SNARE MACHINERY IN MACROPHAGE IN RESPONSE TO LPS

M.Sc. Thesis

(2014)

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

In the Partial Fulfillment for the Requirement of the Degree of Masters in Biotechnology

Tarka Raj Bhatta

Exam Roll No: 083/068

TU Registration No: 5-1-61-233-2005

STUDY THE MODULATION OF SNARE MACHINERY IN MACROPHAGE IN RESPONSE TO LPS

M.Sc. Thesis

(2014)

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

By

Tarka Raj Bhatta

Supervisors

Dr. Rajani Malla
Assoc. Professor, HOD
TU, Nepal

Dr. Niti Puri
Asst. Professor,
JNU, India

TU Registration No: 5-1-61-233-2005

Dedicated to

My Bajai ***Mandharee Devi Bhatta,***

My Aama ***late. Janaki Devi Bhatta,*** *and*

My Buwa ***Bhoj Raj Bhatta***

Acknowledgement

This M.Sc theis, like most research work, is the result of a curious and inquisitive sprit, coupled with plenty of hard work and persistence. Naturally, it was difficult at times, but overall, the fulfilling moments far exceeded the hardship – and I owe that to a world of people, to whom I will always be grateful.

I would like to give my sincere thanks to ***Dr. Rajani Malla****, Head of the Department, Central Department of Biotechnology, Tribhuvan University, Nepal for her supervision, continuous advice, support, encouragement, trust and recommending me to complete my thesis at such a prestigious university without which this research work have not been possible. I will forever be thankful to her for her scientific advice and knowledge and insightful discussions and suggestions and for being a great soul. She always provided me best of everything and inspired me to work hard. I am grateful to her for her time as well as her diligent efforts in significantly improving the overall quality of this study.*

I would like to express my heartfelt gratitude to, ***Dr. Niti Puri****, Assistant Professor, School of Life Sciences, Jawaharlal Nehru University, for her supervision, her support, patience, and encouragement throughout my research period. It is not often that one finds an advisor that always finds the time for listening to the little problems and roadblocks that unavoidably crop up in the course of performing research. I could not have imagined having a better advisor for my research. Her technical and editorial advice was essential to the completion of this dissertation and has taught me innumerable lessons and insights on the workings of academic research in general. As my supervisor, she has taught me more than I could ever give her credit for here. She has shown me, by her example, what a good scientist (and person) should be.*

I would like to thank ***Prof. Dr. Mohan Kharel, Prof. Dr. Ganga Kharel, Prof. Dr. Tribikram Bhattarai, Prof. Dr. Krishna Das Manandhar, Dr. Tilak R. Shrestha, Dr. Sampoornanand Jha****,* ***Dr. Deepak Raj Pant, Dr. Ramchandra Basynat, Ms. Jarina Joshi, Mr. Bal Hari Paudel and Dr. Smeeta Shrestha*** *and all the teachers and staffs of Central Department of Biotechnology for supporting me by providing their valuable suggestions.*

I would like to express my special thanks to PhD scholar, ***Mr. Zaigham Abbas Rizvi, Mr. Anwar Alam, Ms. Seroshi Chatterjee, Mrs. Vasudha Agrawal, Mrs. Nilofer Naqvi, Ms. Pieu Naskar****,* ***Ms. Priyanka Sharma, Ms. Gagandeep Kaur*** *school of life science, JNU, for their excellent guidance, care, support, supervision and answering my endless queries and cooperation throughout my training period.*

*A good support system is important to stay in JNU. I am lucky to get the warm help and care of **Mr. Sandeep Paudel.** I would like to pay my special regard to him. Thank you very much for being my generous friend and guardian.*

*My thanks also go to my senior dai**, Govinda Sha and Santosh Panthi** for providing me their valuable suggestions, caring about me and about my works. My special THANKS go to my sathi **Dr.Bhawana Bhatta**, dear friends **Pratikchya and Rashmi** for their help during my lab works. I would like to thank **Tarun Shardar** for kind help and making the lab very comfortable and ordered to work in.*

*A special thanks to my family. Words cannot express how grateful I am to my Buwa **Bhoj Raj Bhatta**, my uncles **Shiva Raj Bhatta** and **Dharma Raj Bhatta**, for all of the sacrifices that you've made on my behalf. Your prayer for me was what sustained me thus far. I would also like to thank to my dee **Hitaisi Bhatta**, my dai **Surendra Bhatta**, my little cute brother **Navraj Bhatta**. Thank you for supporting me for everything, and especially I can't thank you enough for encouraging me throughout this experience. To my bhai **Dharma**, and babu **Saurav**, I would like to express my thanks for being such a good guys always cheering me up.*

*Last but not least, I thank my god, my aama late. **Janaki Devi Bhatta**, for letting me through all the difficulties. I have experienced Your guidance day by day. You are the one who trust me the must. I will keep on working to make your dream come true. I will keep on trusting You for my future. I am missing you very much aama. Thankyou very much, my Lord.*

TARKA RAJ BHATTA

Glossary Acronyms

Ag : Antigen

AMs : Alveolar macrophages

APCs : Antigen presenting cells

A-SMase: Acid spingomyelinase

BCR : B-cell receptors

Cat S : Cathepsin S

CD : Cluster of differentiation

CpG : Methylated Oligonucleotide

Crt : Calreticulin

CTL : Cytotoxic T- lymphocyte

CTL : Cytotoxic T-lymphocyte

Cxn : Calnexin

DCs : Dendritic cells

DEPC : Diethyl Pyro Carbonate

DNA : Deoxyribo Nucleic Acid

EDTA : Ethylene Diamine Tetra Acetate

ER : Endoplasmic reticulum

ERAD : Endoplasmic reticulum associated protein degradation

FBS : Fetal Bovine Serum

GAPDH : Glyceraldehyde-3-Phosphate Dehydrogenase

IFN-γ : Interferon Gamma

Ig : Immunoglobulin

Ii : Invariant chain

IL : Inter leukin

ITAM : Immunoreceptor tyrosine-based activation motif

LDLR : Low-density lipoprotein receptor

LPS : Lipopolysaccharide

Mg : Milligram

MHC : Major histocompatibility complex

ml : Millilitre

MR : Mannose receptor

MTP : Microsomal triglyceride transfer protein

NCBI : National Centre for Biotechnology Information

NIH : National Institute of Health

NSF : N-ethyl-maleimide sensitive fusion protein

PBS : Phosphate Buffer Saline

PRR : Pattern-recognition receptors

RNA : Ribonucleic Acid

RPMI : Rosewell Park Memorial Institute

SNAP : Soluble N-ethyl-maleimide sensitive factor attachment protein

SNARE : Soluble N-ethyl-maleimide sensitive factor attachment protein receptor

SV40 : Simian virus 40

TAP : Transporter associated with antigen processing

T_c : T cytotoxic

TCR : T-cell receptor

TGN : Trans-Golgi Network

T_H : T helper

TLR : Toll-like receptors

TNF-α : Tumor necrosis factor alpha

t-SNARE : target membrane SNARE

VAMP : Vesicle Associated Membrane Protein

v-SNARE : vesicle-associated SNARE

CONTENTS

List of Figures

List of Tables

ABSTRACTS

Human body is regularly under attack by various pathogens as it provides optimal environment for growth of pathogens. To defend against these pathogens and their toxic compounds, our immune system has developed an intricate method of recognition and responses. Recognition of antigens by T-cells requires uptake, processing and presentation of pathogen-derived antigens on MHC of APCs. While the pathways of antigen processing and presentation in APCs have been defined, not much is known about the molecular mechanisms and regulation of intracellular traffic involved in antigen processing and presentation pathways in response to pathogens. SNAREs are the proteins that play crucial role in vesicle trafficking in cells and therefore in all immune processes involving exocytosis of inflammatory mediators and uptake and killing of pathogens, also in transport, expression and downregulation of various receptors. So the main aim of our research was to study the modulation of expression of SNARE proteins against LPS challenges. MH-S cell line was choosen as the model for our study as it is a macrophage cell line which is capable of phagocytois and antigen presentation of various pathogens as well as exocytosis of various cytokines produced against the pathogens. We standardize RT-PCR, real-time PCR in term of various parameters and using those standardized condition the modulation of the SNARE proteins was studied in presence of LPS. In order to study the modulation of expression of SNAREs at protein level, western blotting was done. We found that expression of the SNAREs proteins studied was not modulated by LPS treatment pointing to some other mode of regulation of SNARE mediated intracellular traffic in response to LPS challenge in macrophages.

Key words: LPS, SNAREs, RT-PCR, Real-time PCR, Western blotting, MH-S cell lines

Chapter I

INTRODUCTION

Human body provides a perfect niche for the survival and growth of various pathogens due to a nutrient-rich, warm and moist environment, which remains at a uniform temperature and constantly renews itself. So, it is not surprising that many microorganisms like various bacteria, viruses and multicellular parasites have evolved the ability to survive and reproduce in this desirable niche. Pathogenic bacteria which can be gram positive or gram negative, gain entry into the body through various routes and cause disease. The ability of the bacteria to enter the human body and their potential to generate toxins (endotoxins and exotoxins) are the two basic factors determining the pathogenicity of the bacteria (Finlay and McFadden, 2006). Many gram negative bacteria like *Vibrio cholera*, *Escherichia coli*, *Chlamydia trachomatis*, *Salmonella typhi* and *Pseudomonas aeruginosa* have emerged as important pathogens causing a variety of human diseases. The pathogenic capability of gram-negative bacteria is often associated with certain components of gram-negative cell envelope, in particular, the lipopolysaccharide (LPS) layer. LPS, the main cell wall component can itself be responsible for a very severe systemic septic shock (Munford, 2008).

To defend against these pathogens and their toxic components, an intricate method of recognition and response comprising our immune system has developed. The immune system controls the entry of these pathogens in the human body by destroying them directly or destroying the infected cells. Generally, it recognizes the pathogen as non-self and protects our body by killing the pathogen or by neutralizing their toxic effects. The innate immune system uses a variety of germline-encoded pattern-recognition receptors (PRRs) as Toll-like receptors (TLRs) that recognize conserved microbial structures or pathogen-associated molecular patterns (PAMS), such as those that occur in the bacterial cell wall components peptidoglycans and lipopolysaccharides and activate the defense system against them (Akira *et al*., 2001). The microbial infections are sensed by TLRs, which lead to activation of transcription factor NF-κB. Thus activated NF-κB induces the expression of inflammatory cytokines (Takeda and Akira, 2005). LPS recognition by TLR4 on innate immune cells is the main initial event starting a whole cascade of defined gene expression and a very specialized immune response to counter the onslaught of these gram negative bacteria.

The recognition of pathogen by innate immune system also sets the stage for activation of the more specific adaptive immune system to tackle the pathogen in more definitive way. T lymphocytes of the adaptive immune system play an important role in this host defense either by providing help to other arms of immune system or by directly killing

the infected cells. The uptake, processing and presentation of antigens by antigen presenting cells (APCs) like macrophages and dendritic cells is a crucial step for activation of adaptive immune system. MHC and CD1 molecules on APCs are involved in presenting proteinaceous or lipid antigen to T cells, respectively.

Macrophages and dendritic cells remove the microorganisms and other foreign particles by phagocytosis. During phagocytosis, microorganisms are taken up by immune cells into phagosomes. Through membrane-trafficking events mediated by SNARE proteins, phagosomes fuse with lysosomes, generating degradative phagolysosomes (Matheoud *et al*., 2013). TNFα and other cytokines that are produced in responses to the antigens are trafficked from the Golgi to the recycling endosome (RE), where R-SNARE-VAMP-3 mediates its delivery to the cell surface after pairing with Q-SNARE (Syntaxin4-SNAP23) on plasma membrane at the site of phagocytic cup formation. The fusion of RE at the cup simultaneously allows rapid release of TNFα and expands the membrane for phagocytosis (Murray *et al*., 2005). In the same way SNARE proteins also play a crucial role for the maturation of the phagolysosome in which degradation of the antigens take place (Matheoud *et al*., 2013). The crucial role of SNARE proteins in membrane fusion and their presence in phagosomal membranes suggest that SNARE proteins have critical roles in phagosome biogenesis. SNAREs are therefore, also likely to be important for uptake and processing of antigens for presentation to T-cells. Hence, defective SNARE proteins may be responsible for deregulated phagocytosis and the resulting pathologies.

SNARE proteins have also important role in exocytosis machinery inside cells for different molecules. SNAP23 is ubiquitously expressed and found to be important for exocytosis of mast cell granules (Puri *et al*., 2003). Syntaxin 4 are present in the plasma membrane as well as on intracellular vesicles in mammalian cells and has its role in the vasopressin-regulated trafficking of aquaporin-2 water channel vesicles to the apical plasma membrane of renal collecting duct cells (Mandon *et al*., 1996). During macrophage activation, Syntaxin 4 is regulated to function in membrane traffic and cytokine secretion (Pang *et al*., 2003). VAMP3 is ubiquitously expressed in all the cells and tissues and is involved in recycling of transferrin receptors to the plasma membrane (Galli *et al*., 1993), secretion of α-granules in platelates (Feng et al., 2002), recycling of T-cell receptors to the immunological synapses (Das *et al*., 2004), and membrane trafficking during cell migration (Tayeb *et al*., 2005). Therefore, it is logical to presume that specific SNARE proteins must also be involved in delivery and presentation of MHC or CD1 and processed antigen to APC membranes for display.

Hypothesis

While there are a lot of studies on the structure and interactions of SNARE proteins, there are hardly any studies on their role in antigen presentation pathway and on their regulation in response to pathogenic challenge. The main aim of present study was to study the modulation of expression of SNARE proteins (mainly SNAP 23, Syntaxin 4 and VAMP 3) in macrophages in response to LPS challenge. Transcriptional regulation of SNAREs was studied by quantitative RT-PCR and real time PCR and the protein levels were assayed by western blotting. These studies will reveal the specific SNARE proteins and the specific intracellular trafficking pathways involved in antigen uptake, processing and presentation and will also help in identifying how these pathways are regulated in response to gram negative pathogen challenges.

Research objectives

General objectives:

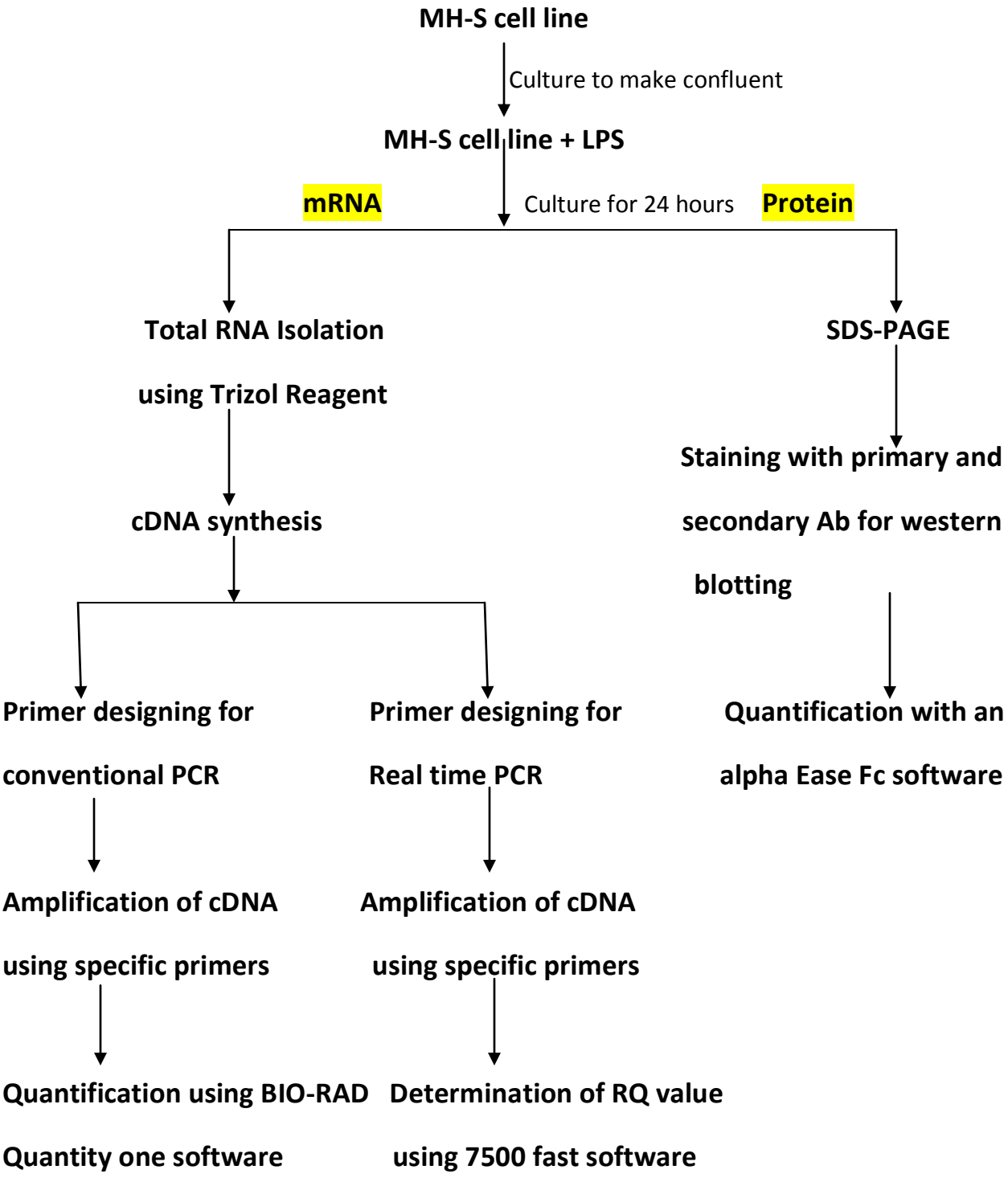
Study the modulation of SNARE machinery in macrophage in response to LPS.

Specific objectives:

1. Innoculation of LPS in MH-S cell line and culture of the cells.
2. Total RNA isolation from the cultured cell line and cDNA synthesis.
3. Designing of primer for the molecules involved in SNARE machinery.
4. Amplification of cDNA using specific primer and quantification of band intensity.
5. Standardization of the parameters like annealing temperature, number of thermocycles and amount of RNA for the molecules SNAP-23, Syntaxin 4 and VAMP 3.
6. Expression analysis of SNAP-23, Syntaxin 4 and VAMP 3 on MH-S cell line using RT-PCR and Real time PCR.
7. Preperation of cell lysates for SDS-PAGE.
8. Expression analysis of SNAP-23 and Syntaxin 4 in protein level using western Blotting.

Research Plan

The present work was planned as following:



Chapter II

REVIEW OF LITERATURE

2.1 The immune system

The immune system is a defense system which defends the body against a wide variety of pathogenic infectious agents such as viruses, bacteria, fungi, protozoa, parasitic worms, etc. The complexity of this task requires a sophisticated repertoire of mechanisms for the recognition of, and defense of the body against, these pathogens. This is achieved by an array of cells (and molecules which they secrete) which are dispersed throughout the body and collectively constitute the immune system (Todd *et al*., 2001).

2.2 Components of immune system

The immune system of higher animals can be simplistically viewed as consisting of three levels: (a) anatomic and physiologic barriers; (b) innate immunity; and (c) adaptive immunity (Figure 2.1). Anatomical and physiological barriers provide the crucial first line of defense against pathogens. These barriers include intact skin, vigorous mucociliary clearance mechanisms, low stomach pH, and bacteriolytic lysozyme in tears, saliva, and other secretions (Turvey *et al*., 2009). Innate immunity augments the protection offered by anatomical and physiological barriers. The innate immune system defends against invading pathogens and is particularly important in warding off bacterial and viral infections presented at the mucosal cell surface (Levy, 2001). Humoral components that include well-characterized components, such as complement proteins, LPS binding protein, C-reactive protein and other, collectins, and antimicrobial peptides, including defensins contribute to the innate immune response (Turvey *et al*., 2009). The cellular elements of the innate immune system are neurophils, monocytes, macrophages, natural killer cells, dendritic cells and mast cells which provide immediate host defense. The highly conserved nature of the response, which is seen in even the less complex organisms, confirms its importance in fighting against many infections. Adaptive immunity is the hallmark of the immune system of higher animals. This response consists of antigen-specific reactions through T lymphocytes and B lymphocytes. The innate response is rapid but lacks specificity, the adaptive response is precise, but takes several days or weeks to develop. The adaptive response has memory, so that subsequent exposure leads to a more vigorous and rapid response, but this is not immediate (Parkins *et al*., 2001).

The characteristic of adaptive immunity is the use of antigen-specific receptors on T and B cells to drive targeted effector responses in two stages. First, the antigen is presented to and recognized by the antigen specific T or B cells leading to cell priming, activation and differentiation, which usually occurs within the specialized environment of lymphoid tissue. Second, the effector response take place, either due to the activated T cells leaving the lymphoid tissue and homing to the disease site, or due to the release of antibody from activated B (plasma cells) into blood and tissue fluids, and hence to the infective focus (Parkin *et al*., 2001).

The innate immune system uses a variety of germline-encoded pattern-recognition receptors (PRRs) as Toll-like receptors (TLRs) that recognize conserved microbial structures or pathogens-associated molecular patterns (PAMPs), such as those that occur in the bacterial cell wall components peptidoglycan and lipopolysaccharide (Akira *et al*., 2001). TLRs are type I transmembrane proteins with ectodomains containing an amino-terminal leucine-rich repeats (LRRs) that mediate the recognition of PAMPs; transmembrane domains; and a carboxy terminal intracellular Toll interleukin 1 (TIR) domains required for downstream signal transduction (Kawai *et al*., 2010). Regarding their role in pathogen recognition, TLR family members are expressed by cells involved in the first line of host defense, including neutrophils, macrophages, dendritic cells, dermal endothelial cells and mucosal epithelial cells. TLR2 and TLR4, which are the major receptors for bacterial lipoproteins and LPS, respectively, are also expressed on B and T cells (Armant *et al*., 2002). Although TLRs are essential for protective immunity against infection, inappropriate TLR responses due to endogenous molecules produced by dying cells, or in certain pathological conditions, contribute to acute and chronic inflammation, as well as to systemic autoimmune diseases (Kawai *et al*., 2010).

Essential to the successful removal of pathogens is the early recognition of microbes by components of the innate immune system. These involve the complement system, specialized receptors expressed on cells of innate immune system like macrophages, dendritic cells, etc and the family of TLRs that are expressed on myeloid as well as lymphoid cells that recognize specific microbially derived molecular structures. The pathogens are processed and by the help of MHC I and MHC II molecules they are presented to T-cells for the activation of adaptive immune response (Vyas *et al*., 2008). Unlike membrane bound antibodies on B cells, which can recognize antigen alone, T-cell receptors can recognize only antigen that is bound to cell-membrane proteins called MHC molecules. There are well-defined subpopulations of T cells: T helper (T_H) and T cytotoxic (Tc) cells. T helper and T cytotoxic cells can be distinguished from one-another by the presence of either CD4 or CD8 membrane glycoproteins on their cell surfaces. There is another subpopulation known as the regualtiory T cells (T regs). The Gram negative bacterial coat component lipopolysaccharide (LPS), the main culprit behind

toxic shock syndrome and sepsis, is a highly potent trigger of cytokine secretion through TLR4.

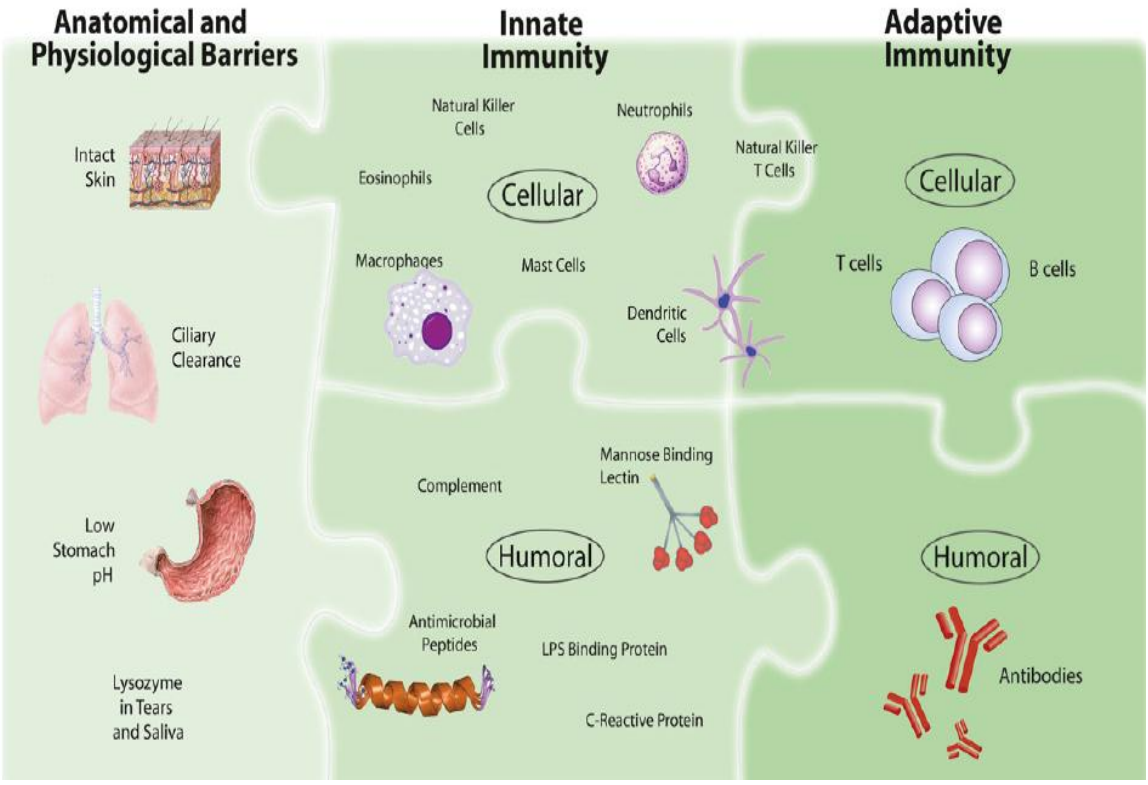


Figure 2.1: Integrated higher animal immune system. The defense system can be simplistically viewed as consisting of three levels: (a) anatomical and physiological barriers; (b) innate immunity; and (c) adaptive immunity. [Source: (Turvey *et al*., 2009)].

The cytokines made by the innate immune cells in response to TLR/PRR activation plays a major role in potentiation of adaptive responses (Lacy and Stow, 2011). Thus, a well orchestrated innate and adaptive immune response will lead to pathogen eradication and host immunity.

2.3 Antigen processing and presentation

Antigens in the peptide-binding groove of surface-expressed MHC class I and class II molecules are recognized by specific T-cell receptors that lead to T-cell activation. To fulfill this condition, the two different classes of MHC molecules first acquire peptide antigens from different sources. Endogenous peptides are generated in the cytoplasm through the action of proteasomes. The exogenous proteins are internalized into the endosomal pathway by a variety of mechanisms, and unfolding and fragmentation are catalyzed by the disulfide reductases and lysosomal proteases. This phenomenon is called as processing of the antigen (Jansen , 2007). MHC class I molecules sample the antigens from the viral infection events and intracellular bacteria and present these antigenic peptides to $CD8^+$ T cells. For MHC class II molecules, the goal is to sample the

extracellular milieu and present antigens to $CD4^+$ T cells for potential activation (Vyas *et al*., 2008). And the lipid antigens are presented by CD1d molecules especially to invariant natural killer T cells (Kunte *et al*., 2013). This process is called as presentation of antigen (Vyas *et al*., 2008).

2.3.1 Professional antigen presenting cells

The term professional antigen presenting cells (APCs) is used to define the bone marrow- derieved cells that have the capability to induce signal 1 and signal 2 for the activation of T-cell. Signal 1 is produced by ligation of the T-cell receptor (TCR)/CD3 complex with antigenic peptides bound to the appropriate major histocompatibility complex (MHC) molecules. It is antigen specific. And signal 2, often termed the 'co-stimulatory' signal is produced by interaction of CD28 on T-cells with one of its ligands, B7-1 or B7-2/B70, on professional APCs. In addition to this inflammatory cytokines like TNF-α and IL-1 induces expression of CD40 on dendritic cells and CD40-dependent signaling can in turn increase B7 expression on APC. Hence, these cytokines have the ability to increase expression of co-stimulatory molecules on APC, thus enhancing the level of signal 2 available to the T cells (Curtsinger *et al*., 1999). It is not antigen specific (Nickoloff *et al*., 1994). There are three main types of professional antigen presenting cells, the macrophage, dendritic cells and B lymphocytes.

2.3.1.1 Macrophages

Monocytes and macrophages originate from a common myeloid precursor and play an important role in innate immune response against invading pathogens. Normally, long lived macrophages in all the organs are formed by the differentiation of short lived bloodstream circulating monocytes in response to differentiation factors or cytokines(M-CSF, GM-CSF, IL-3) (Parihar *et al*., 2010). Although all macrophages have the same origin, their function depends on the tissue in which they reside; in the erythroblast centers in bone marrow they transfer iron to erythroblasts; in spleen they phagocytose erythrocytes; in other tissues they phagocytose microorganisms, cells and residual products (Valledor *et al*., 1998). They are found in almost every organ and were historically given different names based on the tissue in which they reside (Figuer 2.2) (Parihar *et al*., 2010). Langerhans cells are present in the epidermis, osteoclasts in the bone, alveolar macrophages in the lung, microglia, perivascular, meningeal and choroid plexus macrophages in the central nervous system, marginal zone and metallophilic macrophages in spleen, kupffer cells in liver, inflammatory-monoyte-derived macrophages at the site of inflammatory lesion, etc (Gordon and Taypor ,2005). The macrophages constantly survey their surrounding environment for signs of foreign invading organisms or tissue damage and they stimulate lymphocytes and other immune

cells in response to the danger signals detected by cell surface receptors on macrophages.

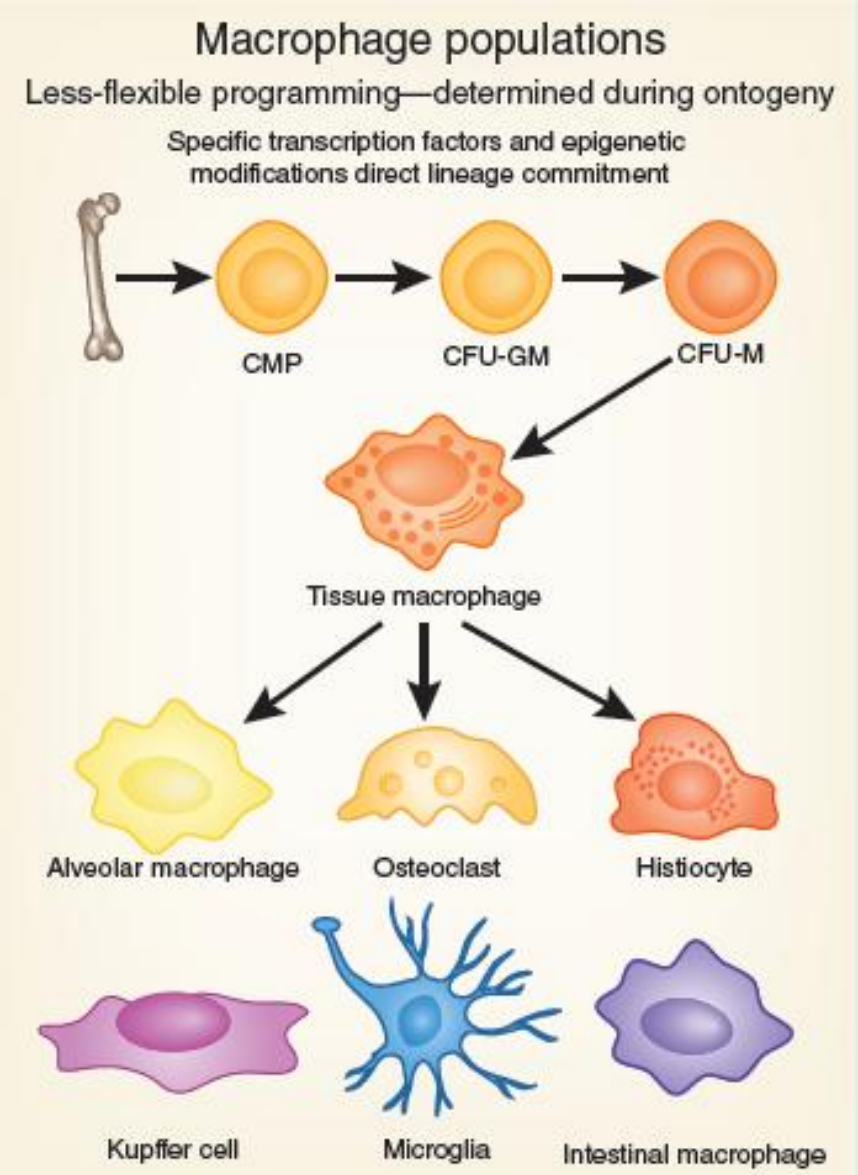


Figure 2.2: Macrophage populations. Macrophages can be subcategorized into specific populations on the basis of their anatomical location. Tissue-resident macrophages include alveolar macrophages (lungs), histiocytes (interstitial connective tissue), osteoclasts (bone), microglia (brain), intestinal macrophages, Kupffer cells (liver) and so on. Mononuclear phagocyte subpopulations in the circulation can also differentiate into tissue macrophages after entering different anatomical sites. [Source: (Galli *et al.*, 2011)]

(CMP: common myeloid progenitors; CFU-GM: colony forming unit granulocyte macrophages; CFU-M: colony forming unit macrophages)

Along with fighting infections, resident tissue macrophages are involved in maintaining healthy tissue by removing dead and dying cells and toxic materials. The alveolar macrophages facilitate the removal of allergens from the lung, the Kupffer cells in liver participate in the clearance of pathogens and toxins from the circulation. In a similar way tissue macrophages also suppress inflammation mediated by inflammatory monocytes, thereby ensuring that tissue homeostatis is restored following infection or injury (Murray *et al*., 2011).

Macrophages have evolved a variety of strategies to internalize particles and solutes, including pinocytosis, receptor-mediated endocytosis, and phagocytosis. Pinocytosis usually refers to the uptake of fluid and solutes, and it is closely related to receptor-mediated endocytosis, the specific process through which macromolecules, viruses, and small particles enter cells. Pinocytosis and receptor-mediated endocytosis share a clathrin-based mechanism and usually occur independently of actin polymerization. By contrast, phagocytosis, the uptake of large particles (>0.5μm) into cells, occurs by an actin-dependent mechanism and is usually independent of clathrin. Macrophages have been referred to as professional phagocytes and are very efficient at internalizing particles. Phagocytosis by macrophages is critical for the uptake and degradation of infectious agents and senescent cells, and it participates in development, tissue remodeling, the immune response, and inflammation (Aderem and Underhill, 1999).

Alveolar macrophages (AMs) play numerous roles in immunity, inflammation, and tissue repair. They are crucial for the clearance and processing of microorganisms, dead cells, and environmental debris in the lung tissue via phagocytosis (Murphy *et al*., 2008). AMs contain toll-like receptor 4 (TLR4), a receptor for lipopolysaccharide (LPS), a cell wall protein on gram negative bacteria, for phagocytosis ((Wong *et al*., 2012). After phagocytosis and internalization of the particulate matter like LPS, the organic components are digested by the endosome into peptide fragments that combine with the MHC class II complex for presentation to CD4+T cells, which are pivotal steps in cell-mediated and adaptive immunity (Alexis *et al*., 2006). The alveolar macrophages then produce the proinflammatory cytokines TNF-α, IL-6 and IL-1β in response to LPS stimulation (Wong *et al*., 2012).

A continuous cell line of murine alveolar macrophage (AM), designated MH-S, has been established following transformation of cells obtained by bronchoalveolar lavage from BALB/cJ mice with simian virus 40 (SV40). MH-S cells are adherent, lacked contact inhibition, and are trypsin-sensitive. MH-S cell line expresses intacellular T-antigen and has a doubling time of approximately 48 hours. MH-S exhibits typical macrophage morphology, showing more than 98% esterase-positivity, is negative for peroxidase, and expresses cell surface Ia and Mac-1 antigens. The cells are Fc receptor-positive as

demonstrated by rosette formation with, and phagocytosis of, antibody coated sheep erythrocytes (Mbawuike *et al*., 1989). This cell line is routinely used as model for alveolar macrophages.

2.3.1.2 Dendritic cells

Dendritic cells (DCs) are bone marrow derived professional antigen-presenting cells (APCs) which are widely distributed, as immature DCs, in both lymphoid and non lymphoid tissues. Immature DCs sample self and foreign antigen. When triggered by pathogens, the pattern-recognition receptors (PRRs) expressed by immature DCs cause them to transform into mature DCs. The expression of high amounts of cell surface major histocompatibility complexes (MHC) and co-stimulatory molecules by the mature DCs initiate primary T cell- mediated immune response (Liu *et al*., 2001). When tissue-resident Dendritic cells (DCs) detect conserved pathogen derived molecules or inflammatory cytokines, they initiate an activation program and migrate to local lymph nodes, where they present antigen to and activate naïve T cells (Hou *et al*., 2004). In addition to this DCs can also induce peripheral tolerance by presenting self- antigen and anergising auto-reactive T cells. Hence, DCs play a unique role in the initiation of the immune response and also as master regulators of the immune system (Gatti *et al*., 2003).

DCs are classified based on the precursor populations from which the various DC subsets originate as conventional DCs and non-conventional DCs. Those DCs which are derived from common DC progenitor and pre-DC populations are conventional DCs that include both migratory and lymphoid-resident DC subsets (Figure 2.3). And those which are derived from monocyte and plasmacytoid DCs are non-conventional DCs and are unique in their ability to secrete high amounts of IFN; this distinguishes them from conventional DCs (Kushwah *et al*., 2011) .

DCs are efficient stimulators of T lymphocytes. They capture and process both intracellular and extracellular antigens, and display large amounts of MHC-peptide complexes at their surface. The complex is then recognized by T-cell antigen receptors (TCRs) on T-cell due to which stimulation of T-cell occurs (Banchereau *et al*., 1998). Unlike most other cell types, DCs have the capacity to transfer exogenously derived fragments of peptides to the MHC class I molecules by a process referred to as cross-presentation to activate naïve CD8+ T cells by two mechanisms; one of them is Transporter associated with antigen processing (TAP) dependent and the other is TAP independent (Lizee *et al*., 2003). DCs use distinct mechanisms for antigen capture. The first is a high level of fluid phase uptake via micropinocytosis and macropinocytosis. Micropinocytosis is the uptake of small vesicles (0.1µm) via clathrin-coated pits.

Macropinocytosis in DCs is constitutive and allows continuous internalization of large vesicles (0.5-3µm) mediated by membrane ruffling driven by the action of cytoskeleton.

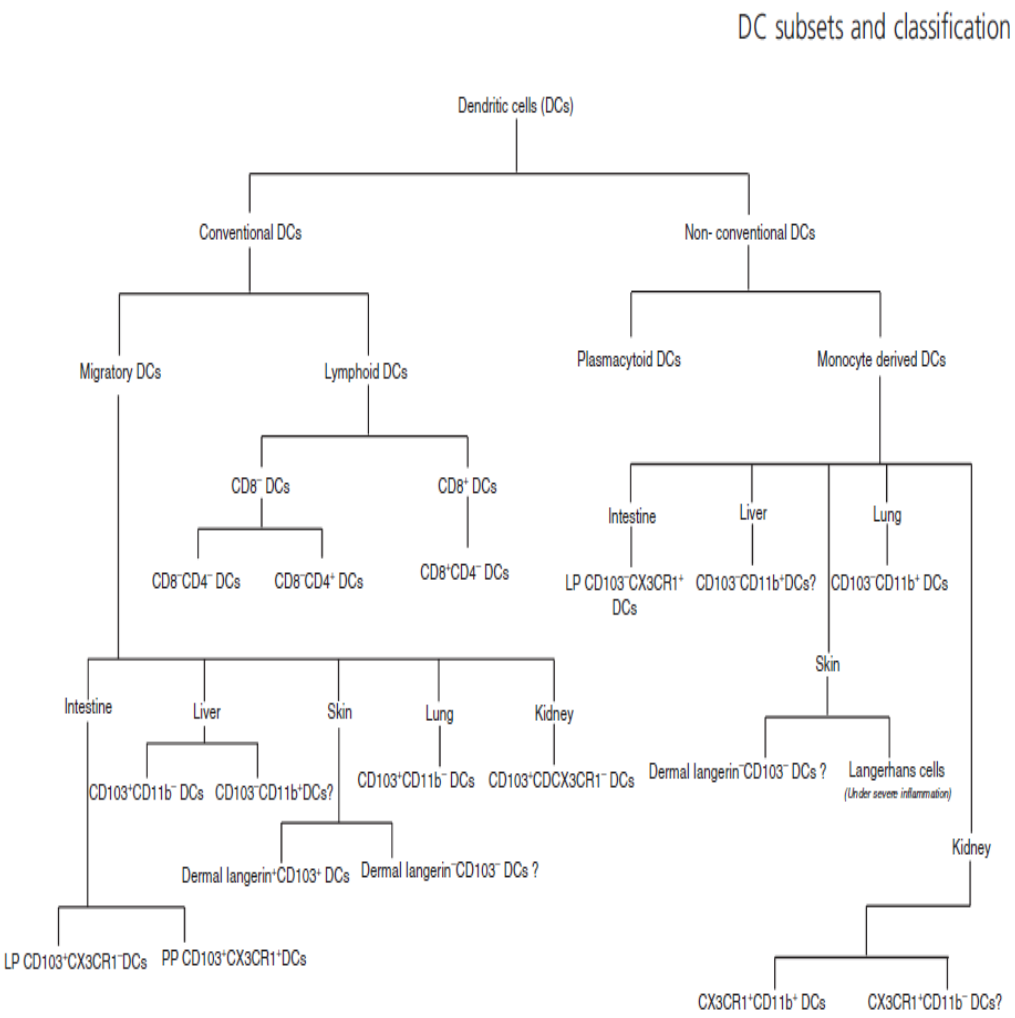


Figure 2.3: Classification of dendritic cell (DC) subsets as conventional and non-conventional DCs. Conventional DCs are derived from common DC progenitor and pre-DC populations and are further divided into migratory and lymphoid DCs. Non-conventional DCs include plasmacytoid DCs, which are derived from the pre-DC population along with monocyte-derived DC subsets, and are found in various peripheral organs. [Source: (Kushwah *et al*., 2011)].

The second mechanism of capture is mediated via the mannose receptor (MR), which is expressed at high levels on DCs (Sallusto *et al*., 1995). DCs are present in all peripheral tissues and accumulate at the sites of pathogen entry. DCs express a large array of phagocytic receptors and efficiently phagocytose pathogens. DCs also express a variety of Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs). Various PRRs are selectively expressed in particular DC subpopulations, which undergo particular maturation programs selectively in response to different pathogens, migrate to

lymphoid organs, and present antigen to T lymphocytes to initiate antigen-specific immune responses (Savina and Amigorena, 2007).

For the detection of pathogens, DCs use a variety of receptors, including those of the Toll-like receptor (TLR) family, which reorganize pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide (LPs), methylated olionucleotides (CpG) and viral double-stranded RNA (Wilson *et al* ., 2006). The stimulation of DCs occurs by the recognition of characteristic patterns of pathogens, inflammatory cytokines and necrotic cells. (Langenkamp *et al*., 2000). The main hallmark of DC maturation is expression of major histocompatibility complex molecules (MHC), T cell co-stimulatory molecules (CD40, CD80 or CD86) and cytokines (for example, interleukin 23; IL-23) in addition to a gene expression program of intracellular factors that enable effective antigen uptake, processing and presentation, and T cell activation (Shih *et al*., 2012).

2.3.1.3 B cells

B cells are the essential components of the humoral immune response. They are produced in the bone marrow and then migrate to the spleen and other secondary lymphoid tissues where they differentiate into mature naive B cells. The main goal of this development process is to generate a population of cells expressing a diverse repertoire of B-cell antigen receptors (BCRs), with different specificities that are capable of recognizing and responding to new and recurring pathogens (Panana *et al*., 2004). IgM, IgD, B220 (CD45R), CD19, CD21, CD23, CD43, CD80 (B7-1), CD86, and MHC class II are the major molecules that are expressed on the membrane of mature B cells (Fairfax *et al*., 2008).

The B cells remain in peripheral tissues until they encounter an antigen and are activated. B cell activation requires two distinct signals, and result in B cell differentiation into memory B cells or plasma cells. The activation signal occurs upon antigen binding to B cell receptors (BCRs). Upon binding to the BCR, the antigen is internalized by receptor-mediated endocytosis, digested and complexed with MHC II molecules on the B cell surface. The second activation signal occurs via either a thymus-dependent or a thymus-independent mechanism. Most B cell responses to antigen require the interaction of B cells with T helper cells (Thymus-dependent activation). Presentation of an antigen-class II MHC complex on a B cell enables it to act as the antigen presenting cell (APC) to T cells. T cell receptor (TCR) on T helper cells binds to the antigen-complexed class II MHC molecules on the B cells surface resulting in T cell activation. The activated T cell then provides a second activation signal to the B cell, which can occur through a variety of proteins. Alternatively, there are a few types of antigens that can directly provide the second B cell activation signal (thymus-independent activation). These antigens include components of some bacterial cell wall

components (e.g., LPS) or other molecules (e.g., bacterial flagellin). Upon activation, B cells proliferate and form germinal centers where they differentiate into memory B cells or plasma cells. Following differentiation into plasma cells, additional signals initiate plasma cell antibody class switching and regulate antibody secretion. The primary function of plasma cells is the secretion of B cell clone-specific antibodies. Each plasma cell secretes antibodies containing a clonally-unique antigen-binding region joined to a constant immunoglobulin (Ig) isotype-defining region (Maddaly *et al*., 2010).

B cells encounter antigen (Ag) within specialized tissues, such as the spleen or lymph nodes, known as secondary lymphoid organs. B cells recognize and respond to Ag through surface B cell receptors (BCRs) that possess a remarkably wide range of potential affinities. The BCR is a heterotirmeric complex containing a membrane immunoglobulin (Ig) responsible for extracellular Ag binding, together with an Igα/β sheath required for mediating intracellular signaling through its immunoreceptor tyrosine-based activation motifs (ITAMs). Engagement of the BCR by specific Ag induces signaling that results in a variety of cellular processes including the internalization of accumulated Ag. In addition to the BCR, B cells express germline encoded Toll-like receptors (TLRs) more usually associated with innate immune cell function. The specificity of TLRs for pathogen associated ligands is dependent on either the restricted expression of the ligand within bacteria or viruses, or the atypical cellular location of more universally expressed ligands (Dorna and Batista, 2009).

2.3.2 Non professional antigen presenting cells

Non professional antigen presenting cells are those cells that do not constitutively express MHC class II molecules, but in some cases initiate primary and secondary immune responses after induction of MHC class II antigen by proinflammatory cytokines (e.g. IFN-γ). Fibroblasts, Hepatocytes, Astrocytes, Epithelial cells, Smooth muscle cells, Myocytes, Chondrocytes, Keratinocytes, Neural cells, etc are some of the examples of non professional antigen presenting cells (Sundstrom *et al*., 1995). Astrocytes, the most abundant glial cells in the central nervous system (CNS), express cell surface MHC class II molecules only upon exposure to the cytokine IFN-γ. Thus activated astrocytes participate in antigen presentation and activation of CD4 helper T-cells in immune-mediated CNS disorders such as multiple sclerosis (Vardjan *et al*., 2012).

MHC class II expression can be induced by the bacterial cell products and inflammatory cytokines on different types of epithelial cells at environmental interfaces including intestinal and skin epithelium. The expression of MHC class II molecules on these non professional APC has been implicated in immune mediated inflammation and progression of or resistance to autoimmunity. In addition lysosomal cysteine protease cathepsin S (Cat S) is a key enzyme regulating MHC class II presentation to CD4 T cells in

intestinal epithelial cells (IECs). Hence, Cat S expression is essential for efficient presentation of endogenous peptides to CD4 T cells by non professional APC *in vivo* (Beers *et al*., 2005). It has been described previously that alveolar type II epithelial cells (AECIIs) show constitutive major histocompatibility complex (MHC) class II surface expression. More recently, Debbabi and colleagues have shown that, upon microbial stimulation, AECIIs become activated, up-regulate expression of MHC class II. This was accompanied with MHC class II presentation of pathogen-derived antigen and activation of antimicrobial T-cell response. Therefore, not only can AECIIs contribute to pulmonary immunity by secreting chemokines that recruit inflammatory cells to the lung, but they can also serve as antigen presenting cells (APCs), suggesting a novel role for AECIIs in the immunological response to respiratory pathogens (Gereke *et al*., 2009).

2.4 Pathways involved in processing and presentation of antigen

Classical studies showed that antigens processing and presentation occurs by two major pathways, MHC class I and II pathways. MHC class I present endogenous peptide antigen to $CD8^+$ T-cells that activate cytotoxicity response to kill infected cells. Whereas MHC class II present exogenous peptides captured from the environment and activate $CD4^+$ helper T lymphocytes to perform their roles in controlling humoral, CTL-mediated and inflammatory immune responses (Sandberg and Glas, 2001). In addition to classical pathway of antigen presentation, there are also non-classical pathways named: cross presentation of antigen and lipid antigen presentation by which processing and presentation of antigens occur. In cross presentation of antigen, presentation of exogenous Ag on MHC class I takes place to stimulate $CD8^+$ T-cells and endogenous Ags are presented on MHC class II to stimulated $CD4^+$ T-cells (Merzougui *et al*., 2011). And the non peptide particles like lipids and glycolipids are presented via CD1 pathway (Sugita *et al*., 1998). Through many years of research the intracellular pathways and various molecules involved in processing and presentation of antigen on MHC I and MHC II have been elucidated in great detail.

2.4.1 MHC class I antigen presentation

MHC class I molecules are expressed by all nucleated cells and present protein fragments of cytosolic and nuclear origin at the cell surface (Neefjes *et al*., 2011). Newly synthesized MHC class I heavy chain associates with binding immunoglobulin protein (BiP) and calnexin (Cxn). Subsequently, assembly with soluble β2-microglobulin (β2m) and exchange of calnexin with calreticulin (Crt) take place. Lectin domain of Crt binds to N-core mono glycosylation at N86 of the heavy chain. This preassembled MHC I sub complex docks to the transporter associated with antigen presentation/ Tapasin and Endoplasmic Reticulum oxidoreductase 57 (TAP/Tsn-ERp57) complex forming a fully

assembled Peptide Loading Complex (PLC). Here, Crt interacts with the b domain of ERp57 via the tip of its P domain, a b-strand hairpin domain. ERp57 and Crt seem to stabilize and support the function of the PLC (Hulpke *et al*., 2013). For the generation of MHC class I peptides, first of all, antigens are degraded by the cytosolic and nuclear proteasomes (Neefjes *et al*., 2011). The polyubiquitinylated endogenous proteins and defective ribosomal products (DRiPs) are degraded into peptide fragments of 8-10 amino acids in length by the 20S proteasome, which is the catalytic core of the 26S proteasome. This 26S proteasome preferentially cleave at the carboxy-terminus after hydrophobic or basic residues thereby generating the C-terminal anchor residue of MHC class I epitopes. And 26S proteasome is built by the association of the 20S core complex with two 19S regulator complexes that are responsible for the activation of the 20S core as well as for the binding and unfolding of ubiqutylated substrates (Sijts *et al*., 2011). Proteases usually cleave off protein fragments, but they can also ligate two peptides, as in the case of protein splicing by the 20S core of the proteasome (Neefjes *et al*., 2011). The resulting peptides are then further degraded by other aminopeptidases in the cytosol. Tripeptidyl-peptidase II (TPP II) is the exopeptidase that helps to remove the tripeptides from a free N-terminal end of longer peptides of 4-41 amino acids in length at neutral pH (Eklund *et al*., 2012). Another cytosolic peptidase is Thimet oligoendopeptidase (TOP) that can degrade several MHC class I epitopes released from protesome cleavage by endoproteolytic cleavage and its preferred substrates are 6-17 residues in length . This makes TOP a significant enzyme in limiting the extent of antigen presentation (Saric *et al*., 2001).

The antigenic peptides thus generated are transported by the transporter associated with antigen presentation (TAP) complex from the cytosol into the endoplasmic reticulum (ER) (Reits *et al*., 2000). The central translocation complex, TAP assembles a maximum of two Tapasin and Endoplasmic Reticulum oxidoreductase 57 (Tsn-ERp57) conjugates via its two transmembrane domain (TMDo) interaction hubs. Tsn is covalently linked to the ERp57 by a mixed, intermolecular disulfide bridge between Cys95 of Tsn and Cys57 of ERp57 that form a functional unit (Hulpke *et al*., 2013). The ER-associated aminopeptidases (ERAAP) might transiently interact with the PLC. ERAAP catalyzes the sequential removal of diverse NH2-terminal residues from NH2-extended antigenic peptides in order to generate the MHC class I specific epitope. ERAAP thus appears to have evolved to generate peptides of eight or nine residues from longer precursors (York *et al*., 2002). Within this multitasking assembly, core TAP acts as the peptide supplier, wheras the TMDo of each subunit is essential in recruiting Tsn, and thus provides proximity between the peptide translocation pore core TAP and the peptide acceptor MHC I, which seems to be important for optimal peptide loading.

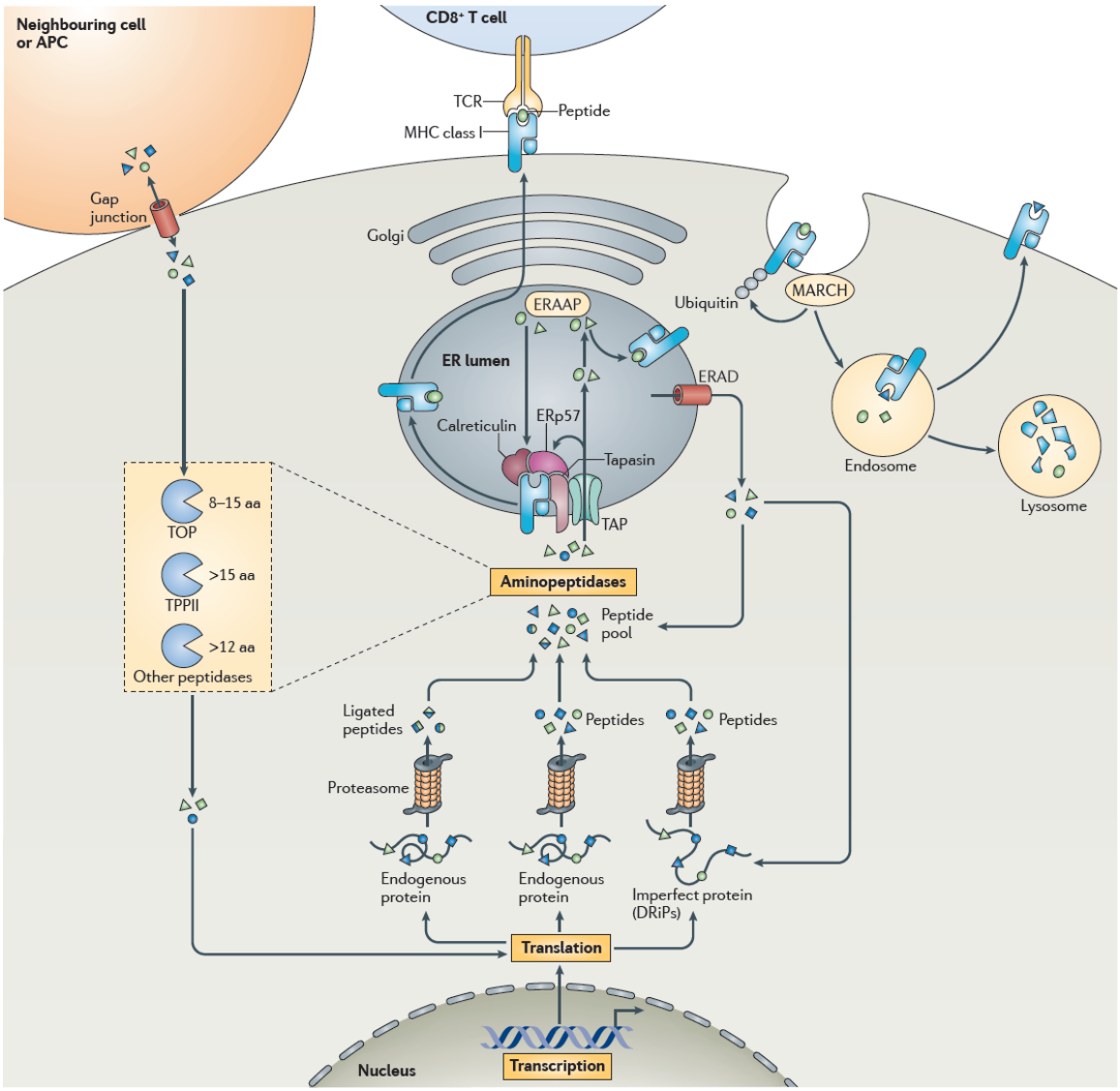


Figure 2.4: MHC class I antigen presentation pathway. Defective ribosomal products (DRiPs) and endogenous proteins are degraded by the proteasome. The proteasome also generate a new peptide by ligation. These peptides are further trimmed and destroyed by thimet oligopeptides (TOP) and tripeptidyl peptidase II (TPPII) and translocate into the endoplasmic reticulum (ER). In ER peptides may further trimmed by ER aminopeptidase associated with antigen processing (ERAAP). Peptide-MHC class I complexs are then transported to the plasma membrane for antigen presentation to CD8+ T cells. Peptides and MHC class I molecules that fail to meet each other are transported back into the cytosol by the ER-associated protein degradation (ERAD) system. A fraction of peptides can enter the MHC class I pathway in neighbouring cells following translocation through gap junctions. Surface MHC class I molecules can be ubiquitylated by MARCH family proteins to promote their internalization and lysosomal degradation. A fraction of endocytosed MHC class I molecules is recycled after peptide exchange with endosomal pepties. APC,antigen-presenting cell. [Source: (Neefjes *et al*., 2011)].

Also, Tsn affinity proof reads peptide-MHC I complexes, and therby catalyzes peptide exchange in favor of petides with very slow dissociation rates (Hulpke *et al*., 2013).

Peptides and MHC I molecules that fail to meet each other are transported back into the cytosol by the translocons called as Sec61p (Koopmann *et al*., 2000) and degraded in cytosol by a process known as ER-associated protein degradation (ERAD) system (Lord *et al*., 2000). Here, they are further trimmed or destroyed by peptidases and the proteasome (Sijts *et al*., 20011). The stable peptide-MHC I complexes are released from the PLC. At last peptide-MHC class I complexes leave the ER for the presentation of peptide at the cell surface to CD8+ T cells (Figure 2.4) (Hulpke *et al*., 2013).

Membrane-associated RING-CH (MARCH) proteins are a novel family of transmembrane ubiquitin ligases that seems to target glycoproteins for lysosomal destruction via ubiqutination of the cytoplasmic tail. These proteins require lysines in the cytoplasmic tail of their target molecules for downregulation. In the presence of MARCH-IV or MARCH-IX, surface MHC-I are ubiqutinated and rapidly internalized by endocytosis, whereas MHC-I molecules lacking lysines in their cytoplasmic tail are resistant to downregulation (Bartee *et al*., 2003).

When MHC class I molecules are internalized into endosomes, they enter the classical MHC class II presentation pathway. Subsequent acidification promotes the release of the associated peptides, which can be exchanged for new peptides generated by the endocytic pathway. A fraction of MHC class I molecules is recycled, along with MHC class II molecules, to the cell surface for the presentation of endosomal antigen fragments (Neefjes *et al*., 2011).

2.4.2 MHC class II antigen presentation

MHC class II molecules are primarily expressed by professional antigen presenting cells (APCs), such as dendritic cells (DCs), macrophage cells and B cells. However, MHC class II expression can be induced by IFN gamma and other stimuli in non-APCs like mesenchymal stromal cells (Mourez et al., 2007). The transcription of the MHC class II locus is controlled by the master regulator named as MHC class II transactivator (CIITA), which in turn is regulated by the combined activity of transforming growth factors β (TGF-β) signaling and chromatin modification (Paul *et al*., 2011). After the translation of α and β chains of MHC class II, the association with a third glycoprotein named as Invariant chain (Ii) takes place in ER (Anderson *et al*., 1991). The MHC class II- peptide interactions are inefficient at the neutral pH of the ER and Golgi, requiring more acidic conditions of the endosomes. In addition to this, the efficient loading of class II molecules in the ER and Golgi is prevented by the invariant chain (Ii). The invariant chain is a transmembrane protein that targets class II molecules to endosomes. Hence, Ii molecule blocks the presentation of a range of endogenously synthesized epitopes (Bodmer *et al*., 1994).

MHC class II molecules are directed by Ii to the endosomal compartments directly from the trans-Golgi network or indirectly via the plasma membrane (PM). The cytoplasmic tail of Ii contains two leucine based sorting motifs which are recognized by the µ-chain of sorting adaptor complexes AP1 and AP2. AP1 is a trans-Golgi network adaptor whereas AP2 is a plasma membrane adaptor (Hofmann *et al*., 1999). Ii chain cleavage is a prerequisite for peptide binding and presentation by MHC class II. The initial stages of Ii chain processing occur in an acidic dense endosomal compartment, an environment favoring aspartic proteinases such as cathepsins D and E. In contrast cysteine proteinase cathepsin B are functional at neutral and acidic pH that catalyzes the final breakdown of Ii chain (Maric *et al*., 1993). In addition to this the cysteinal lysosomal proteases, cathepsin L (CL) and cathepsin S (CS), degrade the Ii until only the fragment called class II-associated leupeptin-induced peptide (CLIP) remains associated with class II. CS and CL proteases also degrade internalized and endogenous proteins that are present in the endosomal compartment (Hsieh *et al*., 2002).

In the endosome membrane, the ubiquitylated MHC class II molecule and HLA-DM are captured by the endosomal sorting complex for transport (ESCRT) machinery. This conserved machinery then transports these molecules to the internal structure of the MIIC (Raiborg *et al*.,2009) (Figure 2.5). Whether 'retrofusion' of internal HLA-DM and MHC class II molecules to the outer membrane of the MIIC occurs is still unknown (Neefjes *et al*., 2011). Human leukocyte antigen DM (HLA-DM) molecules are structurally related to classical MHC class II molecules that are present in MIIC. HLA-DM (H2-DM in mice) is a dedicated chaperone that catalyzes the dissociation of CLIP from MHC class II-CLIP complexes and facilitates the binding of antigenic peptides in MIIC (Denzin *et al*., 1995). In B cells, dendritic cells and thymic epithelial cells, the peptide loading of class II molecules by HLA-DM is modified by the expression of the non- classical class II molecule, HLA-DO (H-2O in mice). Collectively, studied to date support that DO/H-2O expression inhibits the presentation of antigens acquired by cells via fluid phase endocytosis. However, in B cells, the expression of H-2O promotes the presentation of antigens internalized by the B-cell receptor (Denzin *et al*., 2005).

The MIIC or its tubular extensions are transported fast towards the cell membrane by the microtubule-based motor proteins dyenin (for inward transport) and kinesin (for outward transport). These proteins have MIIC-localized receptors, such as RAB7-interacting lysosomal protein (RILP) that control the dynein motor. RILP in turn is then controlled by the cholesterol sensor oxysterol-binding protein (OSBP)-related protein 1L (ORP1L) and the ER-resident protein VAMP-associated protein A (VAPA) (Rocha *et al*., 2009). MHC class II transport was also controlled by the GTPase ADP-ribosylation factor-like protein 14 (ARL14).

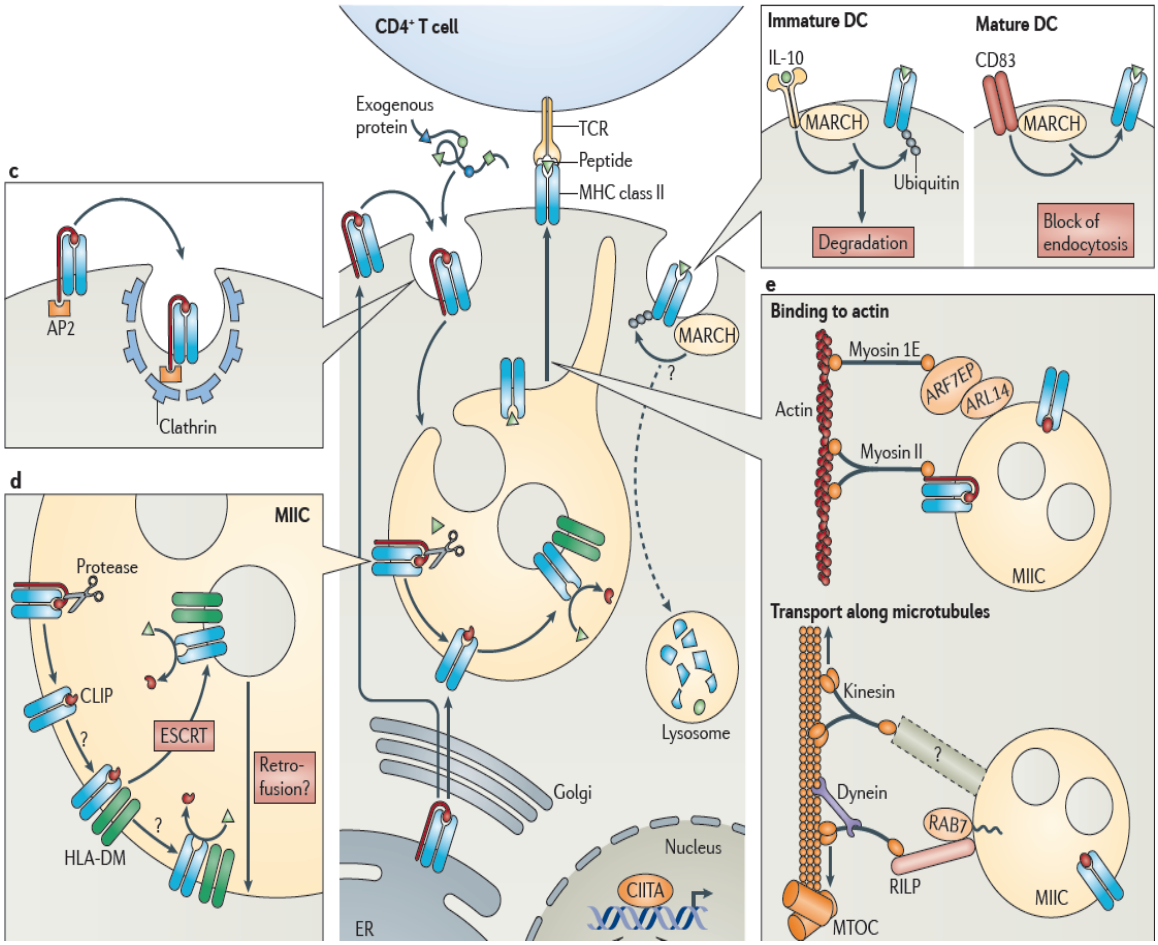


Figure 2.5: Complexity of the MHC class II antigen presentation pathway. Transcription of the MHC class II genes is controlled by the master regulator MHC class II transactvator (CIITA). The adaptor protein AP2 drives the internalization of Ii-MHC class II complexes into clathrin-coated vesicles at the plasma membrane for transport to the MHC class II compartment (MIIC). In the MIIC, Ii is degraded and MHC class II molecules interact with HLA-DM. The MIIC or its tubular extensions are transported to the cell membrane by the microtubule-based motor proteins dynein and kinesin. The final step involves actin-based myosin motors that interact with the MIIC either via Ii or via the GTPase ADP-ribosylation factor-like protein 14 (ARL14). In immature DCs, internalization of MHC class II molecules from the plasma membrane may require the ubiquitin ligase MARCH1, which is controlled by interleukin-10 (IL-10). CD83 on mature DCs prevents this ubiquitylation of MHC class II molecules and thus stabilizes MHC class II molecules on the cell surface. TCR; T cell receptor. [Source: (Neefjes *et al*., 2011)].

And the ARL14 recruits the motor myosin 1E via an effector protein ARF7EP. This complex controls movement of MHC class II vesicles along the actin cytoskeleton in human immature dendritic cells (DCs) (Paul *et al*., 2011). Lastly, MHC class II molecules are transported to the plasma membrane to present antigenic peptides to CD4+T cells.

In immature dendritic cells, Interleukin-10 (IL-10) leads to an accumulation of internalized MHC class II complexes in intracellular vesicles and prevents MHC class II molecules from reaching the plasma membrane. Hence, IL-10 affects antigen presentation by regulating MHC exocytosis and recycling (Koppelman *et al*., 1997). CD83 is an immunoglobulin family protein expressed on the surface of mature DCs, that promotes MHC class II and CD86 expression by blocking MHC class II association with the ubiquitin ligase MARCH1. The transmembrane region of CD83 blocks interleukin 10-driven, MARCH1-dependent ubiquitination and degradation of MHC class II and CD86 in DCs (Tze *et al*., 2011).

2.4.3 Cross-presentation of antigen

Presentation of exogenous antigen (Ag) on major histocompatibility complex (MHC) class I molecules by professional antigen presenting cells (APCs) to stimulate $CD8^+$ T-cell against a variety of pathogens, tumors and viral infections is termed cross-presentation (Merzougui *et al*., 2011). 'Cytosolic' and 'vaculor' pathways are the two main intracellular pathways that have been reported for the cross-presentation of exogenous antigens (Figure 2.6) (Joffre *et al*., 2012). In cytosolic pathway, the antigen is transferred from the phagosome into cytosol, where it is hydrolyzed by proteasomes into oligopeptides that are then transported by the transporter associated with antigen processing (TAP) to MHC class I molecules in the endoplasmic reticulum or phagosomes. The presentation by this pathway is sensitive to proteasome inhibitors and is TAP dependent. The particulate antigens must be transported from phagosome to cytosol, but the mechanism is not known. It is found that a subset of phagosome acquires TAP, MHC class I, Tapasin, and SEC61 from the ER. It has been suggested that this is a consequence of membrane from the ER fusing with the phagosome during phagocytosis. SEC61 is involved in the import and export of proteins from the ER. Therefore, SEC61 has been suggested as a possible mechamism by which proteins are transported out of the pagosome to cytosol (Rock *et al*., 2005).

It is also found that SEC22 B, ER-resident SNARE, is required for the antigen transfer from phagosomes to cytosol. And antigen cross-presentation is inhibited in SEC22 B-deficient cells (Joffre *et al*., 2012). In a vaculor pathway, the antigen is cleaved into peptides by endosomal proteases, particularly cathepsin S, and bound by class I molecules probably in the endocytic compartment itself. The presentation by this pathway is resistant to proteasome inhibitors and generally independent of TAP, but is sensitive to cathepsin S inhibitors (Rock *et al*., 2005).

Antigens for cross presentation can be acquired by phagocytosis (>1µm particulate antigens) and fluid-phase macropinocytosis (<1 µm small particles and fluids). But

antigens in particulate forms are much more efficient in stimulating CTL immunity than soluble antigens.

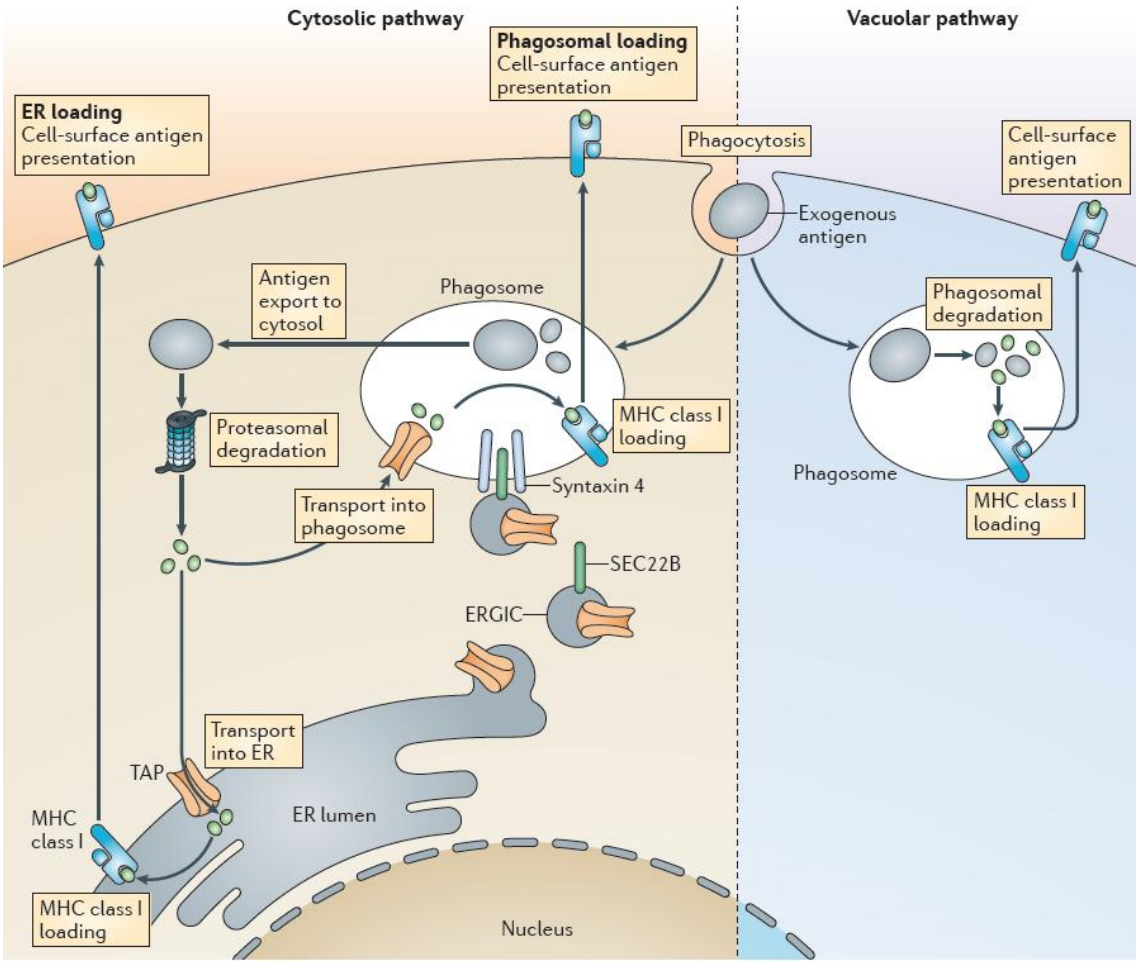


Figure 2.6: Intracellular pathways for cross-presentation in dendritic cells. After phagocytosis, exogenous antigens can be exported into the cytosol, while they are processed by the proteasome. The processed antigens can then be loaded on MHC class I molecules in the endoplasmic reticulum(ER) (the cytosolic pathway with ER loading) or re-imported into the phagosome to be loaded on MHC class I molecules (the cytosolic pathway with phagosomal loading). The SNARE SEC22B, which localizes in the ER-Golgi intermediate compartment (ERGIC) and interacts with syntaxin 4 on phagosomes, mediates the recruitment of a subset of ER components, including transporter associated with antigen processing (TAP), to phagosomes. Alternatively, exogenenous antigens can be degraded into peptides in the phagosome, where they are then loaded on MHC class I molecules (the vaculor pathway). [Source: (Joffre *et al*., 2012)].

CTL responses have also been implicated to destroy the intracellular pathogens such as viruses and some bacteria and in the elimination of cells that have undergone malignant

transformation (Brode and Macary, 2004). To initiate a protective CTL response, the antigens derived from pathogens like *Leishmania* (Matheoud *et al*., 20013) and transformed cells must be processed and presented on professional antigen-presenting cells (APC) in the context of MHC class I molecules. Only professional APC such as dendritic cells (DC) and possibly some macrophages express a combination of co-receptors and MHC-class I molecules at levels high enough to stimulate naive $CD8^+$ T cells (Brode and Macary, 2004).

2.4.3.1 Potential use of cross-presentation for therapy

One of the most efficacious and cost-effective therapies for the prevention of many infectious diseases, such as smallpox and polio, has been the stimulation of specific immune responses through vaccination. However, there remain a number of infectious diseases for which vaccines are unavailable or only stimulate suboptimal immunity. Moreover, there are noninfectious indications, such as cancer, that could be potentially treated with vaccines.

Most current vaccines consist of non-living components of pathogens. Such killed or subunit vaccines are considered safer than ones using live organisms. However, one of the limitations of subunit vaccines is that they generally fail to elicit CD8+ T-cell immunity. This limitation would preclude the use of subunit vaccines for protection against diseases where immunity is mediated by CTLs, e.g. certain viral infections or cancer. It is known that subunit vaccines fail to stimulate CTL immunity, because the antigens in these preparations do not get presented on the MHC class I molecules of the professional APCs. If, however, these antigens were introduced in ways that led to their being cross-presented, then CTL immunity could be generated. One way in which this can be achieved is to generate particulate forms of antigen. Such preparations are taken up efficiently by APCs and presented on both MHC class I and class II molecules. When such particulate preparations are injected into animals, they stimulate both CTL and $CD4^+$ T-cell responses. A single injection subcutaneously could stimulate protective immunity. These responses are sufficiently strong to protect animals against challenge with aggressive tumors. Moreover, it is possible to make particle preparations from biocompatible and biodegradable materials such as polylactide-coglycolide. Therefore, particulate antigens can be used to target antigens into DCs *in vivo* in ways that elicit both $CD4^+$ and $CD8^+$ T-cell immunity. It is similarly possible to introduce antigens into the cross-presentation pathways of DCs *ex vivo* and to then inject these APCs back *in vivo* as a cellular vaccine (Rock and Shen, 2005).

2.4.4 Lipid antigen presentation

Besides MHC class I and II molecules, a third lineage of antigen-presenting molecule that bind lipid and glycolipid antigens rather than peptides exists and is mediated by the family of Cluster of Differentation 1 (CD1) proteins (Sugita *et al*., 1998). The CD1 family comprises type I integral membrane proteins that structurally resemble MHC class I molecules along with the presence of a shared subunit, β2-microglobulin (β2m). Based on amino acid sequence homology, the CD1 molecules are divided into two groups: group 1, compromising CD1a, b and c, group II comprising CD1d. A third CD1 species, CD1e, is intermediate between the two groups. Among these groups the only isoform that is present in mice and rats is CD1d. CD1 is expressed on most of the APCs (e.g. macrophages, DCs, etc) and its expression can be regulated through different pathways like Toll like receptors (TLRs) ligands or cytokines or viral infection mediated mechanisms (Paduraru *et al*., 2010).

Natural killer T (NKT) cells specifically recognize self lipid-based or foreign lipid-based antigens bound to the CD1 antigen presenting molecules. NKT cells are divided into two broad classes: type I or semi-invariant (iNKT) cells and type II or non-invariant NKT cells. iNKT cells are defined by expression of an invariant T cell antigen receptor (TCR) consisting of α-chain (α-chain variable region 24-α chain joining region 18 (Vα24- Jα18) in human; Vα14-Jα18 in mice) paired with a limited repertoire of TCR β chains (V β11 in humans; Vβ8, Vβ7 and Vβ2 in mice) and by their ability to recognize the non-self glycolipid antigen-α galactosylceramide (α- GalCer). In contrast, type II NKT cells express a different and more diverse TCR repertoire (Patel *et al*., 2012).

Glycolipids from lipopolysaccharide of gram negative bacteria such as *Borrelia* are the lipid antigens that can directly activate iNKT cells. In the same way enhanced secretion of interleukin 12 (IL-12) and upregualtion of endogenous lipids by APCs may also activate iNKT cells in CD1d-dependent manner or CD1d-independent manner (Barral *et al*., 2010). Also α -linked sphingolipids, diacylglycerols, lipophosphoglycans from various microorganisms and protozoans were found to bind CD1d and stimulate iNKT cells *in vitro* (Barral *et al*., 2007). Activated iNKT cells rapidly secrete large quantities of cytokines, such as interferon-γ (IFN-γ) and IL-4, and induce downstream activation of variety of other cell types, including DCs, NK cells, B cells and classical T cells. Hence, iNKT cells have a significant role in coordinating both innate and adaptive immune responses (Barral *et al*., 2010).

CD1d is synthesized in the ER and interacts with calnexin, calreticulin and ERp57. After the heavy chain exits the chaperone cycle it interacts with β2m and possibly acquires self-lipids with the help of microsomal triglyceride transfer protein (MTP). MTP was shown to transfer a phospholipid (phosphatidylethanolamine) to mouse CD1 *in vitro*,

and therefore could have a role in the loading of CD1 with endogenous lipids, that are important both for NKT-cell selection in the thymus and antigen presentation by APCs in the periphery. CD1 molecules then follow the secretory route along with MHC class I and MHC class II through the Golgi apparatus to the plasma membrane (Paduraru *et al*., 2010).

From plasma membrane, CD1 molecules are internalized via clathrin-coated pits by the interaction of the adaptor complex AP2 with tyrosine-based sorting motifs present in the cytoplasmic tails of the CD1 (Figure 2.7).

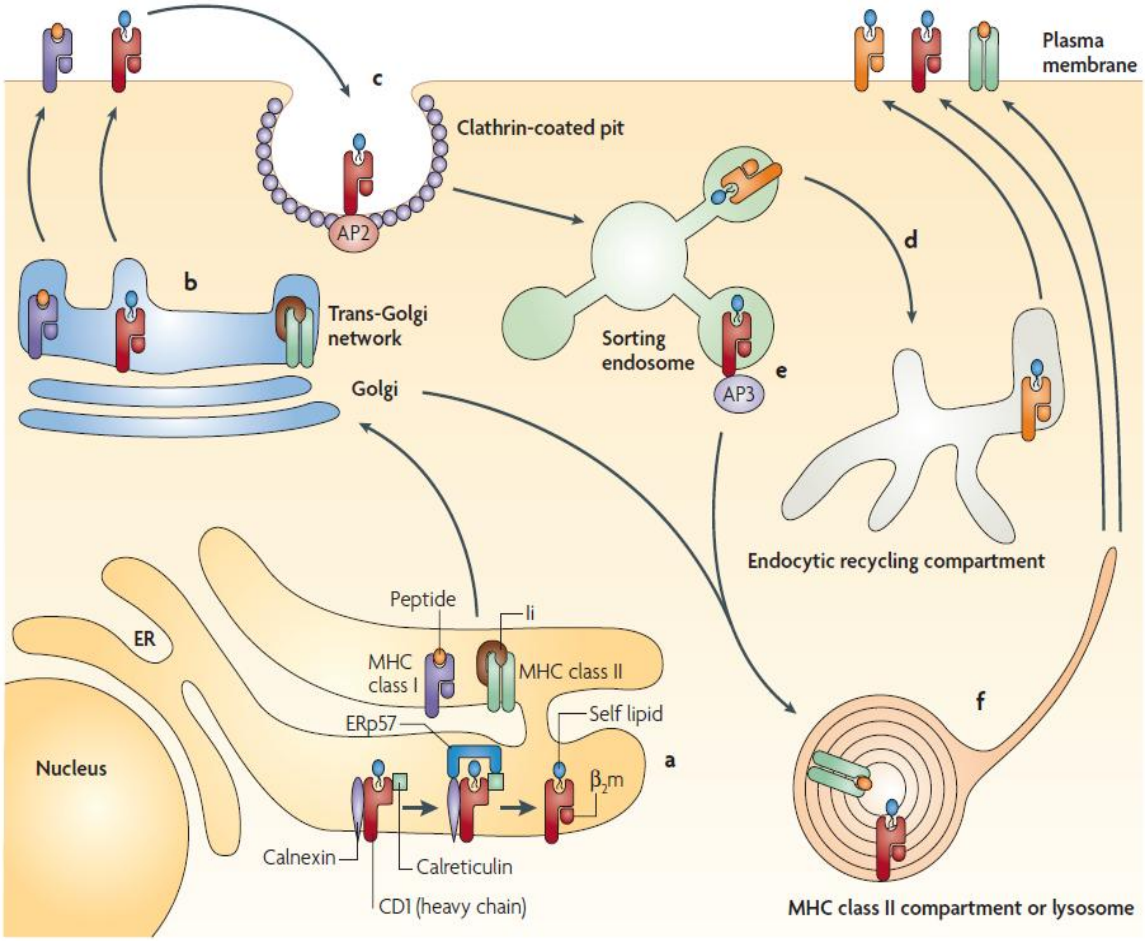


Figure 2.7: Intracellular trafficking of CD1 molecules. (a) CD1 heavy chains are assembled in the endoplasmic reticulum (ER), where they bind the chaperones calnexin, calreticulin and ERp57. They also bind β2-microglobulin (β2m) non-covalently in the ER. (b) CD1 molecules then follow the secretory route through the Golgi apparatus to the plasma membrane (c) CD1 molecules are internalized in clathrin-coated pits via the interaction of the adaptor complex AP2. (d) CD1 molecules follow the slow recycling pathway, back to the plasma membrane, through the endocytic compartments. (e) CD1 molecules traffic to late endosomal and lysosomal compartments via the interaction of AP3 (f) CD1 and MHC class II molecules recycle form lysosomal compartments to the plasma membrane. [Source: (Barral *et al*., 2007)].

From the sorting endosome, CD1 molecules can follow two main routes. CD1 molecules such as CD1a and CD1c can follow the slow recycling pathway back to the plasma membrane, through the endocytic recycling compartment. CD1 molecules such as CD1b and mouse CD1d can traffic to late endosomal and lysososmal compartments via the interaction of AP3 also with tyrosine based motifs contained in the cytoplasmic tails of these CD1 molecules. In the case of mouse CD1d, AP3-deficient mice show a defect in CD1d localization to lysosomes and CD1d-mediated antigen presentation, which is reflected by a significant reduction in the number of NKT cells in these mice (Barral *et al*., 2007).

Within the endocytic pathway the lipid from the binding site can be exchanged with a self or non-self one with the help of lipid transfer proteins such as saposins. In the case of sapsoin B, the protein probably binds lipids, extracts them from membranes and transfers them onto CD1d molecules. The antigen-CD1d- β2m complex traffics to the plasma membrane where it can undergo recycling in the endocytic pathway or recognition by the TCR of the NKT cells (Paduraru *et al*., 2010).

There are four mechanisms by which foreign lipids antigens can be taken up: (a) clathrin-dependent internalization of apolipoprotein E (apoE)- lipid complexes bind to the low-density lipoprotein receptor (LDLR), (b) phagocytosis or particulate material or whole pathogens internalization, (c) c-type lectins, which can bind mannose residues or glycolipids, and (d) internalization through scavenger receptor, which can bind modified forms of LDL and apoptotic cells (Barral *et al*., 2007).

2.5 Membrane fusion and vesicular trafficking

Membrane fusion is one of the most fundamental processes in life that occurs when two separate lipid membranes merge into a single continuous bilayer. It is essential for communication between membrane-delineated compartments in all eukaryotic cells. Exocytosis is one of the best-studied processes involving membrane fusion, whereby vesicles fuse with the limiting membrane of a cell in order to release their contents (for example, hormones or neurotransmitters) into the extracellular milieu (Martens *et al*., 2008). The fusion reactions share common features, but are catalyzed by diverse proteins. Fusion of lipid bilayers in an aqueous environment is a two-step process. First, the membranes are brought into close proximity where counteracting electrostatic forces need to be overcome before the lipids of the proximal leaflets can interact. Second, the boundary between the hydrophilic and hydrophobic portion of the bilayer is destabilized. Non-bilayer transition states are generated that culminate in the formation of an aqueous fusion pore. According to the stalk hypothesis, fusion proceeds by an ordered sequence of steps that include the merging of the proximal monolayers, stalk

formation, generations of hemifusion, intermediates and fusion pore opening (Chernomordik *et al*., 1987).

Membrane fusion can be distinguished into following three types: (1) Extra- and intracellular fusion of pathogens with host cells. Of these, fusion of enveloped viruses in which the entire reaction is carried out by a single protein is best characterized. (2) Extracellular fusion of eukaryotic cells. Example is fusion of sperm with oocyte cell. (3) Intracellular fusion of organelles. These reactions are mediated by the dynamic supramolecular assemblies involving conserved protein families (Jahn *et al*., 2003).

The fusion between vesicles or the plasma membrane is not a spontaneous event. Since the late 1980s, when Soluble NSF attachment protein receptor where NSF stands for *N*-ethyl-maleimide sensitive fusion protein (SNAREs) were characterized, rapid progress has identified SNAREs as key elements of membrane fusion, one of the basic reactions of the intracellular trafficking, and now they appear to be implicated in all of the trafficking steps of the secretory pathway (Wilson *et al*., 1989). SNAREs are a superfamily of small proteins with more than 38 known members in mammals (Stow *et al*., 2006). These proteins have been implicated as central in most, if not all, intracellular membrane trafficking events so far. The synaptic SNARE proteins synaptobrevin1/VAMP 1 (on the vesicle) and syntaxin 1; and SNAP-25 (on plasma membrane) were the first SNAREs to be discovered (Chen *et al*., 2001). Both syntaxin and VAMP are anchored to the membrane by a carboxy-terminal transmembrane domain, whereas SNAP-25 is peripherally attached to the membrane by palmitoylation of four cysteine residues in the central region of the protein (Sollner *et al*., 1993).

Eukaryotic cells contain a variety of membrane enclosed organelles that communicate with each other, i.e. they exchange proteins and lipids via directed trafficking. This membrane trafficking is a basic requirement in order to maintain the identity of the different cell compartments and the inner organization of the cell. For instance, proteins destined for secretion are synthesized at the endoplasmic reticulum (ER), travel through the cis-, medial- and trans- Golgi compartment, before being further sorted and transported via vesicle transport to the plasma membrane where excretion occurs (Tokarev *etl al*., 2009).

Each organelle has a defined localization and function that demands a specific membrane composition of lipids and proteins. During trafficking, a carrier vesicle pinches off from the donor compartment, is transported; and finally fuses with its acceptor compartment. A prerequisite for correct targeting is that the donor and acceptor membranes recognize each other (tethering and docking) before the lipid bilayer fuse. All these processes are mediated by specific soluble and membrane resident proteins and are subject to high degree of regulation.

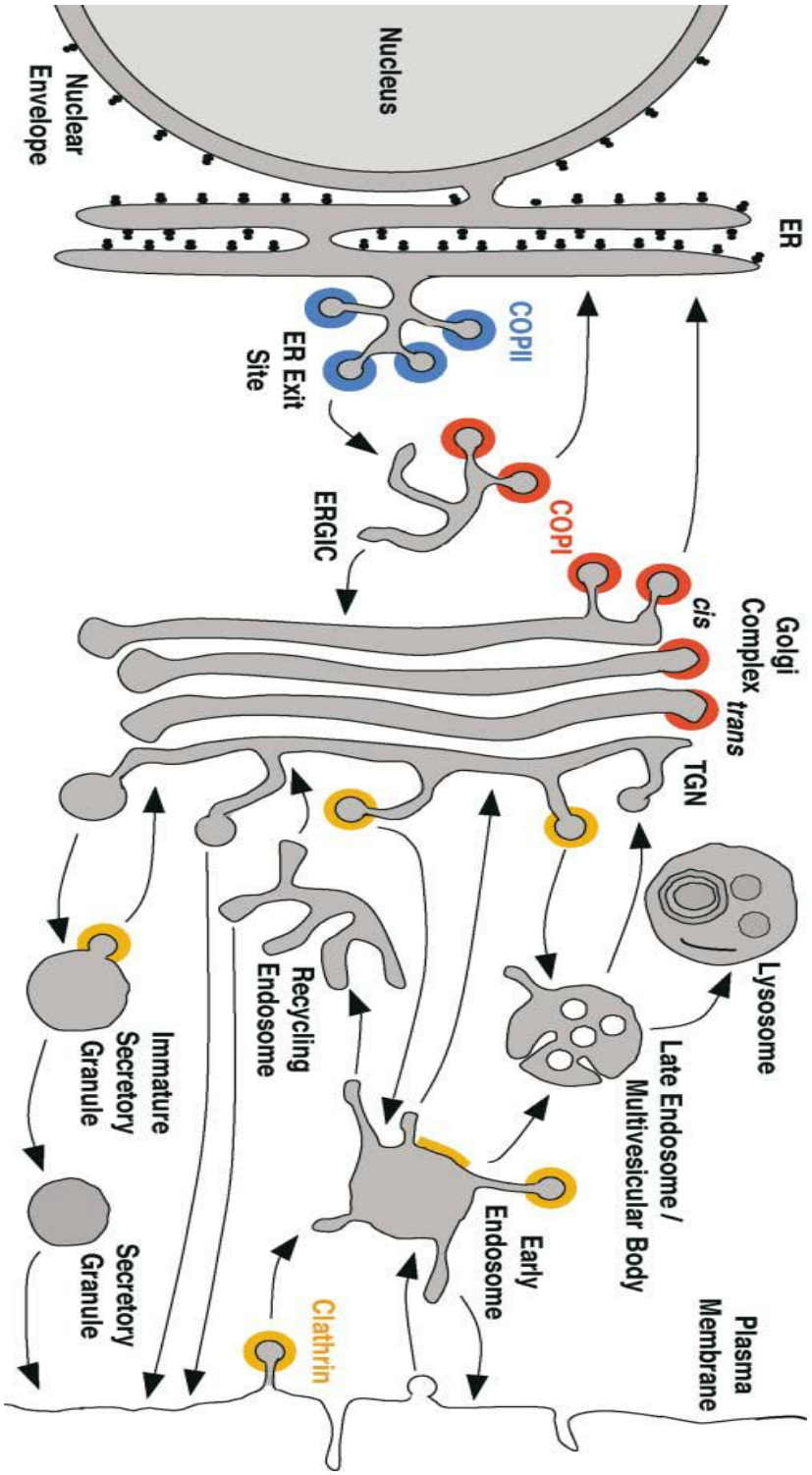


Figure 2.8: Intracellular Transport Pathways in Eukaryotes. The compartments of the secretory, lysosomal/vacuolar, and endocytic pathways are shown. The transport steps are indicated by arrows. [Source: (Bonifacino and Glick, 2004)].

Each vesicle transport reaction can be divided into four steps that include vesicle budding, transport, tethering, and fusion (Bonifacino *et al*., 2004). These steps are tightly regulated to ensure that vesicles generated from a donor compartment are delivered to their correct acceptor compartment. Vesicle budding is mediated by protein coats (McMahon *et al*., 2004). Coats deform flat membranes into rounds buds, which lead to the release of coated vesicles. Coats proteins also participate in cargo selection through the recognition of sorting signals present in the cytoplasmic domain of transmembrane cargo proteins. Clathrin was the first coat to be identified (Pearse, 1975).

Clathrin-coated vesicles are mainly derived from the plasma membrane or the trans-Golgi network (TGN) and are transported to endosomes (Owen *et al*., 2004). Subsequent studies identified two non-clathrin coats, COPI (coat protein complex I) and COPII (coat protein complex II), that mediate vesicle transport in the early secretory pathway. COPI primarily acts from the Golgi to the endoplasmic reticulum (ER) and between Golgi cisternae (Figure 2.8), while COPII mediates traffic from the ER to the Golgi (Barlowe *et al*., 1994; Letourneur *et al*., 1994; Waters *et al*., 1991). After budding, vesicles are transported to their final destination by diffusion or by motor-mediated transport along a cytoskeletal track. The molecular motors kinesin, dynein, and myosin have all been implicated in this process (Hammer *et al*., 2002; Matanis *et al*., 2002; Short *et al*., 2002). The next step in vesicle-mediated membrane traffic is tethering. Tethering is a term used to describe the initial interaction between a vesicle and its target membrane. It precedes the pairing of transmembrane SNAREs on opposing membrane, which leads to membrane fusion (Sollner *et al*., 1993). The last step in vesicle-mediated transport is the fusion of the vesicle with its target membrane. Fusion is thought to occur by the pairing of SNAREs (Rothman, 1994; Sollner *et al*., 1993).

2.6 Classification of SNARE proteins

SNAREs were originally classified as v-(vesicle-associated) or t- (target-membrane) SNAREs, on the basis of their locations and functional roles in a typical trafficking (Sollner *et al*., 1993). Subsequently, the involvement of SNARE proteins in many other membrane fusion events was discovered. The v/t classification was found to be confusing for symmetric fusion events such as the homotypic fusion of yeast vacuoles or the fusion of late endosome and lysosome etc. Hence, SNAREs had to be re-classified into a more comprehendible form (Fasshauer *et al*., 1998).

The presence of one Arginine (R) and three Glutamine (G) residues in the 0^{th} ionic layer of the core of the SNARE complex was a highly conserved feature of the core complexes (Sutton *et al*., 1998). Therefore, it served as a basis of reclassification of SNARE proteins into Q SNAREs and R SNAREs depending on the amino acid residue they contribute to the 0^{th} layer of the core (Fasshauer *et al*., 1998). Further, the three Glutamine

contributing SNAREs were sub classified into Qa, Qb and Qc. In reference to the neuronal SNARE complex, Qa SNAREs are located at the position of syntaxin1 of the neuronal complex and Qb and Qc occupy the position of N- and C-terminal motifs of the SNAP25 (Sutton *et al*., 1998). So far, most of the known v-SNAREs can be placed in the R SNARE family while most of the t-SNAREs are placed under the Q-SNARE family. There are a few exceptions such as the yeast Bet1p which is a Q SNARE and contributes a serine; and Leech synaptorevin (R SNARE) contributes a lysine to the 0^{th} layer (Fasshauer *et al*., 1998). However, both functional classifications into v/t SNAREs as well as structural classifications into R/Q SNAREs are widely used.

2.7 Structure of SNARE complex

All the members of the SNARE family have a characteristic 'SNARE motif' which is a stretch of about 60 amino acid residues, located close to the trans-membrane domain. This domain has a highly conserved heptad repeat pattern which forms an α helical coil (Weimbs *et al*., 1997; Jahn *et al*., 2003). SNAREs like syntaxin and VAMP are anchored to the membrane by a carboxy-terminal transmembrane domain, whereas SNAP-25 is peripherally attached to the membrane by palmitoylation of four cysteine residues in the central region of the protein (Chen *et al*., 2001). The SNARE motifs are unstructured when the SNAREs are free in solution. However, when the proteins on opposing membranes interact, their SNARE motifs undergo spontaneous restructuring into a highly stable, elongated four helical colied coil structure called the 'core complex' (Hanson *et al*., 1997; Lin *et al*., 2000).

For the first time in 1998, Sutton and co-workers crystallized the core complex of the synaptic SNARE complex comprising of syntaxin 1A, synaptovrevin-II and SNAP 25B at 2.4 Aº resolution (Sutton *et al*., 1998). They observed a highly twisted and parallel bundle of four helices, two contributed by SNAP25 and one each by syntaxin 1 and synaptobrevin. The core bundle could be divided into 16 backbone layers numbered -7 to +8 as shown in figure 2.9. The ionic layer at the 0^{th} position of the complex comprised an Arginine residue contributed by VAMP and three Glutamine residues, two from SNAP 25 and one from syntaxin 1 (Sutton *et al*., 1998).

The positively charged guanidino groups of the arginine residue interact with carboxyl groups from each of the three glutamine residues. The flanking leucine-zipper layers act as a water tight seal to shield the ionic interactions from the surrounding solvent. This seal may further stabilize the four-helical oligomeric state and register of the complex by decreasing the local dielectric, thereby enhancing electrostatic interaction within the ionic layer (Sutton *et al*., 1998).

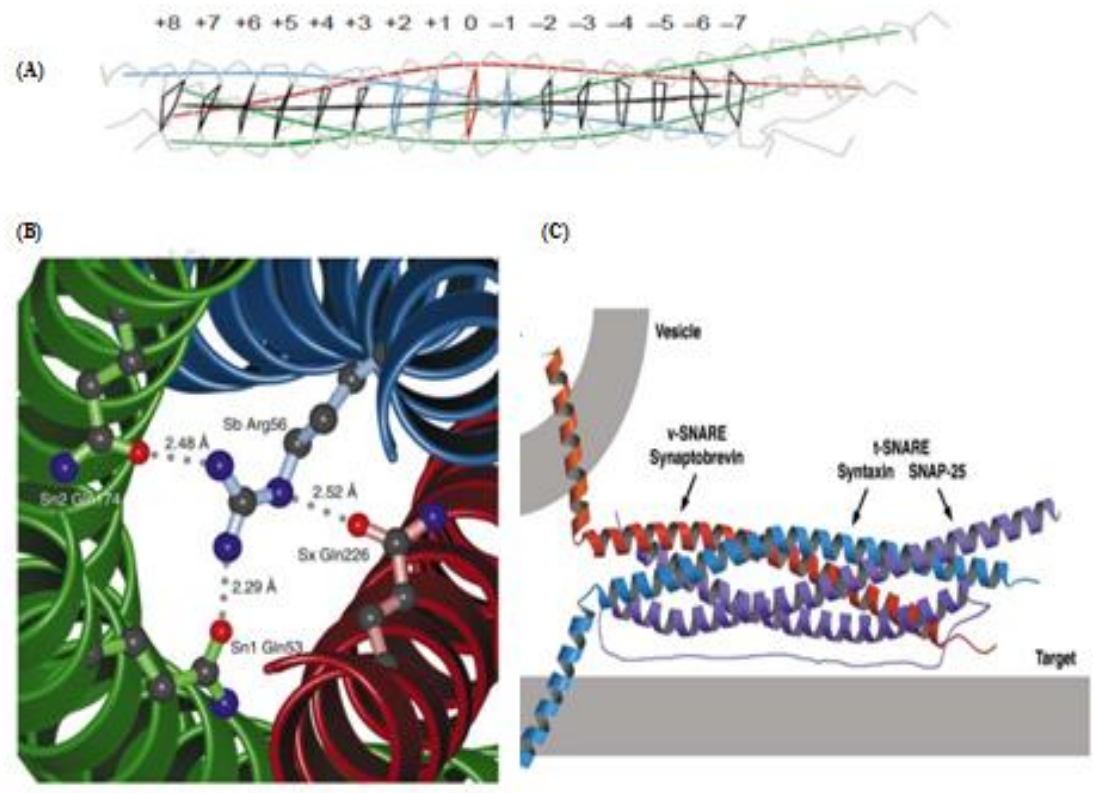


Figure 2.9: Structure of SNARE core complex: (A) 16 layers of the synaptic core complex with 0th ionic layer in red (B) Arginine 56 from synaptobrevin-II, Glutamine 226 from syntaxin1A and Glutamine 53 and Glutamine 174 from the two SNARE motifs of SNA25B form the 0th layer of the core (C) Hypothetical model of the synaptic fusion complex as it joins two membranes, and location of neurotoxin-mediated cleavage sites. [Source: (Sutton *et al*., 1998)].

2.8 SNARE complex formation

SNAREs are the central components of membrane fusion but they are not sufficient on their own to complete the fusion process. There is an ordered process that takes place between several proteins that leads to membrane fusion. Some of these proteins and their functions are discussed below.

2.8.1 Rab proteins:

Rabs are small GTPases of the Ras superfamily that continuously cycle between the cytosol and membranes. Membrane bound Rab with GTP is the active form of Rab where as the GDP bound form is the inactive form (Rybin *et al*., 1996). About 11 Rab proteins have been identified in yeast, and more than 60 in mammalian cells till now (Lazar *et al*., 1997; Pferrer, 2001). The best understood function of Rab proteins is probably their role in vesicle tethering. Rabs in their GTP-bound form appear to facilitate

the recruitment of tethers to specific locations thereby bridging the two membranes (Munson and Novick., 2006). Finally, the GTP is cleaved by GTPase Activating protein (GAP) and the inactivated Rab protein is removed from the membrane by GDP dissociation inhibitor protein (GDI) (Jahn *et al*., 2003).

2.8.2 Tethering and docking factors:

The donor membrane recognizes the correct acceptor membrane in a process called as docking or tethering. Different tethering and docking proteins have been identified. p115 is one of the well studied tethering factor in mammalian cells (Pfeffer, 1999). Proteins as tethers include Uso1p, the exocyst, the HOPS complex and EEA1 (Zerial and McBride, 2001).

2.8.3 The SNARE cycle:

Once the membranes are held together by the docking and tethering factors SNAREs can mediate the actual fusion event. nSec1 is the polypeptide that is present on the surface of the Syntaxin-1. Generally, nSec1 binds to the full length of the Syntaxin-1 in its closed conformation whereas binding of SNAP-25 and VAMP, requires only the H3 domain of syntaxin-1 (Misura *et al*., 2000). So, removal of nSec1 from the surface of syntaxin is required in order to form the SNAREs complex. The action of Rab then causes the conformational change in nSec1, leading to destabilization of syntaxin's linker region between the Hc and H3 helices. This conformational change in syntaxin allows nucleation of the ternary complex (Misura *et al*., 2000). The SNAREs on the opposite membranes 'zip up' together from the distal N terminal to the membrane proximal C terminal forming the core complex as shown in the figure 2.10. The orientation of the *trans*-SNARE complex pulls the two membrane close together (Hanson *et al*., 1997). The force generated in the process overcomes the energy barrier for facilitation fusion (Hanson *et al*., 1997).

The irreversible assembly of SNARE core complex is dependent on $Ca2^{+}$. The binding of calcium to the phosphate head groups of the apposing bilayers displaces the loosely coordinated water at the PO-lipid head groups, resulting in the dehydration, leading to destabilization of the lipid bilayers and membrane fusion (Potoff et al., 2008). After the membranes fuse, all the SNAREs are present on acceptor membrane in a so called *cis*-complex. This complex is dissociated by the chaperone ATPase NSF with SNAP as a co-factor thereby freeing the SNARE proteins for another round of membrane fusion as shown in the figure 2.10 (Jahn *et al*., 2003).

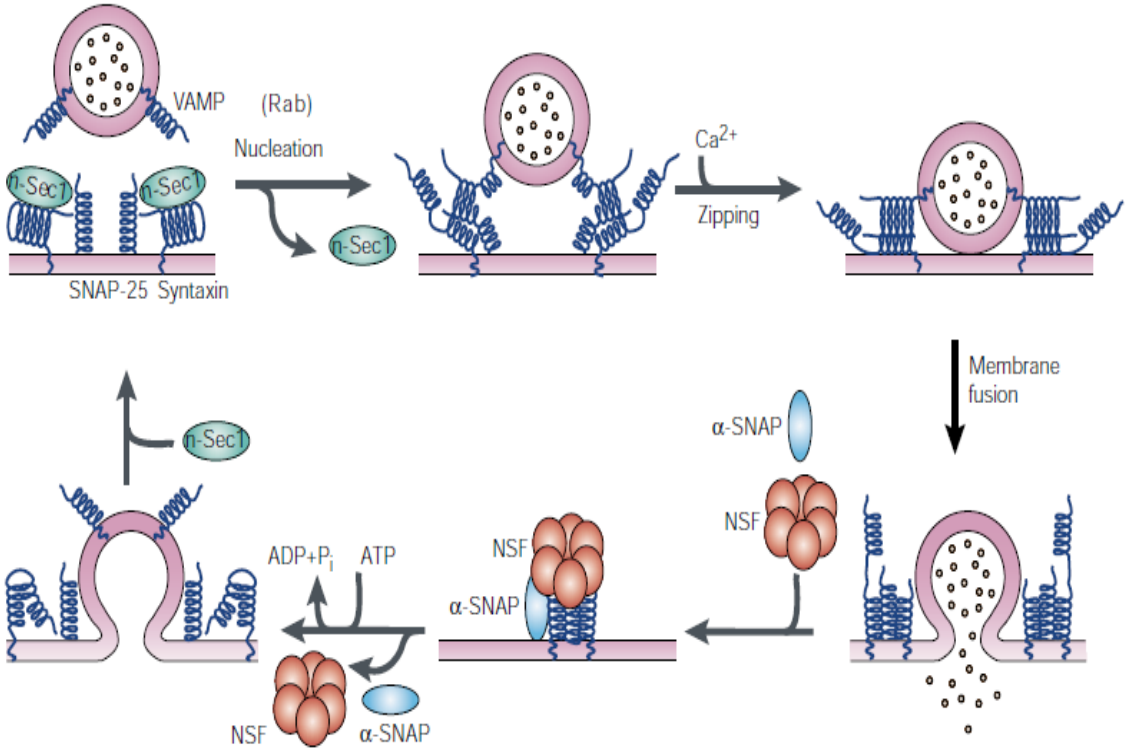


Figure 2.10: Cycle of assembly and disassembly of the SNARE complex in synaptic vesicle exocytosis: Showing the assembly of SNARE complex leading to fusion of membranes and eventual dissociation of the complex by ATPase NSF and SNAP. (NSF; *N*-ethyl-maleimide-sensitive fusion protein; SNAP-25, 25 kDa synaptosome-associated protein; SNARE, soluble NSF attachment protein receptor, VAMP, vesicle-associated membrane protein).[Source (Chen *et al*., 2001)].

2.9 SNAP-25 family

SNAP-25 is the first member of the synaptosomal associated protein family having the molecular weight of 25kD (Hence, the name SNAP25). For the first time, this protein was identified in mouse hippocampus (Oyler *et al*., 1989). It contributes two SNARE motifs Qb and Qc to the neuronal exocytic SNARE complex (Sollner *et al*., 1993). Though SNAP25 lacks a transmembrane domain, it is attached to the membrane by palmitoylation of the cysteine residues in the spacer region between the two SNARE motifs (Chen *et al*., 2001; Jan *et al*., 2003).

There are three more members in the SNAP-25 family, namely SNAP-23, SNAP-29 and SNAP-47. SNAP-47 is ubiquitously distributed on intracellular membrane and most of the tissues with high levels in nervous tissue. The 47kD protein, SNAP-47 was purified from the rat brain synaptic vesicles for the first time. For SNAP-47, SNAP-29 is found to be the closest mammalian homologue protein in which both the SNARE proteins contain two SNARE motifs connected by a linker. Unlike SNAP-25 and SNAP-23, SNAP-47 lacks a

conserved stretch of cysteine residues in the linker region connecting the two SNARE motifs (Holt *et al*., 2006).

SNAP-23 is a 23kD protein with 211 amino acid residues that was identified from the human B lymphocytes. It is homolog of SNAP-25 and bear 59% identity with SNAP-25 (Ravichandran *et al*., 1996). It is ubiquitously expressed and is involved mostly in exocytosis. In the similar way, SNAP-23 was found to be important for excytosis of mast cell granules (Puri *et al*., 2003). Two isoforms of SNAP-23 named as SNAP-23A and SNAP-23B have been identified in human neutrophils. SNAP-23A which is similar to the recently cloned SNAP-23, and a novel isoform, termed SNAP23B. SNAP23B results from a deletion of 159 bp from SNAP-23A cDNA, encoding a protein of approximately 17.8kD. SNAP-23B is identical to SNAP-23A, but lacks fifty three amino acid residues, from 90 to 142. This region of SNAP-23A has a target sequence for post translation fatty acid acylation, suggesting that the two isoforms can differ in their capacity to interact with membranes (Mollinedo *et al*., 1997).

2.10 Syntaxin family

Syntaxins are about 35kD t-SNARE components expressed at the cytoplasmic surface of certain membrane domains that participate in the formation of the SNARE complex, which mediates the docking and/ or fusion of intracellular vesicles to target membranes (Hagiwara *et al*., 2013). There are 15 members of the syntaxin family in the human genome and 7 syntaxin-like genes in the yeast *Saccharomyces cerevisiae* (Teng *et al*., 2001). All the mammalian syntaxins, with the exception of syntaxin 11, are transmembrane proteins anchored by their carboxy-terminal with a type II orientation (that is, with amino terminus and the bulk of polypeptide facing the cytoplasm). The membrane proximal coiled-coil domain is the SNARE domain (H3 domain) that is characteristic of and conserved in all syntaxins which is reqired for binding of both VAMP and SNAP-25. The N-terminal regulatory region Habc domain is also the characteristic feature of syntaxin (Fernandez *et al*., 1998).

Syntaxin1 is distributed in neuronal and secretory cell which is localized in the presynaptic plasma membrane. Generally, it is involved in neuronal exocytosis (Solimena *et al*., 1995). Syntaxin2 and Syntaxin3 are ubiquitously distributed and are localized in the plasma membrane. And they are also involved in exocytosis (Quinones *et al*., 1999; Low *et al*., 2006). The most distant member of the family, Syntaxin5, has been found in the Golgi region and has significant homology (35% identity) with Sed5p, an essential protein in yeast which is required for vesicular transport from the endoplasmic reticulum (ER) to the Golgi stack (Dascher *et al*., 1994). Syntaxin6 is one of the important members of the syntaxin family, whose constituents are required components of several vesiclular trafficking pathway. Syntaxin6 is located in the trans-Golgi network (TGN),

where it partially colocalizes with the TGN adapter protein AP-1 on clathrin-coated membranes. Syntaxin6 mediates a TGN trafficking event, perhaps targeting to endosomes in mammalian cells (Bock *et al*., 1997). Syntaxin7 is mainly localized to vacuolar early endosomes (EEs) and may be involved in protein trafficking from plasma membrane to the EE as well as in homotypic fusion of endocytic organelles. In contrast, Syntaxin8 is likely to function in clathrin-independent vesicular transport and membrane fusion events necessary for protein transport form EEs to LEs (Prekeris *et al*., 1999). Syntaxin10 is localized to the TGN and ubiquitously expressed protein. Syntaxin10 making pair with VAMP3 helps in the transportation of Mannose 6-phosphate receptors (MPRs) from endosomes to the Golgi complex (Ganley *et al*., 2008). Syntaxin11 is 287-amino acid SNARE protein that does not have transmembrane domain present in it. Syntaxin11 is predominantly membrane associated and colocalizes with the mannose 6-phosphate receptor on late endosomes and the trans-Golgi network. Syntaxin11 acts to regulate protein transport between late endosomes and the trans-Golgi network in mammalian cells (Valdez *et al*., 1999). Syntaxin12 is 272-amino acid long and is localized in the Enodosome (Tang *et al*., 1997) whereas Syntaxin13 is found in tubular early and recycling endsosomes, where it colocalizes with transferrin receptor. It has the role in endocytosis-mediated recycling of plasma membrane (Prekeris *et al*., 1998). Syntaxin16 is present in TGN-Golgi and is required for efficient retrograde transport of several exogenous and endogenous cargo proteins (Amessou *et al*., 2007). Syntaxin17 is abundantly expressed in steroidogenic cell types and specifically localizes to smooth membranes of the ER. It has the function in a vesicle-trafficking step to the smooth-surfaced tubular ER membranes that are abundant in steroidogenic cells (Steegmaier *et al*., 2000). Syntaxin18 is the ER localized SNARE protein that has the role in ER-mediated phagocytosis (Hatsuzawa *et al*., 2006)

Syntaxin4 is present in the plasma membrane as well as on intracellular vesicles in the mammalian cells and have the role in phagosomal maturation (Hackam *et al*., 1996). Syntaxin4 and VAMP3 function as target membrane and vesicles SNAP receptors, respectively, for insulin-responsive GLUT4 transloacation to the plasma membrane (Olson *et al*., 1997). In addition to this, Syntaxin4 has its role in the vasopressin-regulated trafficking of aquaporin-2 water channel vesicles to the apical plasma membrane of renal collecting duct cells (Mandon *et al*., 1996). Acid sphingomyelinase (A-SMase) is activated through translocation from intracellular compartments to the plasma membrane in an exocytic pathway requiring the t-SNARE proteins syntaxin4 (Perrotta *et al*., 2010). During macrophage activation, Syntaxin4 is regulated to function in membrane traffic and cytokine secretion (Pagan *et al*., 2003).

2.11 VAMP family

Vesicle Associated Membrane Proteins (VAMPs) are classified as R SNAREs since they contribute an Arginine residue to the 0^{th} layer of the core complex (Sutton *et al*., 1998). Around 7 mammalian VAMPs have been identified till date and are named VAMP 1-5, 7 and 8 (Stow *et al*., 2006). The first member of the R-SNARE VAMP family to be known was a 120 amino acid longer membrane protein, isolated from the synaptic vesicles of *Torpedo californica* (Trimble *et al*., 1988). Using cDNA clone of this VAMP, two genes named VAMP1 and VAMP2 were identified in rat brain (Elferink *et al*., 1989). VAMP1 and VAMP2 are called synaptobrevin1 and 2 respectively and are highly expressed in neuron and endocrine cells. Synaptobrevins are involved in exocytosis of synaptic vesicles (Jahn *et al*., 1994).

VAMP4 has a broad tissue expression profile. It is localized to the Golgi-TGN in the NRK (Normal Rat Kidney) cells (Advani *et al*., 1998) and tubular and vesicular membranes of the TGN in PC12 cells. PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla (Steegmaier *et al*., 1999). VAMP4 was shown to function in the SNARE complex that mediates retrograde trafficking from early endosomes to TGF (Kreykenbohm *et al*., 2002). VAMP5 is found associated with plasma membrane and intracellular vesicular structures in myotubes (Zeng *et al*., 1998). VAMP7 also called toxin insensitive VAMP (TI-VAMP) was identified in EST database search (Advani *et al*., 1998). It was found to be abundant on the membranes of the *trans* golgi network and the late endosomes (Advani *et al*., 1999) and mediates vesicular transport from the late endosome to the lysosomes (Advani *et al*., 1999; Ward *et al*., 2000). VAMP7 was shown to play a critical role in the onset of phagocytosis in macrophages (Braun *et al*., 2004). VAMP8 is a 15kD, found to be present on early endosomes. The amino acid sequence of VAMP8 is found to be 32% identical to VAMP1, 33% identical to VAMP2 and had 31% identity to VAMP3. Since, it does not contain the conserved toxin cleavage site it is insensitive to tetanus toxin and botulinum toxin (Wong et *al*., 1998).

VAMP3 is also called the cellubrevin as it is found ubiquitously expressed in all the cells and tissues. It is the non neuronal homologue of the synaptobrevins. VAMP3 is present in recycling endosomes and endosome-derived vesicles (McMahon *et al*., 1993). It colocalizes with endocytosed transferring receptors (McMahon *et al*., 1993) and the glucose transporter GLUT4 in adipocytes (Volchuk *et al*., 1995), and is also present in α-granules in platelates (Feng *et al*., 2002). Different studies have shown the physiological role of VAMP3. VAMP3 has been involved in recycling of transferring receptors to the plasma membrane (Galli *et al*., 1993), secretion of α- granules in platelets (Feng *et al*., 2002), recycling of T-cell receptors to the immunological synapses (Das *et al*., 2004), and membrane trafficking during cell migration (Tayeb *et al*., 2005). Like VAMP2, it can also

be cleaved by tetanus toxin, a metalloendoprotease which blocks synaptic exocytosis (McMahon *et al*., 1993). Biochemical studies show that VAMP3 forms ternary complexes with syntaxin4/SNAP25 (Polgar *et al*., 2002) and syntaxin4/SNAP23 (Flaumenhaft *et al*., 1999).

2.12 SNARE proteins in antigen presenting cells

Cells of the innate immune system like macrophages remove invading micro organisms and other foreign particles by phagocytosis. During phagocytosis, microorganisms are taken up by immune cells into phagosomes. Through membrane-trafficking events mediated by SNARE proteins, phagosomes fuse with lysososmes, generating degradative phagolysosomes. Phagolysosmes contributes to host immunity by linking microbial killing within these organelles with antigen processing for presentation on MHC- class I or II molecules to T cells (Matheoud *et al*., 2013).

It is known that various microorganisms and exogenous agents like LPS lead to the activation of the macrophages (one of the most potent phagocytic cells of the immune system). Membrane traffic in activated macrophages is required for two critical events in innate immunity: proinflammatory cytokine secretion and phagocytosis of pathogens. It is found that there is joint trafficking pathway linking both actions, which may economize membrane transport and increase the immune response. TNFα, the earliest and most potent proinflammatory cytokine released, has an essential role in immunity. SNARE-mediated fusion of vesicular carriers is a requirement of this and other trafficking pathways. TNFα is trafficked from the Golgi to the recycling endosome (RE), where VAMP-3 mediates its delivery to the cell surface at the site of phagocytic cup formation. In macrophages, endogenous VAMP3 is located on vesicular membranes and plasma membrane and plasma membrane ruffles R-SNARE-VAMP3 on the RE, and then VAMP3 pairs with the Q-SNARE (Syntaxin4-SNAP23) on the plasma membrane. Activated macrophages increase their cell surface area for phagocytosis by using extra membrane from endoplasmic reticulum (ER), lysosomes and endosomes in SNARE mediated fusion events. Thus, TNFα and VAMP3 containing RE membranes translocate to the nascent phagocytic cup for SNARE-mediated fusion during the initial stages of phagocytosis. Fusion of the RE at the cup simultaneously allows rapid release of TNFα and expands the membrane for phagocytosis (Murray *et al*., 2005).

The processing of antigens for cross-presentation in phagosomes is also tightly controlled by the oxidation level generated through the NADPH oxidase activity. In macrophages, SNAP-23 is a major Qbc-SNARE protein located on the plasma membrane that regulates the entire process of phagocytosis through enhanced ROS production and acidification within phagosome in macrophages (Sakurai *et al*., 2012). It is reported that

VAMP3 is also a major candidate to regulate the phagocytosis oxidative activity which is responsible for the degradation of antigen in macrophages (Matheoud *et al*., 2013).

CHAPTER III

MATERIALS AND METHODS

3.1 Reagents

All cell culture reagents including Roswell Park Memorial Institute (RPMI) media, Fetal Bovine Serum (FBS) along with other reagents including Chloroform, isopropanol, ethanol, Ethylene Diamine Tetra Acetic acid (EDTA), trizol (TRI reagent), trypsin, boric acid, Tween-20, Tris, sodium acetate, Diethyl pyro carbonate (DEPC), agarose, primers were purchased from SIGMA chemicals (St. Lous, MO, USA). RNase inhibitor, Oligo(dt)-18, dNTPs mix, M-MulV reverse transcriptase enzyme and its buffer and DNA loading dye were from Fermentas (Lithuania). Taq polymerase and its buffer, ssRNA ladder and DNA ladder (100bp) were purchased from NEB (UK). Syber Green was from G Biosciences. Skim milk powder was purched from Titan. SDS and Glycine were from High media. Sequi blot PVDF membrane and Low Range Protein Marker were purched from Biorad whereas Immobilon Chemiluminiscent substance was from Milipore. X-ray films were purched from Kodak. Anti-SNAP 23 C-terminus primary antibody and anti-Syntaxin 4 primary antibody were gifted from Dr. Paul A. Roche, NIH (USA), anti-β-actin (Chemicon), Anti mouse secondary antibody conjugated to HRP were purchased from Southern Biotechnology.

3.2 Cell culture and activation of cell line *in vitro*

MHS cell line was a kind gift from Prof. R. K Saxena and were maintained in RPMI 1640 media, supplemented with 10% heat inactivated FBS, 300 µg/ml L-glutamine and 60 µg/ml gentamicin at 37°C in a 5% CO_2 incubator. For harvesting the cells, culture media was removed and collected; the culture was further washed with phosphate buffered saline and was collected. Finally 0.25% w/v trypsin in EDTA was added to detach the monolayer. The harvested cells were collected and pelleted down by centrifugation at 1500 rpm for 5 minutes at 4°C. The pelleted cells were then suspended in 1 ml media, counted by haemocytometer using trypan blue exclusion dye and 0.5 million cells seeded in 6 well plate/ 3ml media and cultured overnight. Cells were grown further in presence or absence of lipopolysaccaride (50 µg/ml) for 24 hours. The cells were then harvested, washed, counted and further used for experiments.

3.3 Isolation of RNA

During RNA isolation it is important to maintain a Ribonucleases (RNases) free environment. All plastic wares or glasswares were first treated with DEPC (Diethylpyrocarbonate) water to inactivate RNases. As well as all instruments and working area were wiped with DEPC to inhibit RNase that might be present.

RNA from the cell line was isolated using Trizol (TRI) reagent. 5 million cells were suspended in 1 ml TRI reagent by vigorously vortexing, 0.2 ml chloroform per ml of TRI reagent was then added mixed/ vortexed vigorously for 10 sec. The resulting mixture was allowed to stand at room temperature for 15 min and then centrifugation at 12,000g for 15 min at 4°C.

As a result the solution got separated into 3 distinct phases: a lower phenol-chloroform phase, interphase and the colorless upper aqueous phase. The aqueous phase was carefully transferred to a fresh tube and 0.5ml of isopropanol was added to precipitate RNA. The contents were mixed well and incubated at room temperature for 15 min followed by centrifugation at 12,000g for 10 min at 4°C. The RNA precipitates as a white gel like pellet at the bottom of the tube. The supernatant was discarded and the RNA pellet was washed twice with 75% ethanol and pelleted down by centrifugation at 7500g for 5 min at 4°C. Ethanol was removed by air drying the RNA pellet for 30 min at room temperature.

RNA pellet was then suspended in Tris/ borate/ EDTA (TBE) buffer by mixing the solution properly and incubating for 10 min at 70°C. RNase Inhibitor (20U) was then added in each RNA sample at -80°C until use for RT-PCR.

3.4 Determination of yield and quality of RNA

Nanodrop ND3000 spectrophotometer, is used to estimate the concentration and purity of RNA samples. After setting a reference blank by TBE buffer, concentration and purity ratio measurement of RNA were obtained. The ratio of absorbance at 260/280 and 260/230 provides an estimate of the purity of RNA (260/280 ratio around 2.0 and 260/230 greater than 1.8 denotes RNA purity).

3.5 Electrophoresis of total RNA (Formaldehyde Agarose gel)

RNA was run on 1.2% formaldehyde agarose gel. The gel was prepared by dissolving 1.2 gram agarose in 100ml agarose gel running buffer containing 1X formaldehyde along with 2.0µl of EtBr (Ethedium bromide) (added after cooling to 40-50 °C). Gel was properly polymerized at room temperature. Then it was put into the gel tank containing 1X formaldehyde agarose gel running buffer. RNA samples mixed with RNA loading dye were then loaded along with the ssRNA ladder. The gel electrophoresis was carried out at 60 V, for 3 hours and the bands were visualized under UV light using BIO-RAD gel documentation system.

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

Reverse transcription was performed using Moloney Murine Leukemia Virus (M-MulV) Reverse Transcriptase and oligo (dT) 18 primer in a total reaction mixture of 20µl (Table

3.1). The amount of RNA template used was determined on the basis of different standardization conditions. 1µg of RNA was used for making cDNA for Real Time PCR.

Table 3.1: Reaction mixture for RT-PCR

Components	20µl Reaction	Final Concentration
Nuclease free H_2O	Calculated accordingly (to 20µl)	-
RNA Template	Variable	Variable
Oligo (dT)-18	1.0µl	
(Heated for 10 min at 65°C and thereafter kept at room temperature for 2 mins)		
5X M-MulV RT Buffer	4.5µl	1.125X
dNTPs mix (10 mM)	2.0µl	1 mM
M-Mul V RT Enzyme (200 U/µl)	1.0µl	20 U/ µl

The sample was incubated in a thermal cycler at 37 °C for 60 min. The reaction was terminated by heating at 94 °C for 2 min. Finally, the reaction product was held at -20 °C till further use.

3.7 Agarose gel electrophoresis of cDNA

1.8% agarose gel was prepared by melting 1.8 gram agarose powder in 100 ml of 0.5X agarose gel running (Tris-Borate-EDTA (TBE)) buffer and adding 2 µl of EtBr after it cools down to 40-50 °C. Thereafter the gel was cast and transferred into gel tank containing 0.5X agarose gel running buffer. cDNA samples mixed with DNA loading dye (1 volume 6X DNA loading dye + 5 volume of cDNA), were loaded on the agarose gel. The gel electrophoresis was carried out at 90 V, for 1.5 hours and the gel was visualized in BIO-RAD gel documentation System.

3.8 Primer designing for conventional PCR

Primers were designed for the molecules for various SNAREs proteins using Primer-BLAST. PCR product size was choosen to be between 250-500 bp, primers melting tempreture (Tm) were calculated according to nucleotide content of the primers. Primers were 18-22 bases long with GC contents 45-55%. Bioinformatics tools such as “Oligo Calc” was used to calculate oligonucleotide properties. It detects the possible formation of internal hairpin loop within primers and also with each other and check for 3´ and 5´ complementarity of primers.

Table 3.2: Bioinformatics tools and their link:

S.N	Bioinformatics Tools	URL Link
1.	Oligo Calc	http://www.basic.northwestern.edu/biotools/OligoCalc.html
2.	Primer-BLAST	http://www.ncbi.nlm.nih.gov/tools/primer-blast/)
3.	MultaAlin	http://multalin.toulouse.inra.fr/multalin/)
4.	Sequence editor	http://www.fr33.net/seqedit.php)

"Primer-BLAST" is a NCBI based tool which is used to design primers specific to the PCR template sequence from Consensus coding sequences (CCDS) of molecules that offers a level of sensitivity and usability not found in other tools. The input can be a Genbank accession, a FASTA file, or even primers from another source. The blast results are automatically analyzed to avoid primer pairs (all combinations including forward reverse primer pair, forward-forward as well as reverse-reverse pairs) that can cause amplification of targets other than the input template. Finally, it displays alignments between primers and targets found, allowing the user to make a decision on whether or

not to use the primer pairs when potentially unintended targets exist. The above table shows bioinformatics tools used with their links. “MultAlin” (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. “Sequence editor”, convert DNA and RNA sequences and also generate antiparallel, complement and inverse sequence.

3.9 Amplification of cDNA with conventional PCR

The first strand cDNA obtained after RT-PCR was amplified by conventional Polymerase chain reaction (PCR) using different primers (Table 4.1) for different molecules in individual reaction mixture of 25µl (Table 3.3).

Table 3.3: Reaction mixture for conventional PCR

Components	25µl Reaction	Final Concentration
Nuclease free H_2O	Calculated accordingly (to 25 µl)	-
Taq Buffer (10X)	2.5 µl	1X
dNTPs mix (10 mM)	0.5 µl	200 µM
Forward Primer (10 µM)	0.5 µl	0.2 µM
Reverse Primer (10 µM)	0.5 µl	0.2 µM
Template (cDNA)	3.0 µl	-
Taq. Polymerase (150 U/ ml)	0.125 µl	0.75 U/ ml

The samples were put in thermocycler for PCR amplification. The reaction starts with initial denaturation step by heating the sample to 94 °C for 30 sec, followed by repeated cyles of denaturation at 94 °C for 30 sec, annealing for 1 min at different temperatures, and extension for 45 sec at 68 °C. The last cycle was followed by an additional extension for 5 min at 68 °C and final hold at 4 °C.

3.10 Agarose gel electrophoresis of amplified cDNA

1.8% agarose gel was prepared by melting 1.8 gram agarose powder in 100 ml of 0.5X agarose gel running (Tris-Borate-EDTA (TBE)) buffer and adding 2 µl of EtBr after it cools down to 40-50 °C. Thereafter the gel was cast and transferred into gel tank containing 0.5X agarose gel running buffer. Amplified cDNA samples mixed with DNA loading dye (25 µl sample + 5 µl 6X gel loading dye), were loaded on the agarose gel. DNA ladder was prepared by mixing 4 µl gel running buffer, 1 µl DNA ladder (100 bp) (500 µg/ ml) and 1

µl 6X gel loading dye. The ladder was then loaded on the agarose gel. The gel electrophoresis was carried out at 90 V, 2 hours and the gel was visualized in BIO-RAD gel documentation System.

3.11 Quantitation of band intensity

Band intensity of PCR- products were measured by using QUANTITY ONE software (BIO-RAD), as band intensity is directly proportional to amount of DNA present in gel.

3.12 Primer designing for Real Time PCR

Real time PCR primers for quantifying selected genes were designed using Primer-BLAST. PCR product size was chosen between 80-100 bp. Primers were 18-22 bases long with GC contents 45-55%. The primers that produced more than one melting peak were discarded.

3.13 Amplification of cDNA with Real time PCR

The first strand cDNA obtained after RT-PCR was amplified by Real time Polymerase chain reaction (PCR) using different sets of primers (Table 4.2) for different molecules in individual reaction mixture of 10 µl (Table 3.4)

Table 3.4: Reaction mixture for real time PCR

Components	10µl Reaction	Final Concentration
Syber Green (2X)	5 µl	1X
Template (cDNA) (1:4 dil)	4 µl	-
Forward Primer (10µM)	0.5 µl	500 nM
Reverse Primer (10µM)	0.5 µl	500 nM

The samples were put in 96 well RT plate sealed with optically active adhesion cover and the plate was put in the 7500 Fast Real Time-PCR for amplification. The reaction starts with initial denaturation step by heating the sample to 95 °C for 15 minutes, followed by repeated cyles of denaturation at 95 °C for 30 sec, annealing for 30 sec at 55 °C, and extension for 45 sec at 72 °C, final extension for 7 min at 72°C and hold at 4°C.

3.14 Preparation of cell lysates

About 1 x 10^7 cells in the culture dish were washed with 1X PBS and were detached after incubating for 5 min at 37°C by 0.25% w/v trypsin and collected. The cells were subjected for centrifugation at 1500 rpm at 4°C for 5 min. The supernatant was removed and cells were dissolved in 1 ml cell lysis buffer in ice with continuous stirring for 30 min.

Protein samples (e.g. cell lysates) were mixed with 2X Sample Buffer to make a final concentration of 1X. Thus prepared samples were heated at 95°C for 5 min prior to loading onto SDS-PAGE gels.

3.15 SDS polyacrylamide gel separation of proteins

Gel assembly was prepared by aligning two glass plates with spacers in between them. Freshly prepared separating gel was poured between the two plates. The gel was layered with 1 ml of water and allowed to set for around 30 minutes at room temperature. Later water was removed and stacking gel solution was poured and the comb was fitted in between the plates. The gel was left undisturbed till the stacking gel was set. Then, 45 µl of samples prepared were loaded in the wells on the gel. 20 µl Low Range Protein Marker was loaded in the first well. The gel was run at at 80 V in stacking and at 120 V in resolving gel till the dye front reaches the end in 1X SDS PAGE running buffer.

3.16 Western Blotting

Stacking portion of the gel was cut and removed. The separating portion of the gel was transferred to prechilled transfer buffer in glass tray for equilibration. PVDF membrane and blot filter paper were cut to the dimension of the gel. The PVDF membrane was wet in methanol for 2 min and then transferred to 1X Transfer buffer. The Blot filter paper and fiber sponge were also transferred to Transfer buffer for equilibration. The gel, membrane, filter paper and fiber sponge were maintained in the required orientation. The assembly was closed and it was subjected to the 1X Transfer Buffer at 250 mA for 90 min at RT. PVDF membrane was removed and was then blocked overnight in Phosphate Buffer Saline/ Tween-20/ Milk (PBS/T/M) (20 ml) at 4°C. The PBS/T/M was removed and the membrane was washed twice with 20 ml PBS/T at RT (15 min each). One hour incubation with primary antibody with the dilution of 2000 in PBS/T (2.5 µl in 5 ml) was done. Then, the PVDF membrane was blocked twice with 20 ml PBS/T/M for 20 min each at RT. Then, 30 min incubation with HRP-Tagged secondary antibody in the dilution of 5000 in PBS/T/M (i.e 2.0 µl in 10 ml) was done at RT. The membrane was blocked three times with 20 ml PBS/T/M for 20 min each. Again the membrane was washed two times with 20 ml PBS/T for 20 min each.

3.17 Detection of the Blots:

ECL reagents were cooled down to room temperature before use. In the dark room with red light, two ECL reagents (H_2O_2 + Lumiol) were mixed 0.5 ml each in an eppendroff. The ECL mix was then spread on a membrane with pipette. X-ray films were given multiple exposures on the membrane at different time interval. The X- ray films were developed in the developer. The films were rinsed in water for sometimes and the bands

thus developed were fixed in the fixer. The film were again rinsed in water and dried after that (Puri and Roche, 2006). The quantitation of the X-ray film blots was done on alpha Ease Fc software.

CHAPTER IV

RESULTS

MH-S is a macrophage cell line which is capable of both phagocytosis, and antigen presentation of the invading pathogens. It also helps in the exocytosis of various cytokines which are produced in response to different foreign invaders. In this study we used MH-S cells as a model cell line as it responds to the surrounding challenges and plays an important role in immune response. It is also self-renewing, immortalized cell line that grows easily in culture. MH-S cells are adherent, lacked contact inhibition, and are trypsin-sensitive. Since MH-S cells are professional antigen presenting cells and are easy to isolate and handle they are quite appropriate for large scale screening process in a biotechnology setting. The main aim of the present study is to study the modulation of expression of SNARE machinery in presence of pathogenic challenges; we first set out to isolate the RNA from the cell line in presence or absence of LPS treatment. LPS being a constituent of Gram-negative bacteria acts as an endotoxin.

4.1 Isolation of RNA from untreated and treated cell line

RNA was isolated from the cell line using the Trizol reagent, which permits the complex dissociation of nucleoprotein complex. The concentration of RNA was measured by using the Nanodrop Spectophotometer at 260 nm. The impurities of protein and organic content were checked at 230 nm and 280 nm. There was little or no impurity found in the RNA preparation. The amount of RNA isolated from 10 million Control and LPS treated MH-S cells was comparable (Figure-4.1).

4.2 RNA and cDNA gel

RNA isolated from the cell line (treated and untreated) was run on a 1.2% agarose formaldehyde gel (Figure 4.2A). The appearance of two distinct bands of rRNA i.e. 28S and 18S confirmed the integrity of RNA. The RNA preparation was used to synthesize the first strand of cDNA and the product was run on 1.5% agarose gel. The appearance of a cDNA smear and the disappearance of RNA bands confirm the perfect preparation of cDNA (Figure 4.2B).

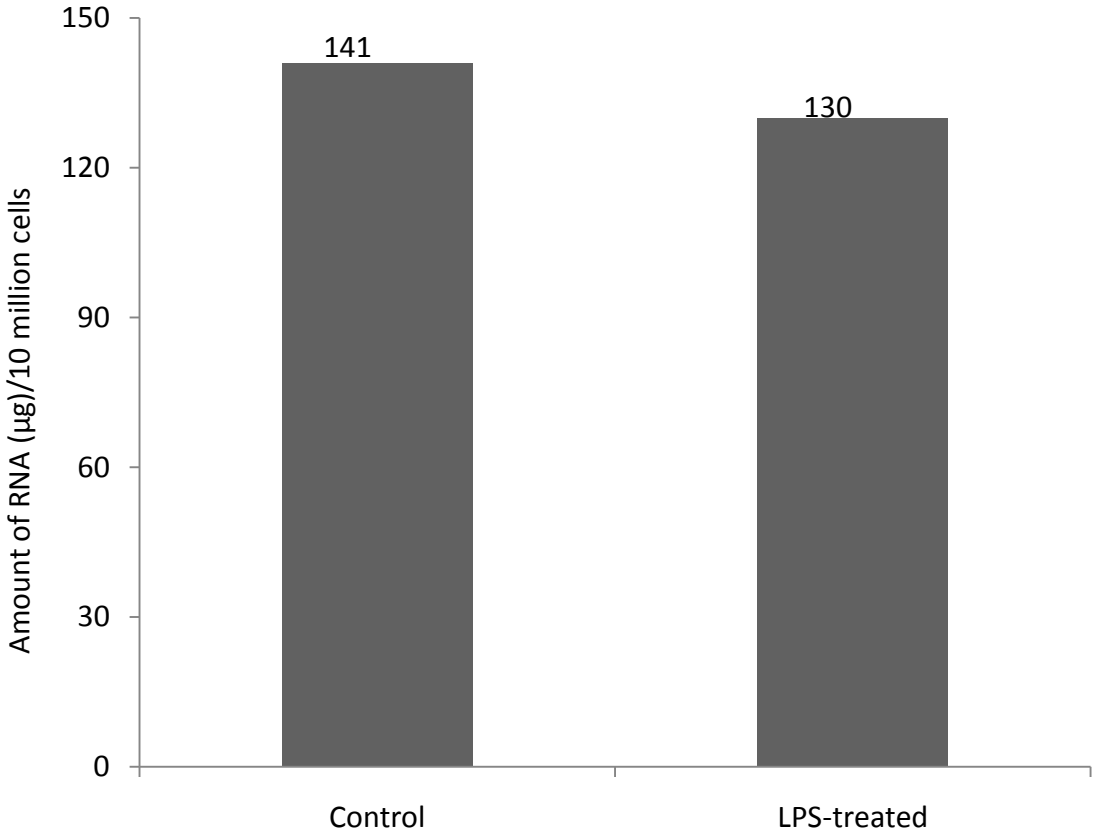


Figure 4.1: The amount of RNA isolated from Control and LPS treated MH-S cells. RNA was isolated from 10 million untreated and LPS treated MH-S cells using Trizol reagent and its yield and purity was determined using Nanodrop (ND-3000) Spectrophotometer. The amount of RNA thus obtained was then plotted as Bar Graph as shown in the figure.

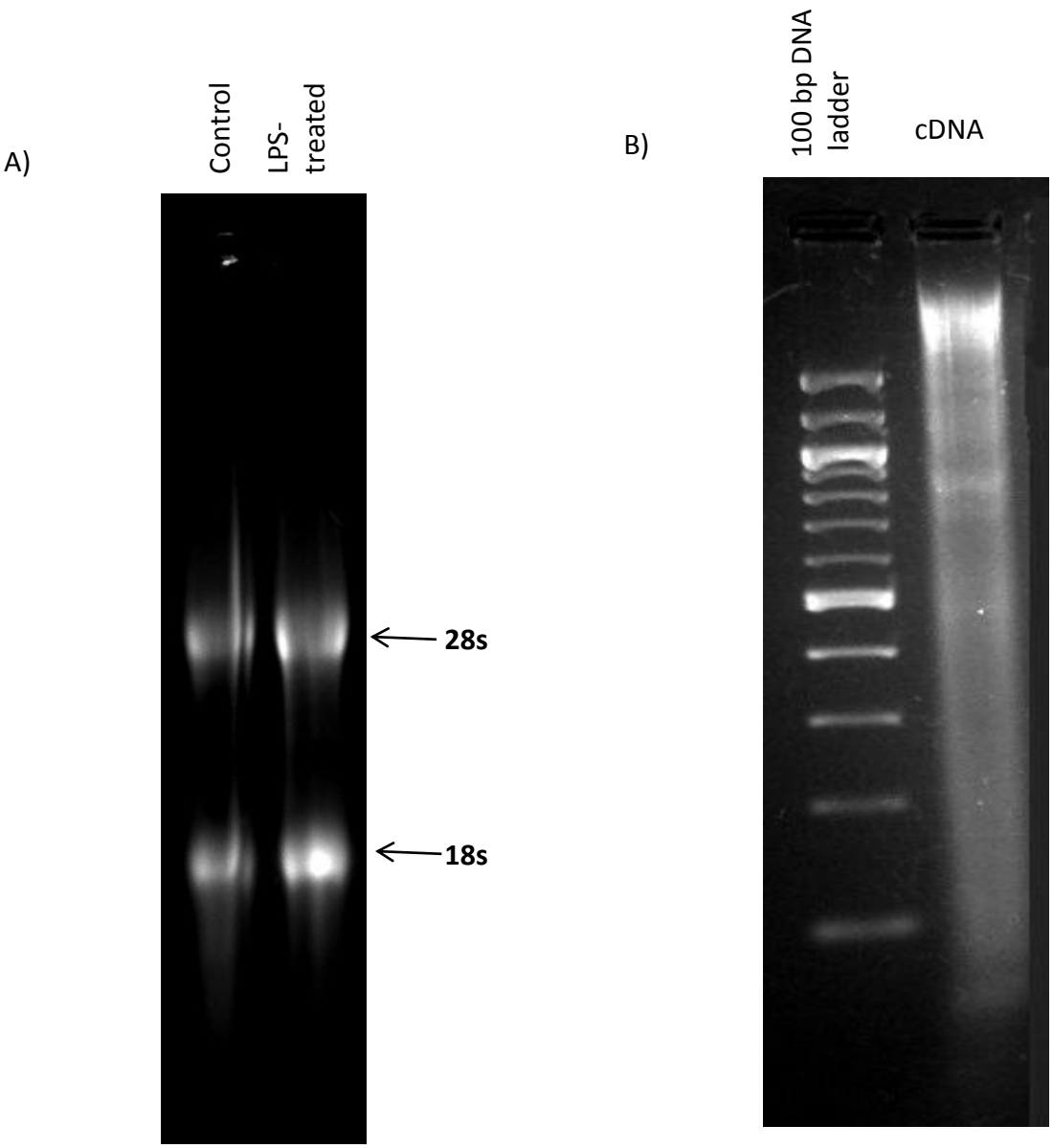


Figure 4.2: Agarose gel electrophoresis of RNA and cDNA isolated from MH-S cell lines. A) 3 µg of RNA isolated from untreated and treated MH-S cells were run on 1.2% formaldehyde agarose gel, visualized under UV in Gel Doc System (BIORAD) in which there was presence of two distinct bands of 28s and 18s rRNA, which confirm the integrity of RNA. B) cDNA was synthesized by reverse transcription of RNA using oligo dT primer. 10µl of cDNA was run on 1.5% agarose gel along with 100 bp DNA ladder and visualized under UV in Gel Doc System (BIORAD).

4.3 Standardization of quantitative RT-PCR for studying modulation of expression of SNAREs

Reverse transcription polymerase chain reaction (RT-PCR) is one of the easiest approaches of RNA detection. This technique is commonly used in molecular biology to detect RNA expression levels. RT-PCR is used to qualitatively detect gene expression through creation of complementary DNA (cDNA) from RNA through the use of reverse transcriptase enzyme. Subsequently, the newly synthesized cDNA is then used as a template for exponential amplification using PCR. The use of RT-PCR for the detection of RNA transcript has revolutionalized the study of gene expression. Its potential for high-throughputs, more reliable instrumentation and improved protocols, has also made this a method of choice in the research field. Qunatitative RT-PCR is characterized by the linear phase during the thermocycle reaction when a PCR product is amplified. In order to quantitate the levels of specific mRNAs transcripts and to compare the level of expression of molecules under different experimental conditions, standardization of quantitative RT-PCR in terms of its parameters such as annealing temperature, number of amplification cycles in PCR, and the amount of RNA template used for RT-PCR was performed. Annealing temperature is the one at which the primers anneal to the templates in PCR reaction, generally this is taken to be 4-5˚C less than the melting temperature of that primer template pair. As said above for low copy number of nucleic acid, higher number of thermocycles (30-40) is required, whereas for high copy number, a lower number of thermocycles (15-25) are sufficient for amplification. Ultimately conditions were standardized to get a broad linear range of mRNA detection.

4.3.1 Designation of RT-PCR primers

Primers were designed for the molecules for various SNAREs proteins using Primer-BLAST, Oligo Calc, MultAlign and Sequence editor tools. The list of primers along with their melting temperature (Tm), GC content and amplicon size are shown in the table 4.1.

Table 4.1: List of Primers for conventional PCR

S.N	Name of molecules	Primers Forward primer (FP) Reverse primer (RP)	Primer length (bp)	Melting Temperature Tm°C	GC content	Product length (bp)
1.	SNAP-23	FP:5'-CCTGGGTTTAGCCATTGAGTCT-3' RP:5'-GAGCCATGTTCTTTAGGTTGCC-3'	22 22	60 °C 59 °C	50 50	461
2.	VAMP3	FP:5'-AATCGAAGACTCCAGCAGACAC-3' RP:5'-GAGACACACCACACGATGATGA-3'	22 22	60 °C 60 °C	50 50	263
3.	Syntaxin 4	FP:5'-GAGAGAAGAATGTGGAGCGGAT-3' RP: 5'-GCCAATGATGACAGCCAAGATG-3'	22 22	59 °C 60 °C	50 50	431
4.	GAPDH	FP: 5'-GTCGGTGTGAACGGATTTGG-3' RP: 5'-CTAAGCAGTT GGTGGTGCAG-3'	20 20	60 °C 60 °C	55 55	457
5.	MHC II	FP: 5'-TTCCAACAACACTCCAGATGCC-3' RP: 5'-TCTCTTCAAACTCCCAGGTCTT-3'	22 22	58 °C 58 °C	55 55	540

4.3.2 Standardization of Quantitative RT-PCR for SNAP23 molecule

A quantitative RT-PCR method was standardized for studying the mRNA expression level of SNAP-23 in MH-S cells. A range of annealing temperatures, number of thermocycles and amount of template RNA were tried in order to standardize conditions for obtaining a broad linear range of quantitaion. The PCR products were then run on 1.8% agarose gel and the bands were quantified using BIO-RAD Quantity One Software. After quantification, the optimum conditions were found to be 55˚C as the annealing temperature (Figure-4.3), 25 numbers of thermo cycles (Figure-4.4) and 0.5µg -1.5µg as the RNA template amount (Figure-4.5).

4.3.3 Standardization of Quantitative RT-PCR for Syntaxin4 molecule

A quantitative RT-PCR method was standardized for studying the mRNA expression level of Syntaxin4 in MH-S cells. A range of annealing temperatures, number of thermocycles and amount of template RNA were tried in order to standardize conditions for obtaining a broad linear range of quantitaion. The PCR products were then run on 1.8% agarose gel and the bands were quantified using BIO-RAD Quantity One Software. After quantification, the optimum conditions were found to be 55˚C as the annealing temperature (Figure-4.6), 20 numbers of thermo cycles (Figure-4.7) and 0.75µg -1.5µg as the RNA template amount (Figure-4.8).

4.3.4 Standardization of Quantitative RT-PCR for VAMP3 molecule

A quantitative RT-PCR method was standardized for studying the mRNA expression level of VAMP3 in MH-S cells. A range of annealing temperatures, number of thermocycles and amount of template RNA were tried in order to standardize conditions for obtaining a broad linear range of quantitaion. The PCR products were then run on 1.8% agarose gel and the bands were quantified using BIO-RAD Quantity One Software. After quantification, the optimum conditions were found to be 55˚C as the annealing temperature (Figure-4.9), 25 numbers of thermo cycles (Figure-4.10) and 0.5µg -1.5µg as the RNA template amount (Figure-4.11).

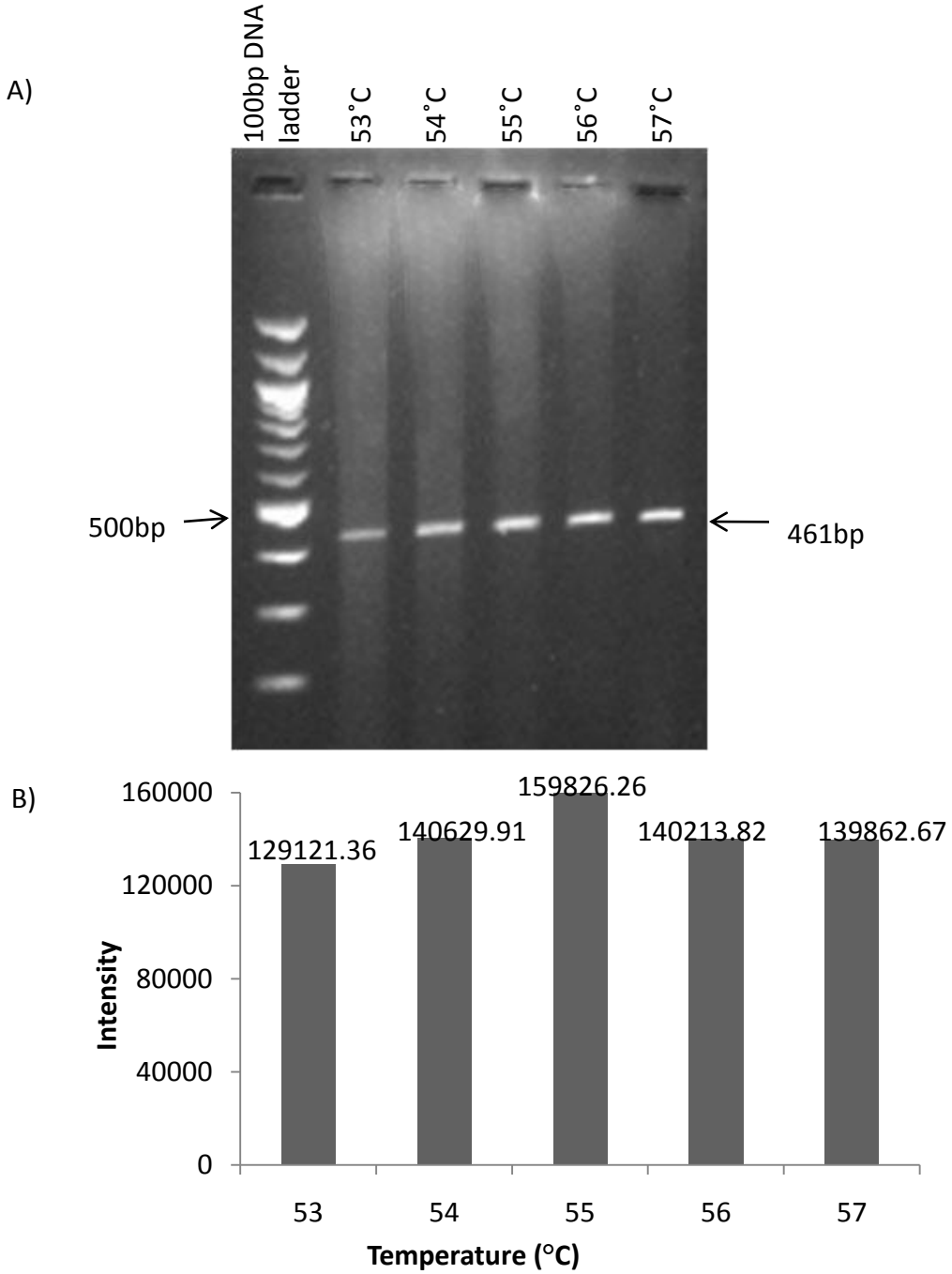


Figure 4.3: Standardization for annealing temperature for SNAP23. 3µg of RNA isolated from MH-S cell lines was converted to cDNA and used for the standardization of RT-PCR reaction for annealing tempreature for SNAP23 molecule using 25 cycles within a gradient of 53-57˚C. The PCR products were then run on 1.8% agarose gel, visualized under UV in GelDoc system (BIORAD) as shown in figure A. The bands thus obtained were quantitated by using the Quantity One software (BIORAD) and intensity plotted as Bar Graph as shown in figure B.

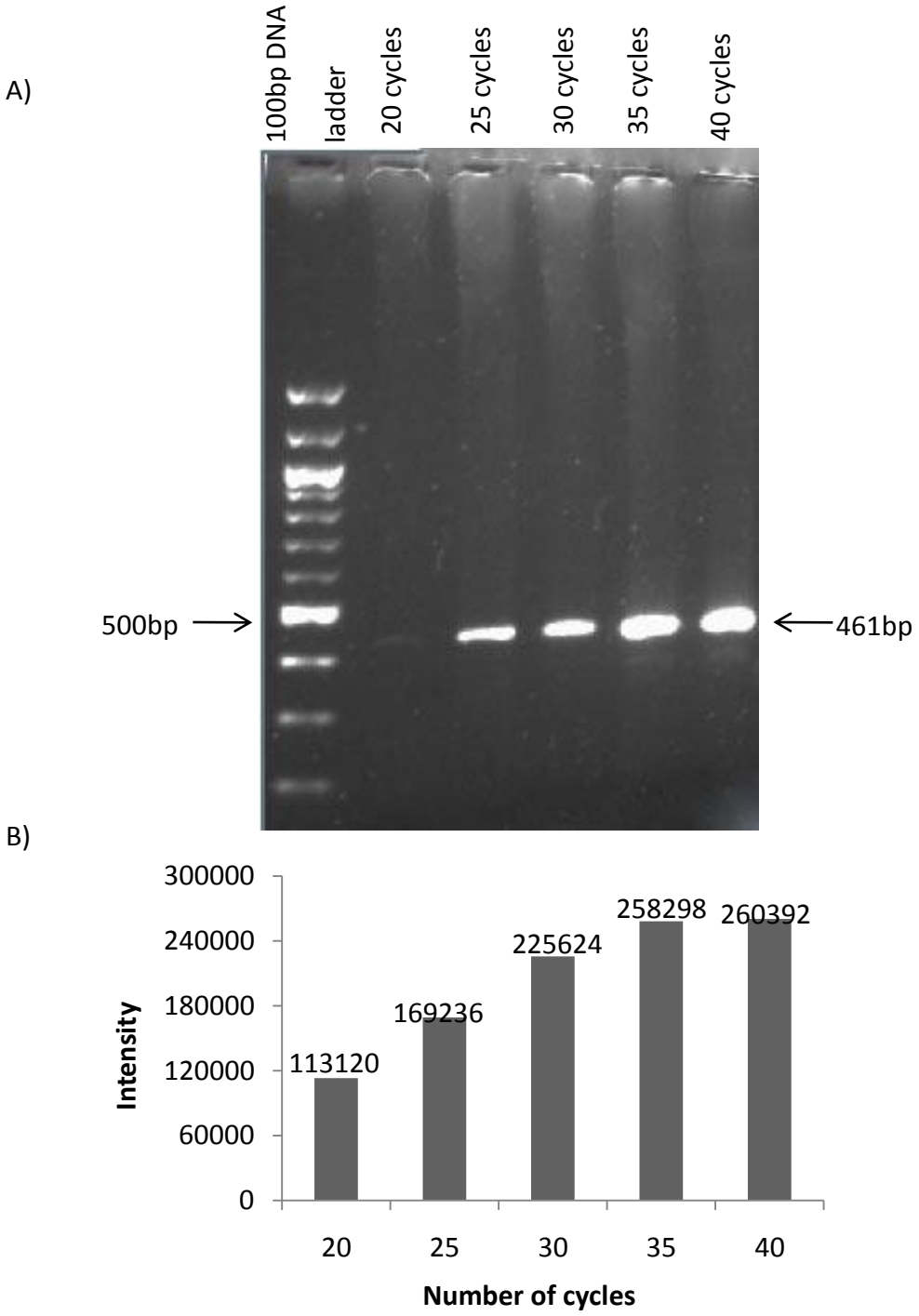


Figure 4.4: Standardization of number of thermocycles for SNAP23. 3µg of RNA isolated from MH-S cell lines was converted to cDNA and used for the standardization of RT-PCR reaction for number of thermocycles for SNAP23 molecule at 55˚C. The PCR products were then run on 1.8% agarose gel, visualized under UV in GelDoc system (BIORAD) as shown in figure A. The bands thus obtained were quantitated by using the Quantity One software (BIORAD) and intensity plotted as Bar Graph as shown in figure B.

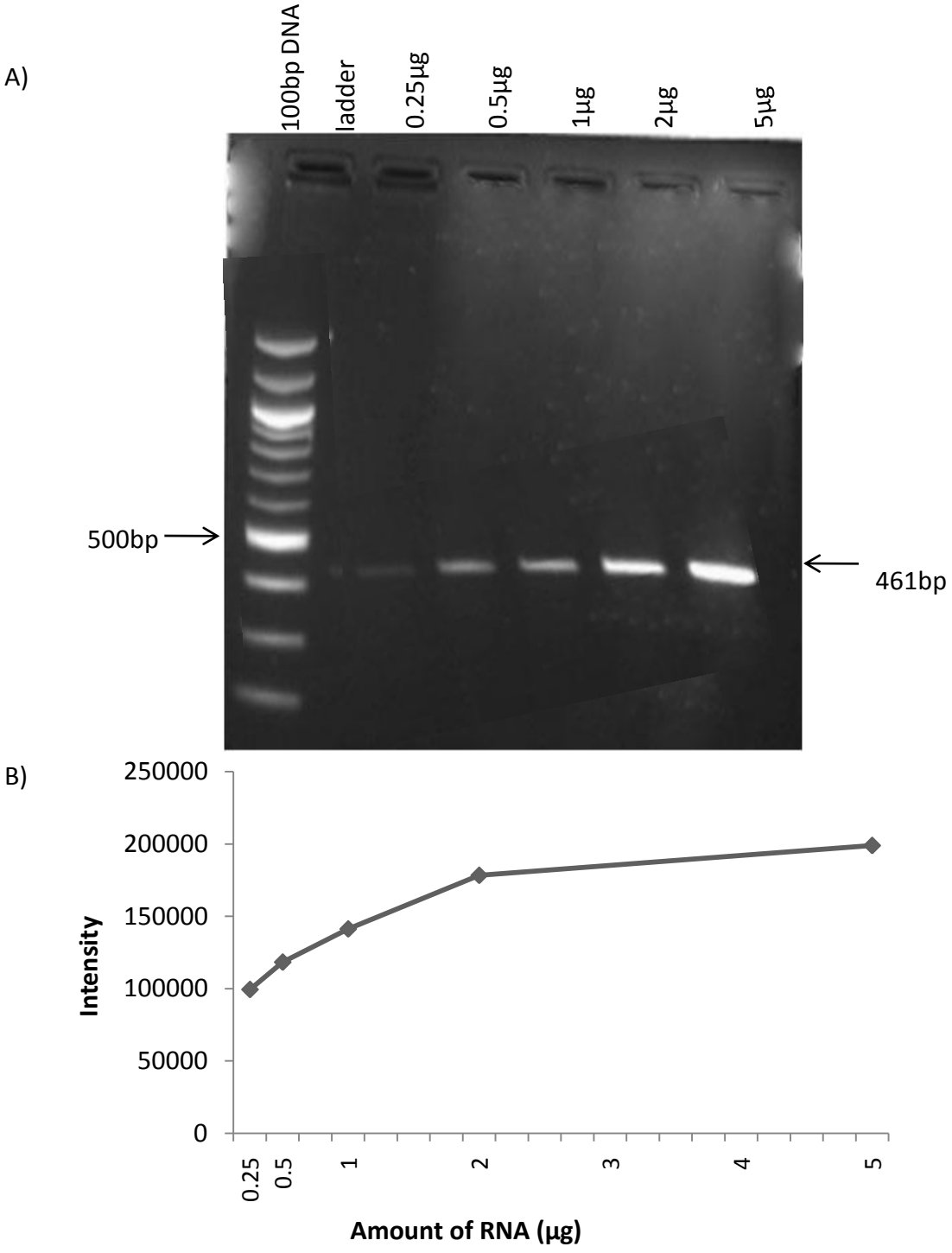


Figure 4.5: Standardization for amount of RNA template for SNAP23 molecule. Different amounts of RNA isolated from MH-S cell line was used for the RT-PCR reaction for 25 number of cycles at 55˚C. The PCR products were then run on 1.8% agarose gel, visualized under UV in GelDoc system (BIORAD) as shown in figure A. The bands thus obtained were quantitated by using the Quantity One software (BIORAD) and intensity plotted as LineGraph as shown in figure B

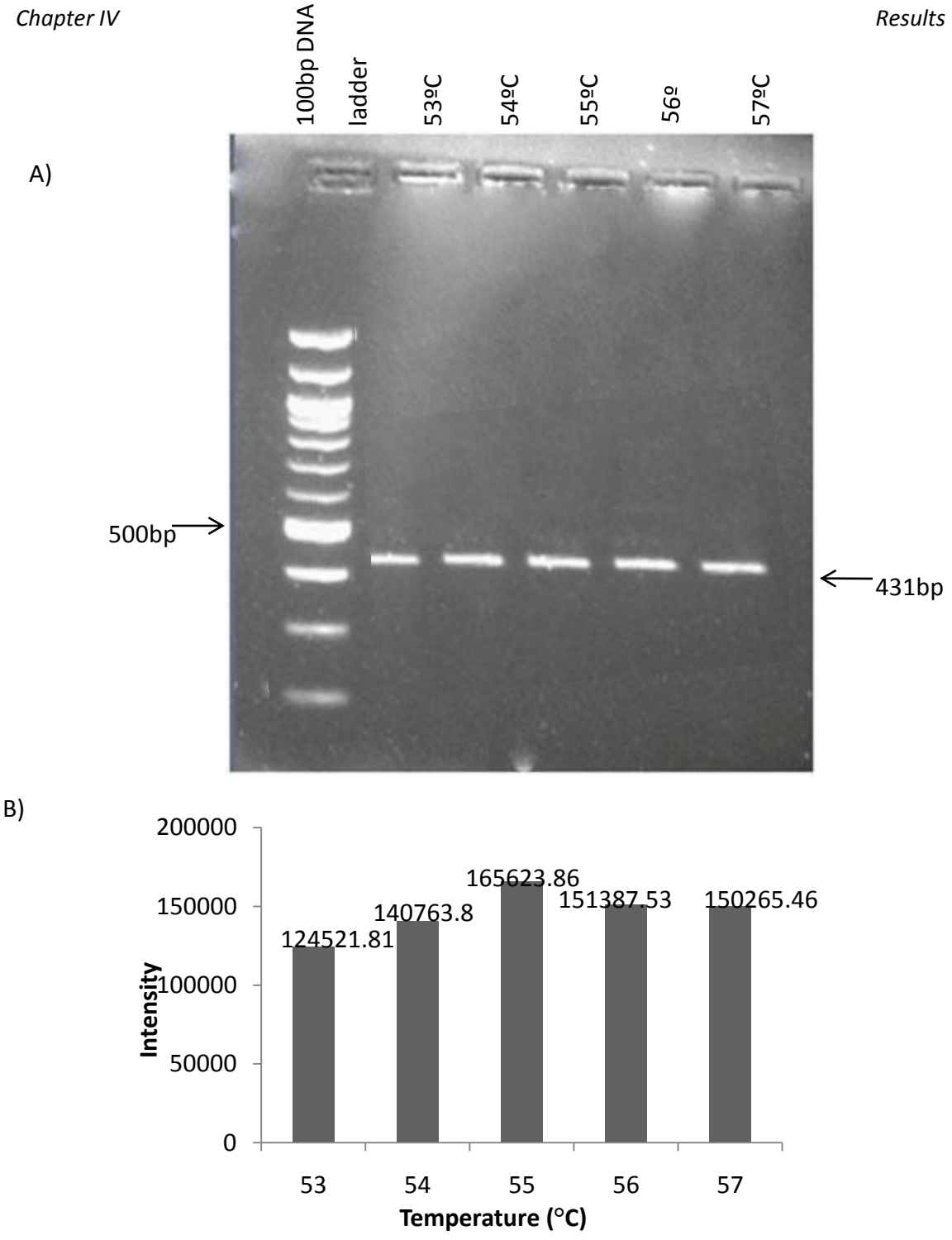


Figure 4.6: Standardization for annealing temperature for Syntaxin4. 3µg of RNA isolated from MH-S cell lines was converted to cDNA and used for the standardization of RT-PCR reaction for annealing tempreature for Syntaxin4 molecule using 20 cycles within a gradient of 53-57˚C. The PCR products were then run on 1.8% agarose gel, visualized under UV in GelDoc system (BIORAD) as shown in figure A. The bands thus obtained were quantitated by using the Quantity One software (BIORAD) and intensity plotted as Bar Graph as shown in figure B.

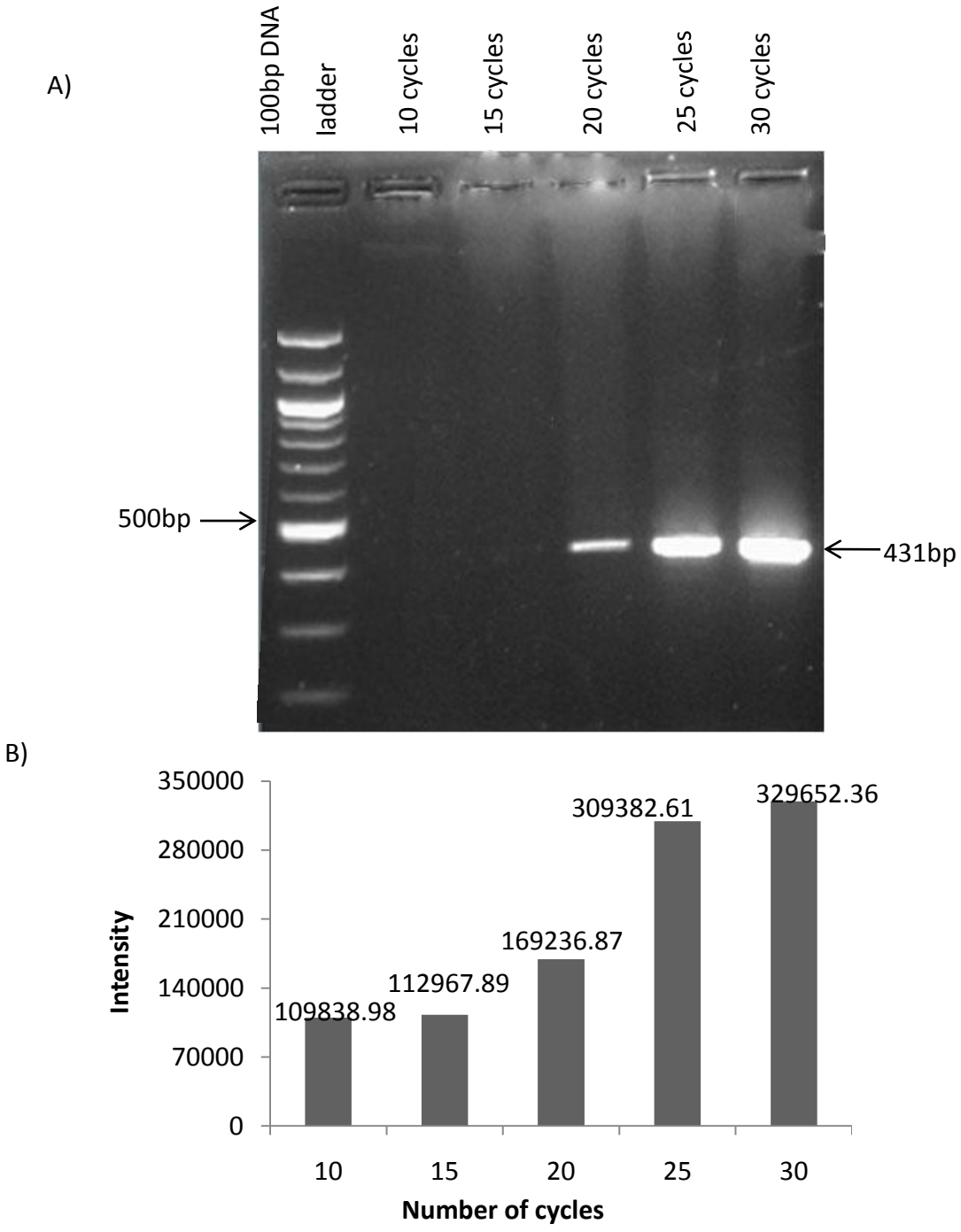


Figure 4.7: Standardization for number of thermocycles for Syntaxin4. 3µg of RNA isolated from MH-S cell lines was converted to cDNA and used for the standardization of RT-PCR reaction for number of thermocycles for Syntaxin4 molecule at 55˚C. The PCR products were then run on 1.8% agarose gel, visualized under UV in GelDoc system (BIORAD) as shown in figure A. The bands thus obtained were quantitated by using the Quantity One software (BIORAD) and intensity plotted as Bar Graph as shown in figure B.

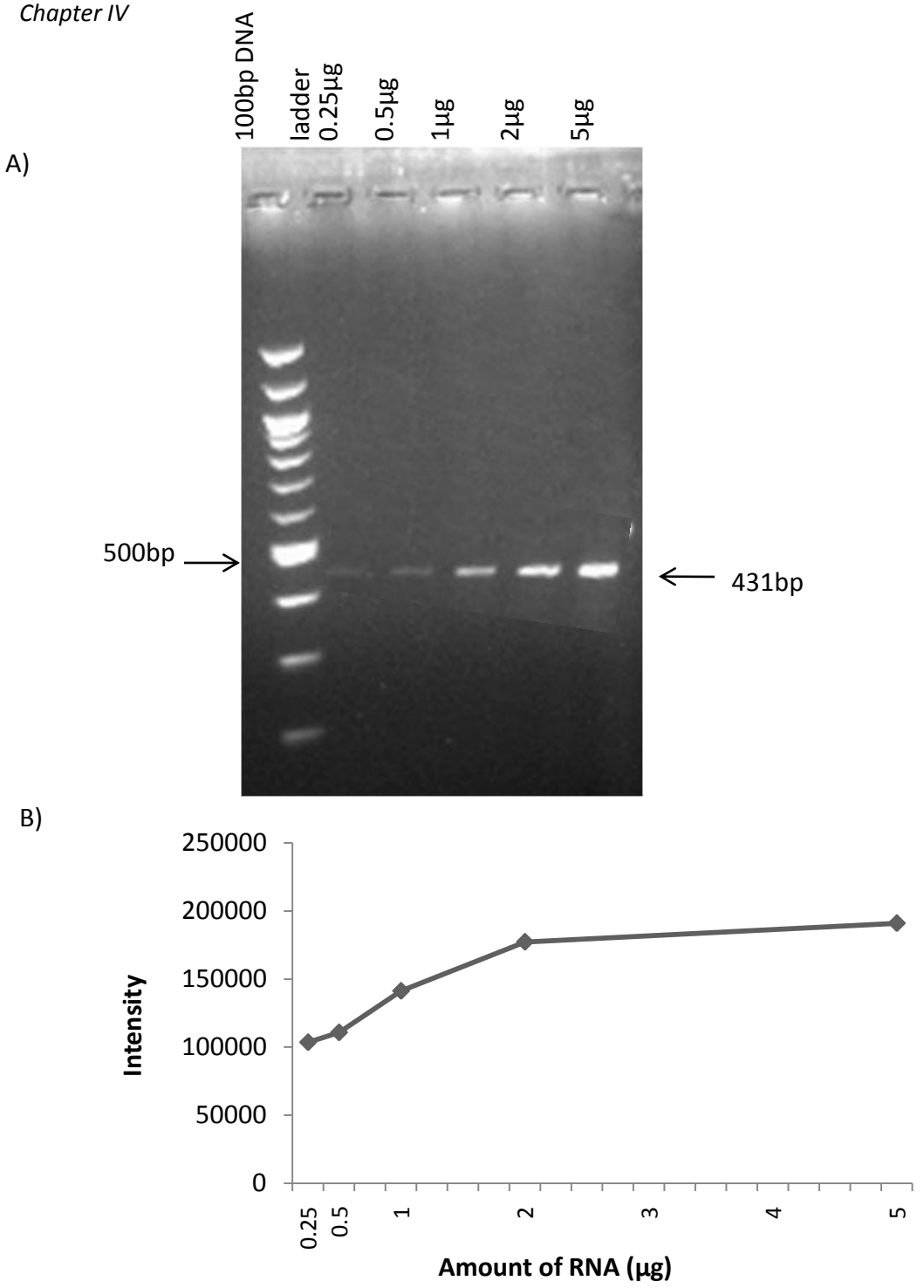


Figure 4.8: Standardization for amount of RNA template for Syntaxin4 molecule. Different amounts of RNA isolated from MH-S cell line was used for the RT-PCR reaction for 20 number of cycles at 55˚C. The PCR products were then run on 1.8% agarose gel, visualized under UV in GelDoc system (BIORAD) as shown in figure A. The bands thus obtained were quantitated by using the Quantity One software (BIORAD) and intensity plotted as Line Graph as shown in figure B.

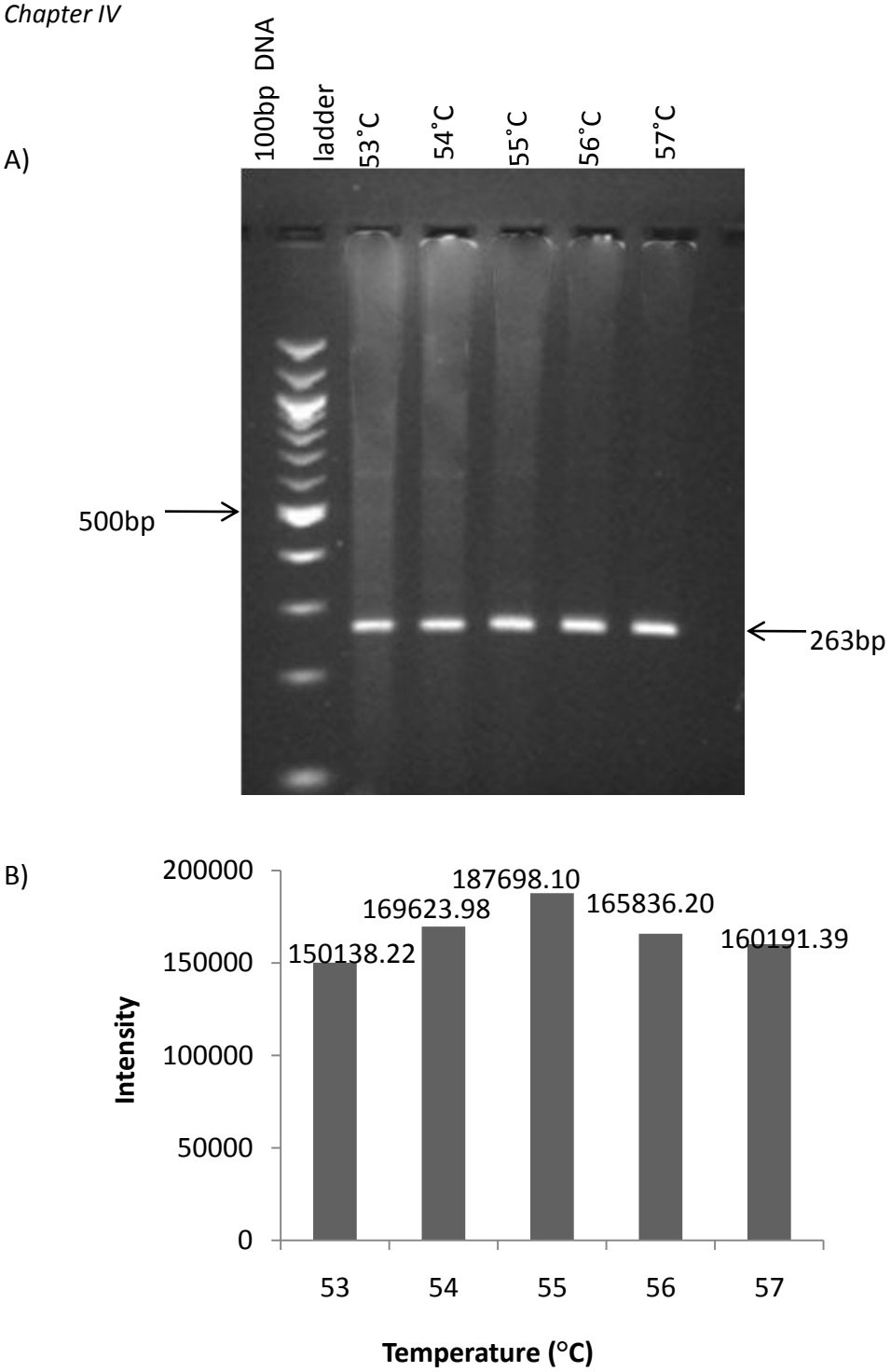


Figure 4.9: Standardization for annealing temperature for VAMP3. 3µg of RNA isolated from MH-S cell lines was converted to cDNA and used for the standardization of RT-PCR reaction for annealing tempreature for VAMP3 molecule using 25 cycles within a gradient of 53-57˚C. The PCR products were then run on 1.8% agarose gel, visualized under UV in GelDoc system (BIORAD) as shown in figure A. The bands thus obtained were quantitated by using the Quantity One software (BIORAD) and intensity plotted as Bar Graph as shown in figure B.

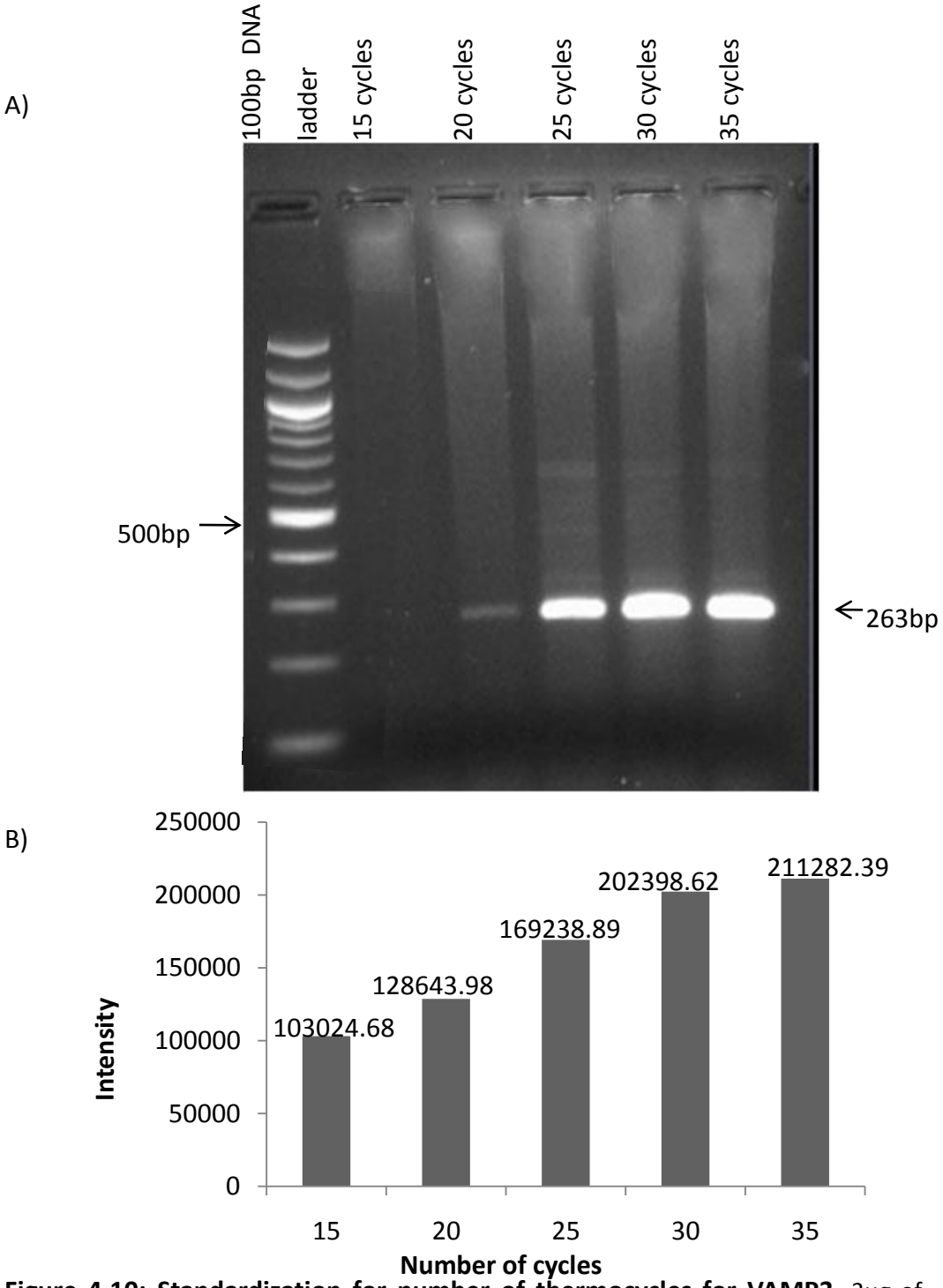


Figure 4.10: Standardization for number of thermocycles for VAMP3. 3µg of RNA isolated from MH-S cell lines was converted to cDNA and used for the standardization of RT-PCR reaction for number of thermocycles for VAMP3 molecule at 55˚C. The PCR products were then run on 1.8% agarose gel, visualized under UV in GelDoc system (BIORAD) as shown in figure A. The bands thus obtained were quantitated by using the Quantity One software (BIORAD) and intensity plotted as Bar Graph as shown in figure B.

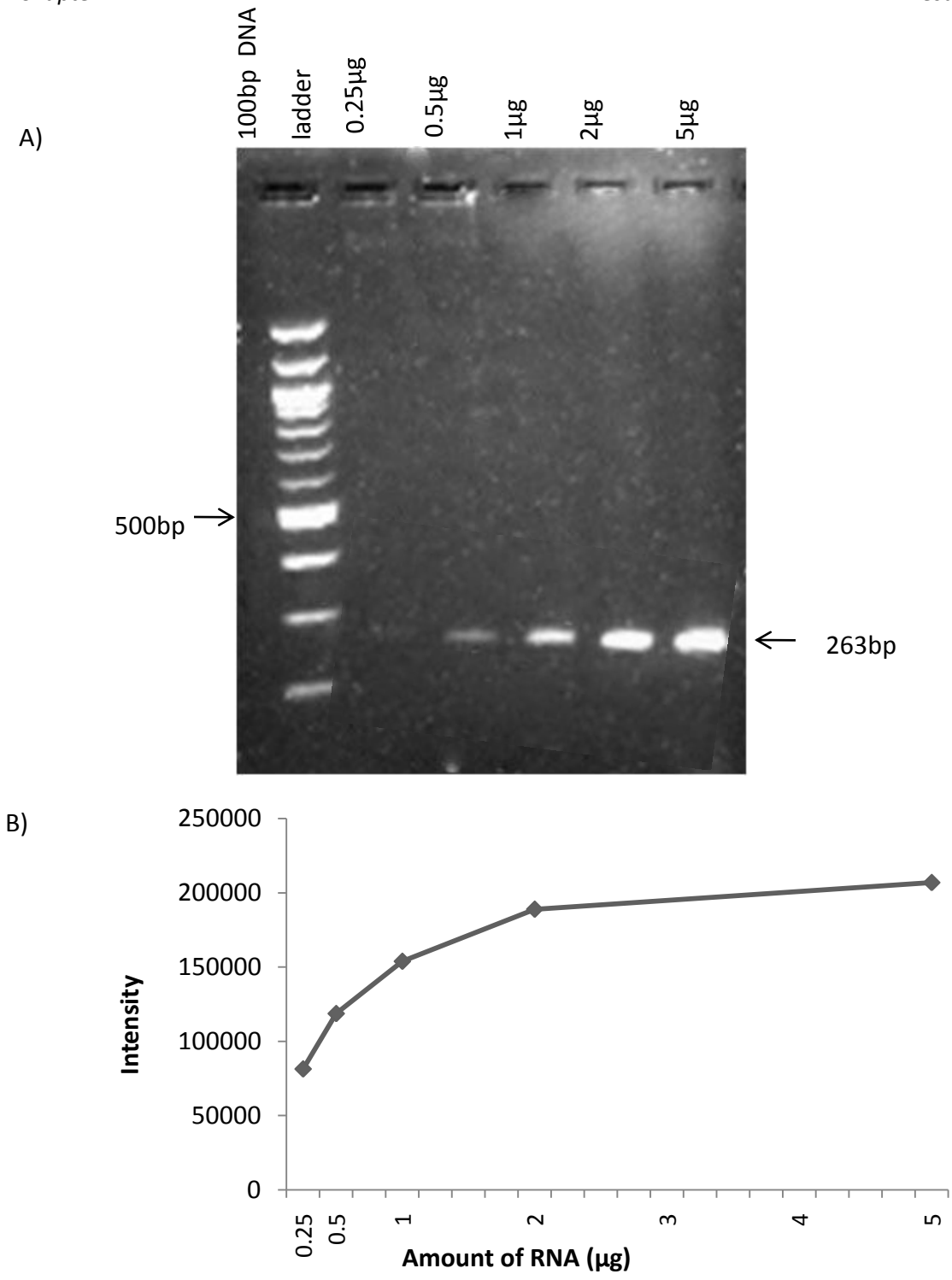


Figure 4.11: Standardization for amount of RNA template for VAMP3 molecule. Different amounts of RNA isolated from MH-S cell line was used for the RT-PCR reaction for 25 number of cycles at 55˚C. The PCR products were then run on 1.8% agarose gel, visualized under UV in GelDoc system (BIORAD) as shown in figure A. The bands thus obtained were quantitated by using the Quantity One software (BIORAD) and intensity plotted as Line Graph as shown in figure B.

4.3.5 Studies on the modulation of SNAREs expression on MH-S cell line in presence of LPS

After standardization of parameters for qRT-PCR for the molecules of SNARE machinery, the optimal conditions for SNAP-23, Syntaxin4 and VAMP3 molecules were used to study the effect of LPS on expression level of mRNA for SNAREs in MH-S cell lines. It was observed that there was very marginal or no modulation in the expression of all these SNAREs (Figure-4.12, 4.13, 4.14). MHC II was used as the positive control which showed a significant upregulation in LPS treated condition with comparison to that of untreated one (Figure-4.16). GAPDH was used as an endogenous control which showed no modulation in expression upon LPS treatment (Figure-4.15). The use of positive control validates the results of other molecules studied by this method. The intensity plotted as bar graph showing the expression of these molecules.

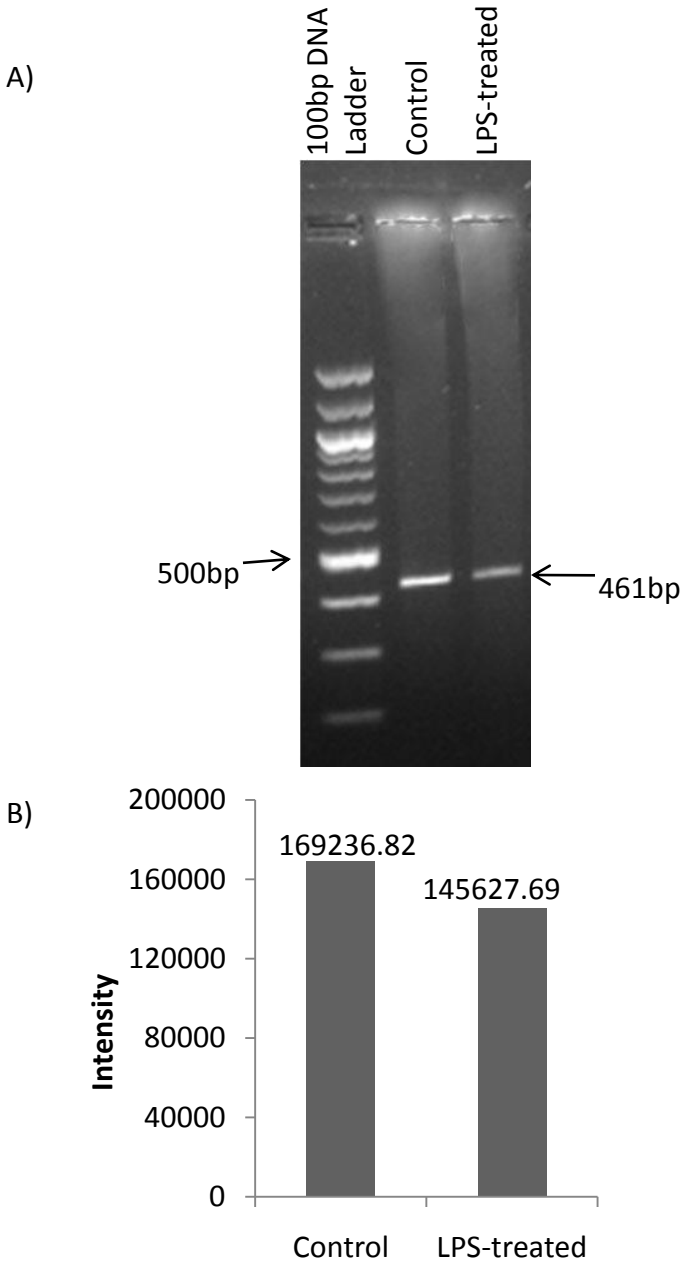


Figure 4.12 : Effect of LPS treatment on the expression of SNAP-23 molecule in MH-S cells. cDNA was prepared by reverse transcribing 1.5µg of RNA isolated from untreated and LPS treated MH-S cell lines and amplified for 25 number of cycles at 55°C. The PCR products were then run on 1.8% agarose gel, visualized under UV in Gel DOC System (BIORAD) as shown in figure A. The bands thus obtained were quantitated by using the Quantity One Software (BIORAD) and intensity plotted as Bar Graph as shown in figure B.

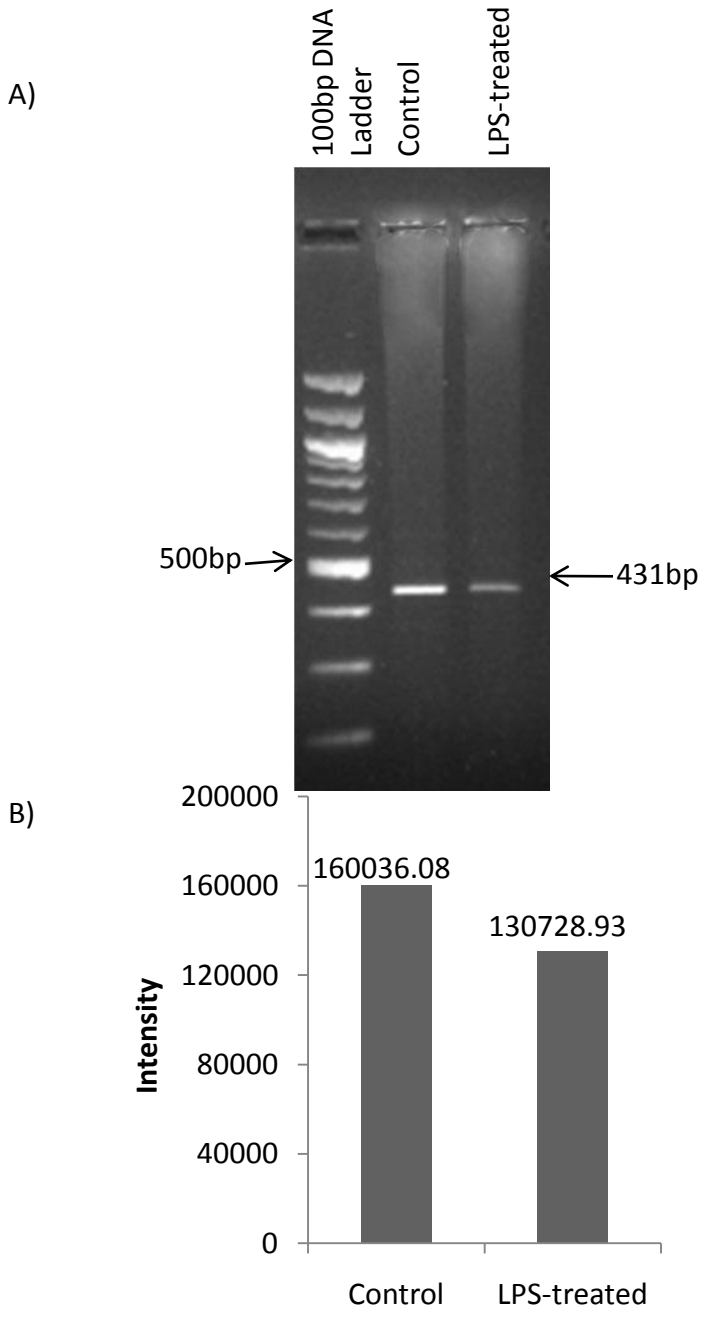


Figure 4.13 : Effect of LPS treatment on the expression of Syntaxin4 molecule in MH-S cells. cDNA was prepared by reverse transcribing 1.5µg of RNA isolated from untreated and LPS treated MH-S cell lines and amplified for 20 number of cycles at 55°C. The PCR products were then run on 1.8% agarose gel, visualized under UV in Gel DOC System (BIORAD) as shown in figure A. The bands thus obtained were quantitated by using the Quantity One Software (BIORAD) and intensity plotted as Bar Graph as shown in figure B.

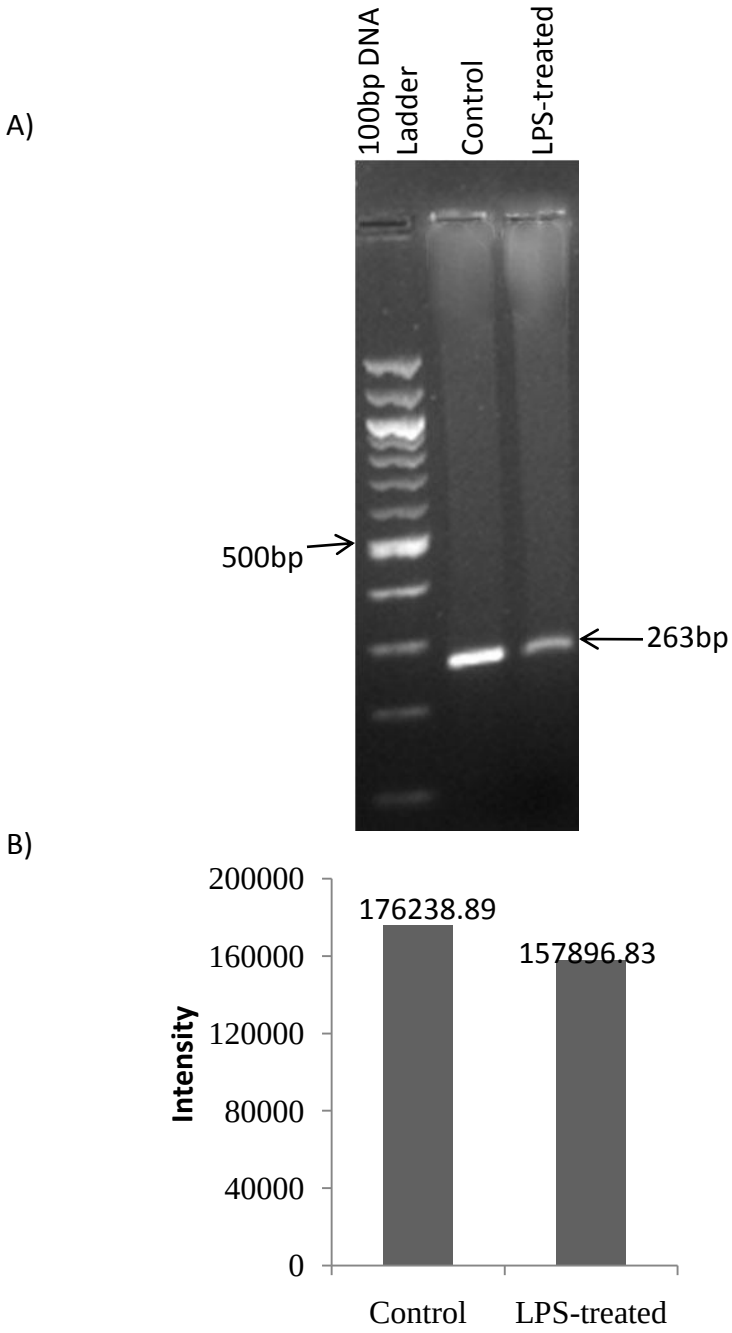


Figure 4.14 : Effect of LPS treatment on the expression of VAMP3 molecule in MH-S cells. cDNA was prepared by reverse transcribing 1.5µg of RNA isolated from untreated and LPS treated MH-S cell lines and amplified for for 25 number of cycles at 55°C. The PCR products were then run on 1.8% agarose gel, visualized under UV in Gel DOC System (BIORAD) as shown in figure A. The bands thus obtained were quantitated by using the Quantity One Software (BIORAD) and intensity plotted as Bar Graph as shown in figure B.

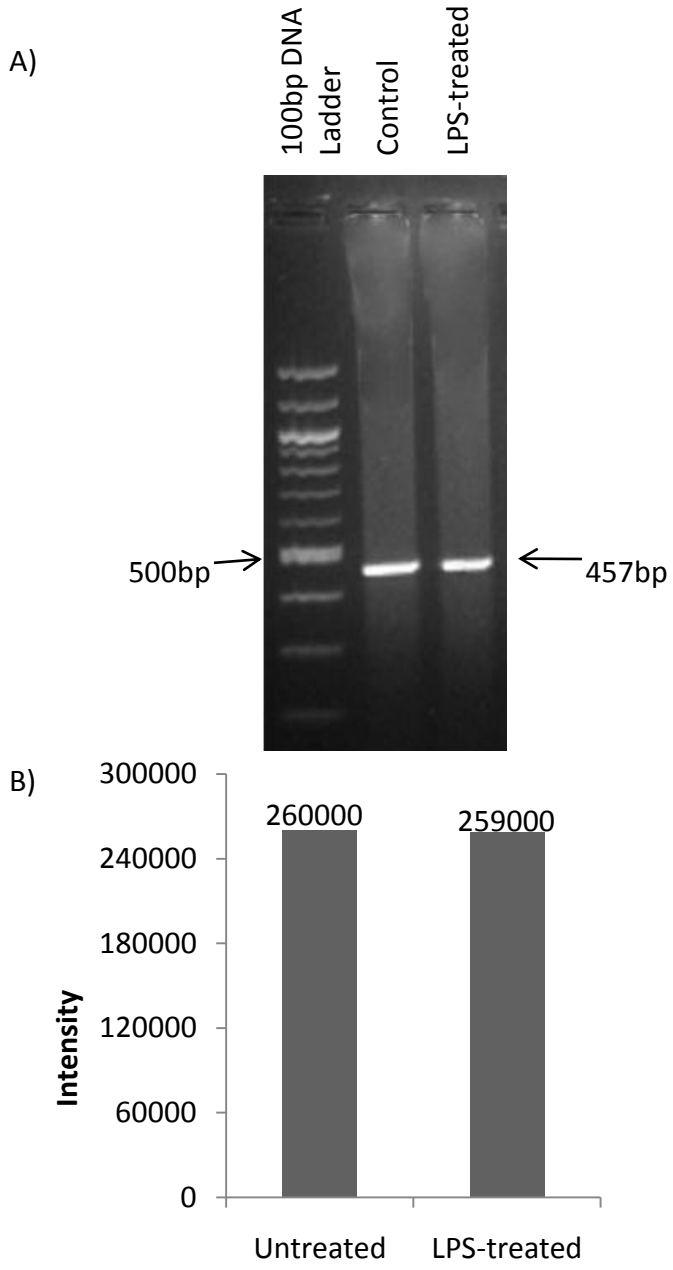


Figure 4.15: Effect of LPS treatment on the expression of GAPDH molecule in MH-S cells. cDNA was prepared by reverse transcribing 1.5µg of RNA isolated from untreated and LPS treated MH-S cell lines and amplified for 20 number of cycles at 55°C. The PCR products were then run on 1.8% agarose gel, visualized under UV in Gel DOC System (BIORAD) as shown in figure A. The bands thus obtained were quantitated by using the Quantity One Software (BIORAD) and intensity plotted as Bar Graph as shown in figure B.

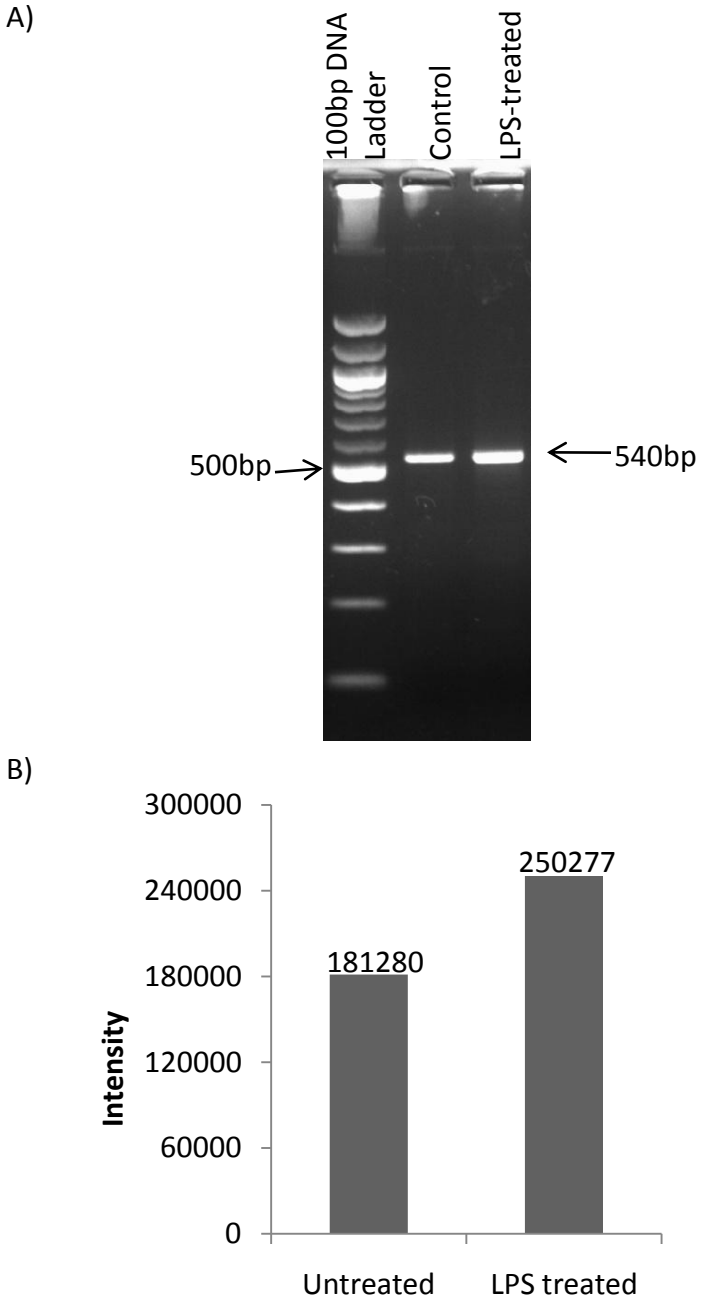


Figure 4.16 : Effect of LPS treatment on the expression of MHC II molecule in MH-S cells. cDNA was prepared byreverse transcribing 2µg of RNA isolated from untreated and LPS treated MH-S cell lines and amplified for 30 number of cycles at 53°C. The PCR products were then run on 1.8% agarose gel, visualized under UV in Gel DOC System (BIORAD) as shown in figure A. The bands thus obtained were quantitated by using the Quantity One Software (BIORAD) and intensity plotted as Bar Graph as shown in figure B.

4.4 Standardization of real time PCR for studying modulation of expression of SNAREs

Real-time polymerase chain reaction (PCR) is a powerful tool to quantitate gene expression which is now routinely used in molecular biology to study low abundance gene expression. It offers high sensitivity, good reproducibility and wide quantification range. Real-time PCR is easy to perform, provides the necessary accuracy and produces reliable as well as rapid quantification results. Real-time PCR has found immense application in studying relative gene expression, where the expression of a target gene is normalized by a non-regulated reference gene (endogenous control). In order to quantitate the levels of specific mRNAs transcripts and to compare the level of expression of the same gene under different experimental conditions, standardization of real time PCR is necessary. Dilution of cDNA defines one of the important parameter to be standardized as the Cycle-Threshold (C_T) value of individual molecule depends on it. The C_T is the numeric value for the number of cycle at which the product fluorescence crosses the threshold. Ideally this C_T value should be within the range of 10 and 30 cycles. Molecule with lower C_T values indicates abundance of mRNA of that gene and vice versa. In order to obtain the C_T within this range standardization for the concentration of cDNA was done. In order to check the efficiency of the primers, melting curve analysis of primers was performed.

4.4.1 Designation of Real Time PCR primers

Primers were designed for the molecules for various SNAREs proteins using Primer-BLAST, Oligo Calc, MultAlign and Sequence editor tools. The list of primers along with their melting temperature (Tm), GC content and amplicon size are shown in the table4.2.

Table 4.2: List of Primers for Real Time PCR

S.N	Name of molecules	Primers Forward primer (FP) Reverse primer (RP)	Primer length (bp)	Melting Tempreature Tm°C	GC content	Product length (bp)
1.	SNAP-23	FP: 5'-TCAGGATGCAGGAATCAAGACC-3' RP: 5'-TGGTCCATGCCTTCTTCTATGC-3'	22 22	60 °C 60°C	50 50	81
2.	VAMP3	FP: 5'-GGTGGAAGAACTGCAAGATGTG-3' RP: 5'-AGAGACACACCACACGATGATG-3'	22 22	59 °C 60°C	50 50	83
3.	Syntaxin4	FP: 5'-AATGCAGTCCGAATACCGAGAG-3' RP: 5'-GTCAGACACCATTCCAGCATTG-3'	22 22	60 °C 59°C	50 50	82
4.	GAPDH	FP: 5'-TCTTCCAGGTATCCTCTCTTGTC-3' RP: 5'-CCTGAATGGA GAGATCTCTG C-3'	23 21	60 °C 60°C	55 55	82
5.	MHC II	FP: 5'-CTTGAACAGCCCAATGTCGTCA-3' RP:5'-CTGAGCAGACCAGAGTGTTGT-3'	22 21	60 °C 60 °C	55 55	82

4.4.2 Confirmation of primer specificity

Melting curve analysis was performed which resulted in a single and product specific melting temperature. No primer-dimers as well as non-specific products were generated during the real-time PCR amplification cycles (Figure-4.17).

4.4.3 Standardization of amount of cDNA

The conc. of cDNA used while performing real time PCR is a crucial parameter. In order to get the optimal conc. of cDNA, its standardization was done by diluting the amount of cDNA. Prior to each quantitative Real Time-PCR, cDNA was prepared using 1µg of RNA. Thus obtained cDNA was diluted and tested for optimal C_T value. After performing various dilutions, 1:4 dilution of cDNA was found to be the optimal for all the molecules studied. And, this dilution of the cDNA was further used to do quantitative real time PCR of LPS treated and untreated MH-S cell lines.

4.4.4 Real Time Polymerase Chain Reaction and RQ value

cDNA gererated were utilized for performing Real Time-PCR with gene specific primer (given in table no.4.2) in 7500 Fast Real Time PCR System using SYBR green universal PCR master mixture from G Biosciences as per the instructions of the supplier. Prior to each quantitative Real Time-PCR, cDNA was prepared using 1µg of RNA. Thus obtained cDNA was diluted in 1:4 ratios. For all experiments, cDNA of the untreated cells and treated cells assayed for the expression of GAPDH, which showed no modulation in the expression in the two samples. The expression of other molecules i.e. SNAP-23, Syntaxin4 and VAMP3 was normalized using this endogenous control (GAPDH) and the RQ (Relative Quantification) value was generated by the software (7500 fast) against a scale of 1 taken for the untreated expression. Expression of MHC class II molecule in presence of LPS was taken as the positive control.

4.4.5 Studies on the modulation of SNAREs expression on MH-S cell line in presence of LPS

After standardization of the concentration of cDNA for the molecules involved in SNARE machinery, the optimal conditions for SNAP-23, Syntaxin4 and VAMP3 molecules were used to study the effect of LPS on mRNA level expression in MH-S cell lines. As seen with RT-PCR method, real-time PCR also revealed little or no modulation of SNAP-23, Syntaxin4 and VAMP3 (Figure-4.20, 4.21, 4.22). MHC II was used as the positive control which showed the upregulation on treatment with LPS (Figure-4.23B) whereas GAPDH was a endogenous control whose level remained the same (Figure-4.23A).

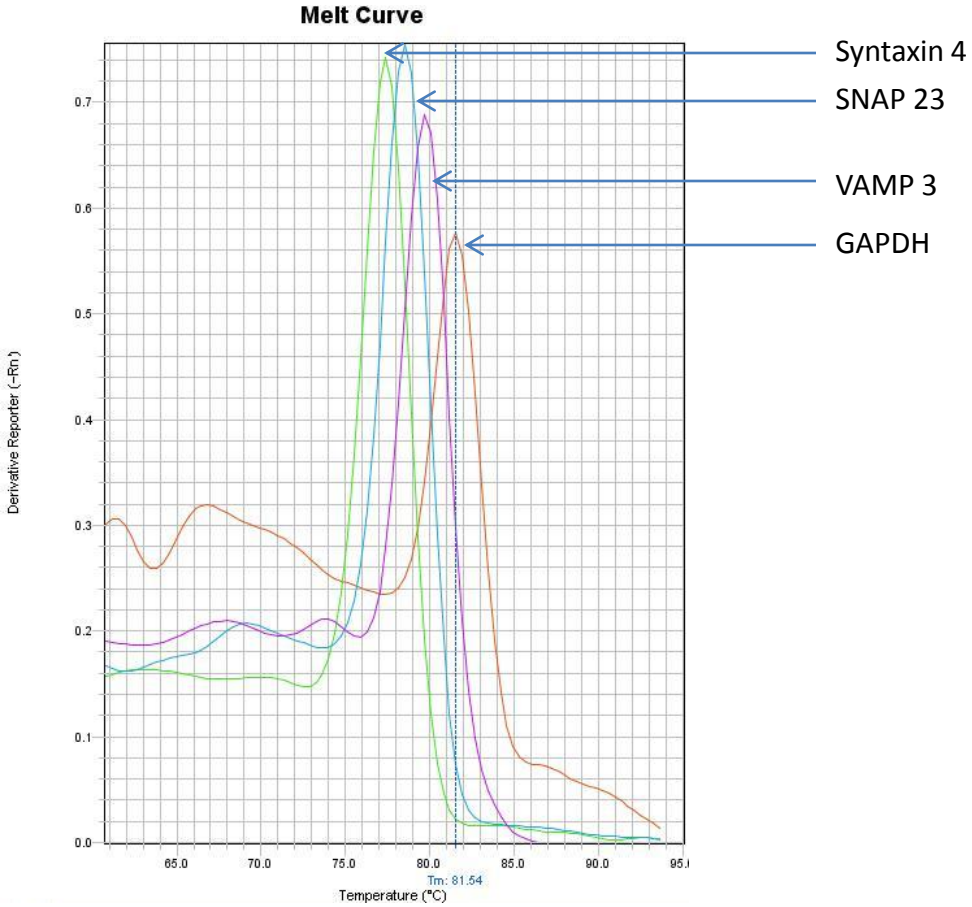


Figure 4.17: Melting curve analysis to check the specificity of the primers in MH-S cells. 1µg of RNA was converted into cDNA from control and LPS-treated MH-S cells. Thus obtained cDNA was diluted in 1:4 dilution and the 4 µl of cDNA and 0.5µl of forward primer and 0.5µl of reverse primer was used to perform the Real-time PCR . The melting curve was then obtained in 7500 Fast Real-time PCR system as shown in the figure above.

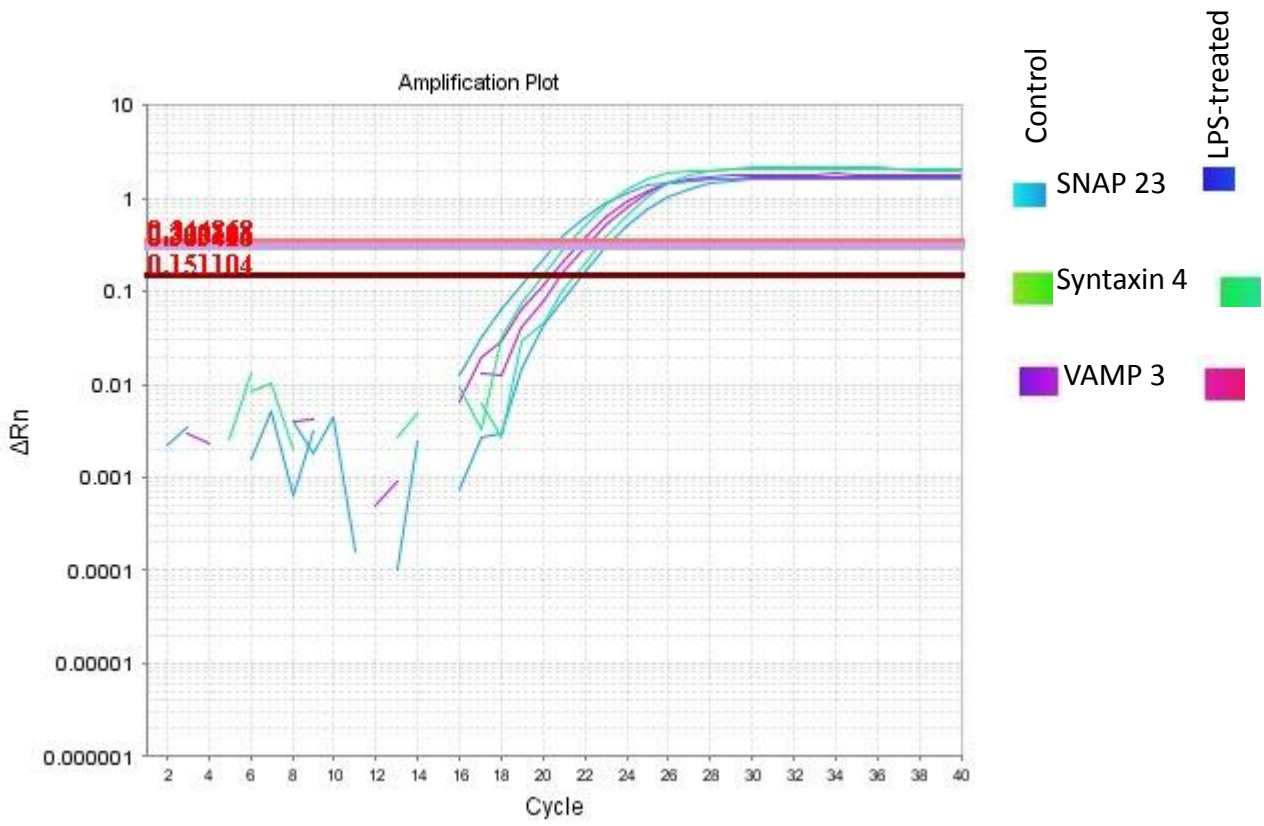


Figure 4.18: **Amplification plot showing the corresponding C_T values of SNAP-23, Syntaxin4 and VAMP3 in Control and LPS-treated MH-S cells.** 1µg of RNA was converted into cDNA from control and LPS-treated MH-S cells. Thus obtained cDNA was diluted in 1:4 dilution and the 4 µl of cDNA was used to perform the Real-time PCR . The amplification plot was then obtained in 7500 Fast Real-time PCR system as shown in the figure above.

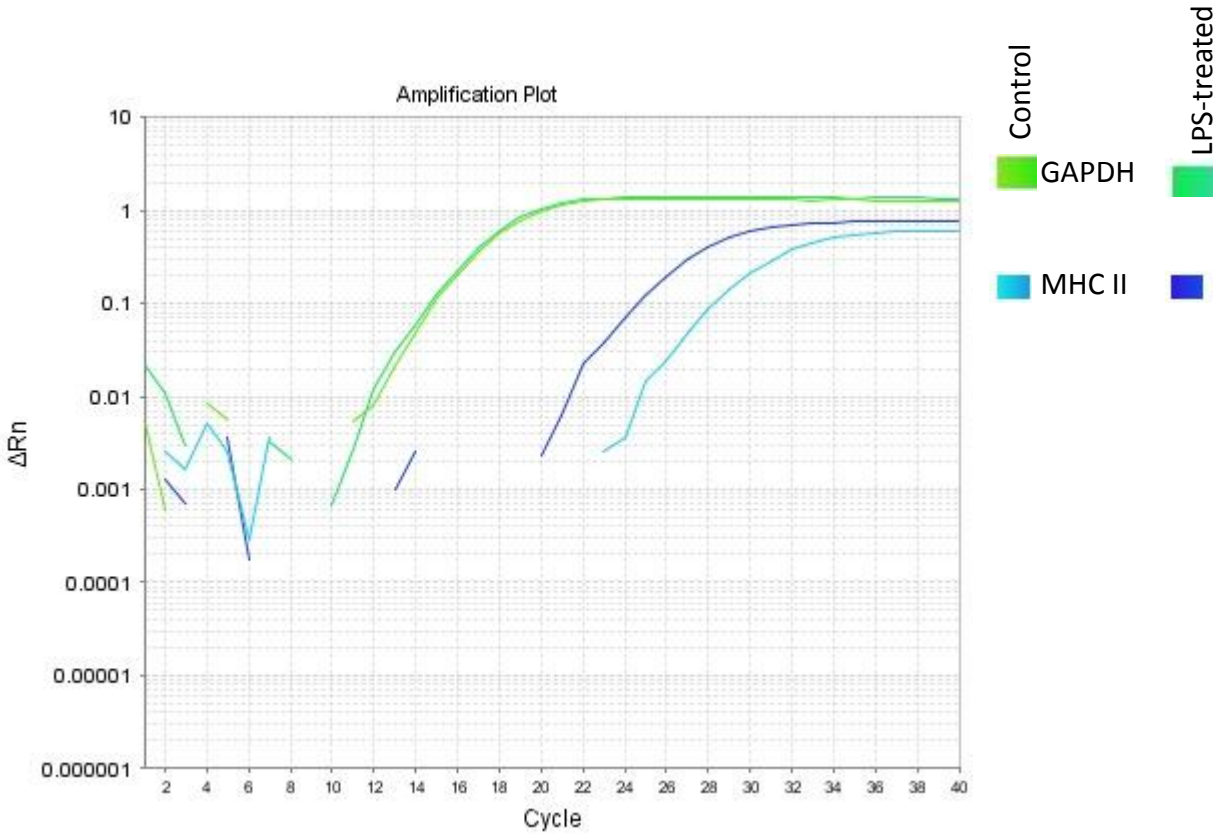


Figure 4.19: **Amplification plot showing the corresponding C_T values of GAPDH and MHC II in Control and LPS-treated MH-S cells.** 1µg of RNA was converted into cDNA from control and LPS-treated MH-S cells. Thus obtained cDNA was diluted in 1:4 dilution and the 4 µl of cDNA was used to perform the Real-time PCR . The amplification plot was then obtained in 7500 Fast Real-time PCR system as shown in the figure above.

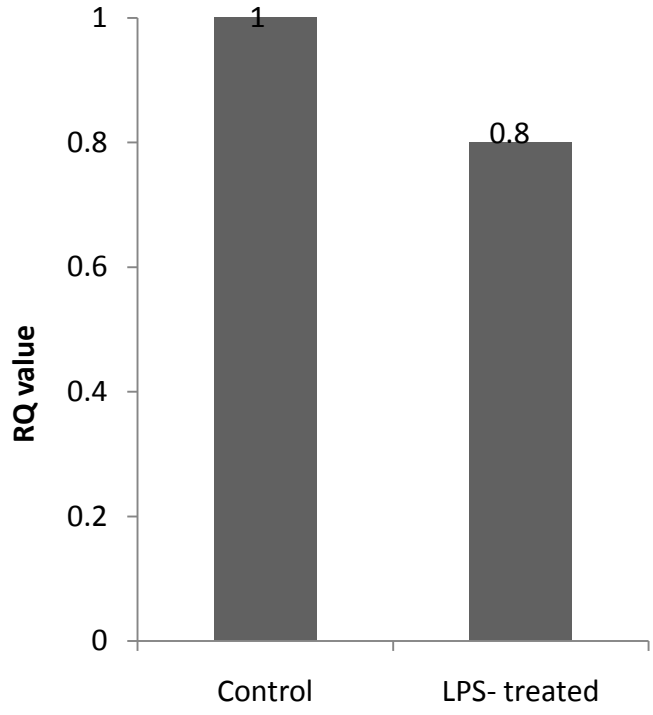


Figure 4.20 : RQ value of SNAP-23 molecule in presence of LPS in MH-S cell lines. cDNA was prepared from 1µg of RNA from untreated and LPS treated MH-S cell lines. Real-time PCR was carried out for this molecule using 1:4 diluted cDNA with SYBR green reaction mixture in 96 well real-time PCR plates and analyzed using 7500 fast software. The RQ value obtained was plotted as Bar Graph as shown in the figure above.

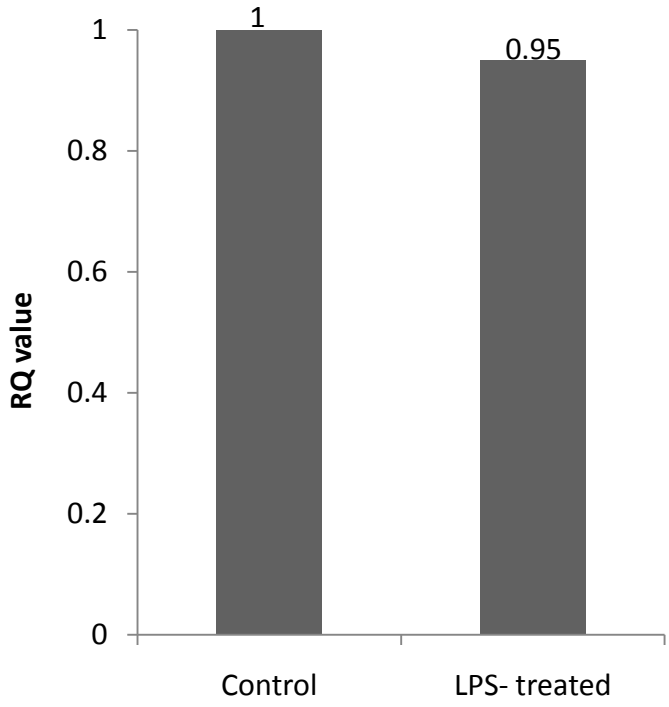


Figure 4.21 : RQ value of Syntaxin4 molecules in presence of LPS in MH-S cell lines. cDNA was prepared from 1µg of RNA from untreated and LPS treated MH-S cell lines. Real-time PCR was carried out for this molecule using 1:4 diluted cDNA with SYBR green reaction mixture in 96 well real-time PCR plates and analyzed using 7500 fast software. The RQ value obtained was plotted as Bar Graph as shown in the figure above.

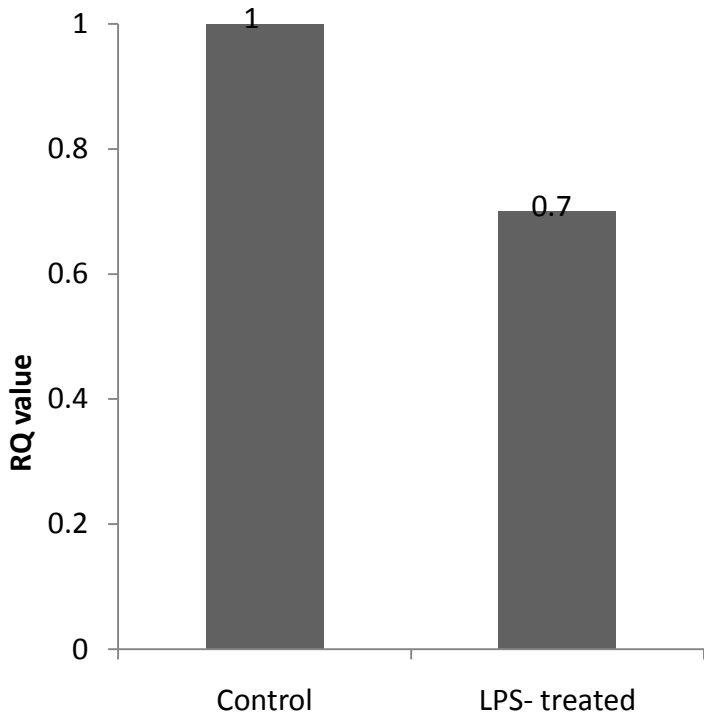


Figure 4.22: RQ value of VAMP3 molecule in presence of LPS in MH-S cell lines. cDNA was prepared from 1µg of RNA from untreated and LPS treated MH-S cell lines. Real-time PCR was carried out for this molecule using 1:4 diluted cDNA with SYBR green reaction mixture in 96 well real-time PCR plates and analyzed using 7500 fast software. The RQ value obtained was plotted as Bar Graph as shown in the figure above.

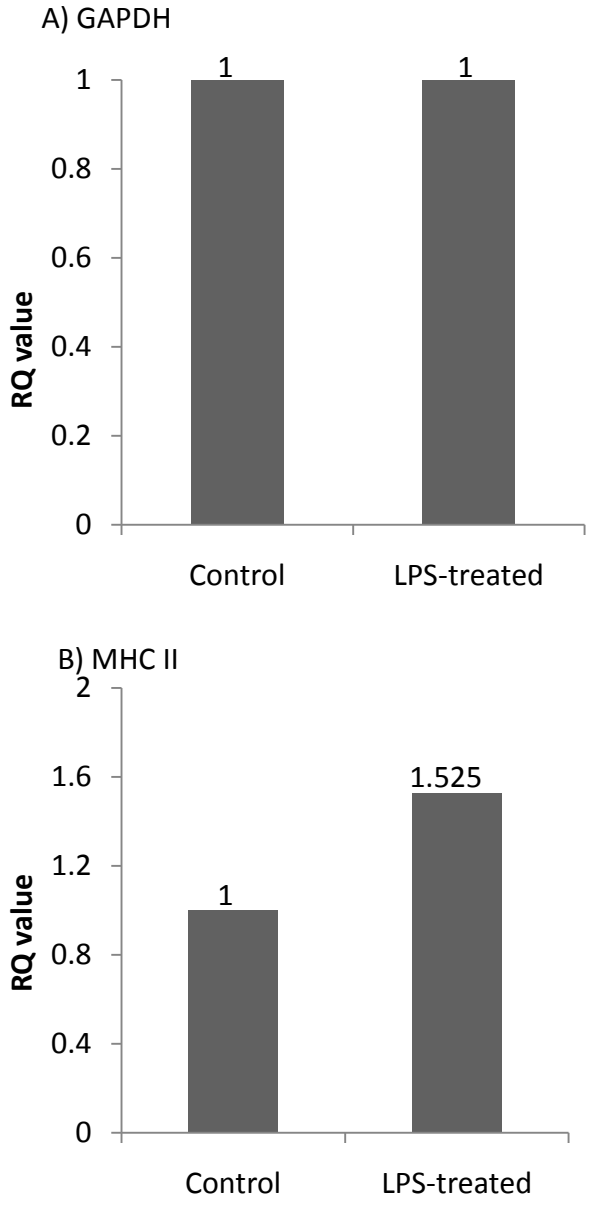


Figure 4.23 : RQ value of GAPDH and MHC II molecules in presence of LPS in MH-S cell lines. cDNA was prepared from 1µg of RNA from untreated and LPS treated MH-S cell lines. Real-time PCR was carried out for individual molecules using 1:4 diluted cDNA with SYBR green reaction mixture in 96 well real-time PCR plates and analyzed using 7500 fast software. The RQ value obtained was plotted as Bar Graph as shown in the figure above.

4.5 Studies on modulation of expression of SNAREs on MH-S by Western Blotting

Western blotting is an analytical method wherein a protein sample is electrophoresed on an SDS-PAGE and electrotransferred onto PVDF membrane. The transferred protein is detected using specific primary antibody and secondary enzyme labeled antibody and substrate. Thus the amount and modulation in expression of the desired protein can be characterized from a complex mixture (e.g. cell lysates) of proteins by western blotting followed by densitometric quantitation of specific bands.

Expression of SNAREs (SNAP-23 and Syntaxin4) was studied on MH-S cell lines by western blotting. Figure-4.24 shows the expression of SNAP23 and figure-4.25 shows the expression of Syntaxin4 on both untreated MH-S cells as well as LPS treated MH-S cells. MH-S cells were cultured overnight in RPMI media constituted with 10% FBS in 5% CO_2 incubator. Cells were then cultured for next 24 hrs with or without LPS. Thereafter cells were harvested and cell lysates were prepared and the expression of the selected protein was done by western blotting. There was no change in the expression of both SNAP-23 and Syntaxin4 in LPS treated condition with respect to that of untreated conditions. β - actin acts as the endogenous control in the experiment. It was shown that there was no change in its expression in untreated and LPS treated condition as shown in the figure 4.26.

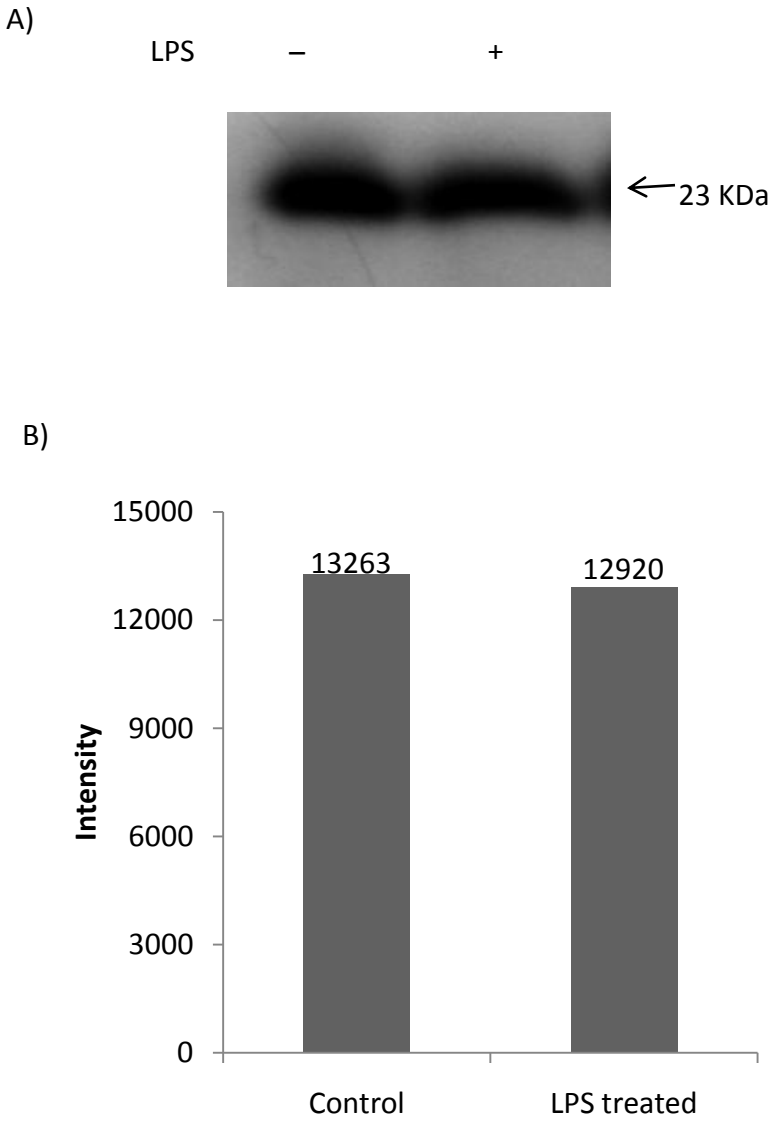


Figure 4.24: Western blotting to show the regulation of SNAP-23 in presence of LPS in MH-S. Cell lysates were run on SDS-PAGE. The bands were transferred on PVDF membrane and labelled with primary and secondary antibody and finally luminiscence were captured on X-ray film. The X-ray film were then analyzed in GelDOC system (BIORAD) as shown in figure A. The quantitation results were plotted as Bar Graph as shown in figure B.

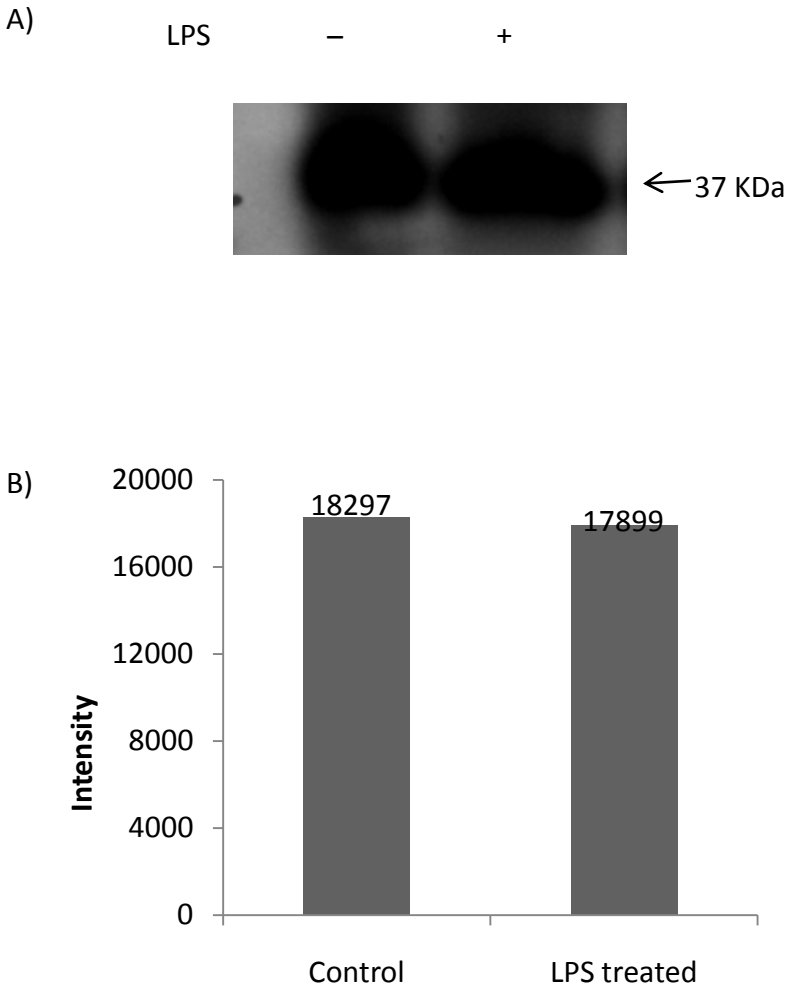


Figure 4.25 : Western blotting to show the regulation of Syntaxin4 in presence of LPS in MH-S. Cell lysates were run on SDS-PAGE. The bands were transferred on PVDF membrane and labelled with primary and secondary antibody and finally luminiscence were captured on X-ray film. The X-ray film were then analyzed in GelDOC system (BIORAD) as shown in figure A. The quantitation results were plotted as Bar Graph as shown in figure B.

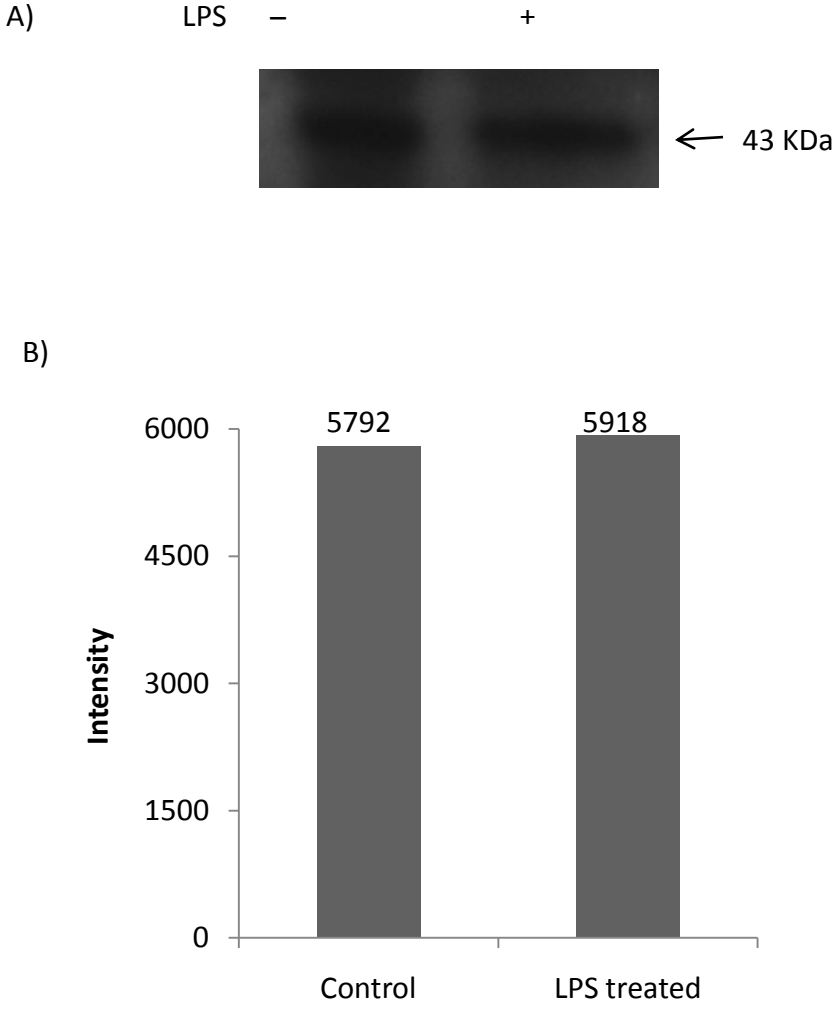


Figure 4.26 : Western blotting to show the regulation of β-actin in presence of LPS in MH-S. Cell lysates were run on SDS-PAGE. The bands were transferred on PVDF membrane and labelled with primary and secondary antibody and finally luminiscence were captured on X-ray film. The X-ray film were then analyzed in GelDOC system (BIORAD) as shown in figure A. The quantitation results were plotted as Bar as shown in figure B.

CHAPTER V

DISCUSSION

Various bacteria have the ability to enter the human body by different routes and cause diseases. This ability of the bacteria to cause the diseases can be called its pathogenicity. To defend against these pathogens and their toxicity, an intricate method of recognition and response has been developed. Our innate immune system uses TLRs that recognize conserved microbial structures or PAMPS and activate the defense system against them (Akira *et al*., 2001). These events lead to the activation of transcription factor NF-κB. Thus activated NF-κB induces the expression of inflammatory cytokines and many other genes leading to an effective immune response (Takeda and Akira, 2005). TLR4 on innate immune cells recognizes LPS of gram negative bacteria and then starts a whole cascade of induced gene expression and a very specialized immune response to counter the onslaught of these gram negative bacteria.

Antigen processing and presentation by APCs have fundamental role in host immune response. Most of the recent studies involving antigen presentations have been done on dendritic cells, a well known APC. Macrophages in comparision to DCs are numerous, easy to handle and do not have an immature-mature phase to deal with like that DCs. Macrophages cells have important role in antigen processing and presentation to T cells. Macrophages remove microorganisms and other foreign particles by phagocytosis. Phagosomes and lysosomes are the important compartments whose fusion is necessary to form the phagolysosomes. Through membrane-trafficking events mediated by SNARE proteins, phagosomes fuse with lysosomes to form degradative phagolysosome (Matheoud et al., 2013). SNARE protein like VAMP3 mediates the delivery of TNF-α and other cytokines at the site of phagocytic cup formation by making the complex with SNAP-23 and Syntaxin4 which are present on plasma membrane. These events show the importance of SNARE proteins in phagolysosome maturation as well as exocytosis of the cytokines which indicate their role in antigen processing and presentation. So, it is presumed that specific SNARE proteins must also be involved in delivery and presentation of MHC or CD1 and processed antigen to APC membrane for display.

In order to better understand the regulation of SNARE proteins during pathogenic challenges especially gram negative bacterial LPS, we exploited quantitative RT-PCR, Real-time PCR method and western blotting as the tool for our study. The RT-PCR method can be used not only to detect specific mRNA but also to quantitate their level. This can be done by quantitating the mRNA level of gene of interest against an endogenous control, housekeeping gene such as GAPDH and β-actin. Transcription of housekeeping gene is believed to be unaffected by almost

all experimental conditions. By including an endogenous control in the assay, reliability of any RT-PCR experiment can be certified. The signals produced are sensitive in determining the relative expression of the gene in every cycle. The main advantage of using the real time PCR over RT-PCR is that it is more sensitive and can detect the gene at low expression also and the PCR provides the result of every cycle when the reaction is on the process.

Three molecules i.e. SNAP-23, Syntaxin4 and VAMP3 which are crucial for SNARE machinery for antigen presentation were identified by literature mining. A quantitative RT-PCR based method was standardized for different parameters like annealing temperature, number of cycles and amount of RNA. After standardization of these parameters, the modulation of the expression of these SNARE proteins were checked in LPS treated and untreated MH-S cell lines. Little or no modulation of the expression of these SNAREs was observed in LPS treated condition in comparision with that of non treated conditions. In order to validate the result obtained from RT-PCR, Real-time PCR was performed for all these molecules. For this, standardization of Real-time PCR was performed in term of concentration of cDNA. Thereafter the real-time PCR results showed little or no modulation in the expression in all the molecules expression level in LPS treated cells in comparision with non treated MH-S cells. MHC class II was used as the positive control for this study. It was found that there was significant upregulation in expression of MHC class II at mRNA level in presence of LPS in MH-S cells by using both RT-PCR and Real-time PCR (as expected) which validates the results of other molecules studied by this method.

In order to study the expression of SNAP-23 and Syntaxin4 at protein level, western blotting was done. β-actin molecule was used as the endogenous control. At protein level also SNAP-23 and Syntaxin4 showed very marginal or no modulation in presence of LPS in MHS cell lines.

It was observed that at mRNA level as well as at protein level, there was no modulation in the expression of these SNAREs. This is similar to the previous observation of expression analysis of Syntaxi4 (Pagan *et al.*, 2003) in which expression of Syntaxin4 was unchanged after 24 hrs of LPS treatment. It might be due to the reason that LPS might not be playing any significant role in the regulation pathway of these proteins. It can also be said that in these cells, SNAREs are present in the abundance level, so in presence of LPS they do not show any significant effect in their expression though they have role in the delivery of cytokines and TNF-α to the cell surface which are produced against pathogens.

Though expression levels may not change- activation status may change, for example, many SNAREs are activated by post-translational modifications like phosphorylation or they may be activated by removal of inhibitory proteins. Also many times they may be recruited to the right subcellular compartment or membrane microdomain. Therefore, change in expression level

doesn't mean that these SNAREs do not have a role. It may be also possible that other SNAREs may be involved for the expression or other mechanism of activation may be involved.

More studied are required to answer these questions. But atleast we have standardize a reliable method to study up/down regulation of SNAREs in macrophages during Ag challenge and can be utilized to study the details of intracellular molecular mechanisms of Ag processing and presentation in APCs.

CHAPTER VI

CONCLUSION

Cellular immunity depends on the interaction of T-lymphocytes with APCs. Recognition of antigens by T-cells requires uptake, processing and presentation of pathogen-derived antigens on MHC of APCs. While the pathways of antigen processing and presentation in APCs have been defined, not much is known about the molecular mechanisms and regulation of intracellular traffic involved in antigen processing and presentation pathways in response to pathogens.

SNAREs are the proteins that play crucial role in vesicle trafficking in cells and therefore in all immune process involving exocytosis of inflammatory mediatiors and uptake and killing of pathogens, also in transport, expression and downregulation of various receptors. In order to understand the regulation of SNARE proteins during pathogenic challenges especially gram negative bacterial LPS, we exploited quantitative RT-PCR, Real time PCR method and Western blotting as the tool for our study. The RT-PCR method can be used not only to detect specific mRNA but also to quantities their level. Real-time polymerase chain reaction (PCR) is a powerful tool to quantitate gene expression which is now routinely used in molecular biology to study low abundance gene expression. It offers high sensitivity, good reproducibility and wide quantification range. Thus, one can compare levels of transcript in different samples.

Molecules that are crucial for SNARE machinery of antigen processing and presentation were identified by literature mining as SNAP23, Syntaxin4 and VAMP3 and a quantitative RT-PCR based method was standardized in terms of annealing temperature, number of thermocylces and amount of RNA. In the same way Real time PCR method was standardized in term of concentration of cDNA. After standardization of parameters for PCR for molecules involved in antigen presentation by SNARE machinery were used to study the expression level. MHC-II was used as the positive control and GAPDH was used as the endogenous control.

The expression of protein level was observed by performing western blotting. SNAP23 and Syntaxin4 were the molecules whose protein level of expression was observed. β-actin was choosen as the molecules for endogenous control.

It was observed that mRNA level as well as protein level, there was no modulation in the expression of these SNAREs by LPS treatment pointing to some other mode of regulation of SNARE mediated intracellular traffic in response to LPS challenge in macrophages.

This is the preliminary study for the modulation of these SNARE molecules. In order to know their complete regulation and modulation, more studied are required about other SNARE

molecules too which might play the important role for vaccine development process. But atleast we have standardize a reliable method to study up/down regulation of SNAREs in macrophages during Ag challenge and can be utilized to study the details of intracellular molecular mechanisms of Ag processing and presentation in APCs.

REFERENCES

Aderem A and Underhill DM (1999) Mechanisms of Phagocytosis in Macrophages. *Annu Rev Immunol*. **17**:593-623

Akira S, Takeda K and Kaisho T (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol*. **2**(8):675-680

Alexis NE, Lay JC, Zeman K, Bennett WE, Peden DB, Soukup JM, Devlin RB and Becker S (2006) Biological material on inhaled coarse fraction particulate matter activates airway phagocytes *in vivo* in healthy volunteers. *J Allergy Clin Immunol*. **117**(6):1396-1403

Amessou M, Fradagrada A, Falguieres T, Lord JM, Smith DC, Roberts LM, Lamaze C and Johannes L (2007) Syntaxin 16 and syntaxin 5 are required for efficient retrograde transport of several exogenous and endogenous cargo proteins. *J Cell Sci*. **120**(8):1457-1468

Anderson MS and Miller J (1992) Invariant chain can function as a chaperone protein for class II major histocompatibility complex molecules. *Proc Natl Acad Sci USA*. **89**(6):2282-2286

Armant MA and Fenton MJ (2002) Toll-like receptors: a family of pattern-recognition receptors in mammals. *Genome Biol*. **3**(8):REVIEWS3011

Banchereau J and Steinman RM (1998) Dendritic cells and the control of immunity. *Nature*. **392**: 245-252

Barlowe C, Orci L, Yeung T, Hosobuchi M, Hamamoto S, Salama N, Resach MF, Ravazzola M, Amherdt M and Schekman R (1994) COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell*. **77**(6):895-907

Barral DC and Brenner MB (2007) CD1 antigen presentation: how it works. *Nat Rev Immunol*. **7**(12): 929-941

Barral P, Polzella P, Bruckbauer A, Van Rooijen N, Besra GS, Cerundolo V and Batista FD (2010) CD169(+) macrophages present lipid antigens to mediate early activation of *i*NKT cells in lymph nodes. *Nat Immunol*. **11**(4): 303-312

Bartee E, Mansouri M, Hovey Nerenberg BT, Gouveia K and Fruh K (2004) Downregulation of major histocompatibility complex class I by human ubiquitin ligases related to viral immune evasion proteins. *J Virol*. **78**(3): 1109-1120

Beers C, Burich A, Kleijmeer MJ, Griffith JM, Wong P and Rudensky AY (2005) Cathepsin S controls MHC Class II-Mediated Antigen Presentation by Epithelial Cells In Vivo. *J Immunol*. **174**: 1205-1212

Bock JB, Klumperman J, Davanger S and Scheller RH (1997) Syntaxin 6 functions in trans-Golgi network vesicle trafficking. *Mol Biol Cell*. **8**(7):1261-1271

Bock JB, Matern HT, Peden AA and Scheller RH (2001) A genomic perspective on membrane compartment organization. *Nature*. **409**(6822):839-841

Bodmer H, Viville S, Benoist C and Mathis D (1994) Diversity of Endogenous Epitopes Bound to MHC Class II Molecules Limited by Invariant Chain. *Science.* **263**: 1284-1286

Brode S, Macary PA (2004) Cross-presentation: dendritic cells and macrophages bite off more than they can chew!. *Immunology.* **112**: 345-351

Chen YA and Scheller RH (2001) SNARE-mediated membrane fusion. *Nat Rev Mol Cell Biol*. **2**(2):98-106

Chernomordik LV, Melikyan GB, Chizmadzhev YA (1987) Biomembrane fusion: a new concept derived from model studies using two interacting planar lipid bilayers. *Biochim Biophys Acta*. **906**(3):309-352

Cresswell P (1996) Invariant chain structure and MHC class II function. *Cell*. **84**(4):505-507

Curtsinger JM, Schmidt CS, Mondino A, Lins DC, Kedl RM, Jenkins MK and Mescher MF (1999) Inflammatory Cytokines Provide a Third Signal for Activation of Naïve CD4+ and CD8+ T cells. *J Immunol*. **162**:3256-3262

Dascher C, Matteson J and Balch WE (1994) Syntaxin 5 regulates endoplasmic reticulum to Golgi transport. *J Biol Chem*. **269**(47):29363-29366

Denzin LK and Cressweli P (1995) HLA-DM Induces CLIP Dissociation from MHC Class II αβ Dimers and Facilitates Peptide Loading. *Cell*. **82**:155-165

Denzin LK, Fallas JL, Prendes M and Yi W (2005) Right place, right time, right peptide: DO keeps DM focused. *Immunol Rev*. **207**:279-292

Eckl-Dorna J and Batista FD (2009) BCR-mediated uptake of antigen linked TLR9 ligand stimulates B-cell proliferation and antigen-specific plasma cell formation. *Blood*. **113**:3969-3977

Eklund S and Tomkinson B (2012) Structure, Function and Evolution of a Gaint Enzyme, Tripeptidyl-peptidase II. Nova Science Publishers, Inc. Uppsala, Sweden. 55-70 pp

Fairfax KA, Kallies A, Nutt SL and Tarlinton DM (2008) Plasma cell development: from B-cell subsets to long-term survival niches. *Semin Immunol*. **20**(1):49-58

Fasshauer D, Sutton RB, Brunger AT and Jahn R (1998) Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc Natl Acad Sci USA*. **95**(26):15781-6

Fernandez I, Ubach J, Dulubavo I, Zhang X, Sudhof TC and Rizo J (1998) Three-dimensional structure of an evolutionarily conserved N-terminal domain of syntaxin 1A. *Cell*. **94**(6):841-849

Finlay BB and McFadden G (2006) Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. *Cell*. **124**(4):767-782

Fuentes-Panana EM, Bannish G and Monroe JG (2004) Basal B-cell receptor signaling in B lymphocytes: mechanisms of regulations and role in positive selection, differentiation, and peripheral survival. *Immunol Rev*. **197**: 26-40

Galli SJ, Borregaard N and Wynn TA (2011) Phenotypic and functional plasticity of cells of innate immunity: macro phages, mast cells and neutrophils. *Nat Immunol*. **12**(11):1035-44

Ganley IG, Espinosa E and Pfeffer SR (2008) A syntaxin 10-SNARE complex distinguishes two distinct transport routes from endosomes to the trans-Golgi in human cells. *J Cell Biol*. **180**(1):159-172

Gatti E and Pierre P (2003) Understanding the cell biology of antigen presentation: the dendritic cell contribution. *Curr Opin Cell Biol*. **15**(4): 468-73

Gereke M, Jung S, Buer Jan and Bruder D (2009) Alveolar Type II Epithelial Cells Present Antigen to CD4+ T cells and Induce Foxp3+ Regulatory T cells. *Am J Respir Crit Care Med* **179**: 344-355

Gordon S and Taylor PR (2005) Monocyte and Macrophage heterogeneity. *Nat Rev Immunol*. **5**:953-964

Gou Z, Turner C and Castle D (1998) Relocation of the t-SNARE SNAP-23 from lamellipodia-like cell surface projections regulates compound exocytosis in mast cells. *Cell*. **94**(4):537-548

Hackam DJ, Rotstein OD, Bennett MK, Klip A, Grinstein S and Manolson MF (1996) Characterization and subcellular localization of target membrane soluble NSF attachment protein receptors (t-SNAREs) in macrophages. Syntaxin 2, 3, and 4 are present on phagosomal membrane. *J Immunol*. **156**(11): 4377-4383

Hagiwara N, Kadono N, Miyazaki T, Maekubo K and Hirai Y (2013) Extracellular syntaxin4 triggers the differentiation program in teratocarcinoma F9 cells that impacts cell adhesion properties. *Cell Tissue Res*. **354**(2):581-591

Hammer JA 3rd and Wu XS (2002) Rabs grab motors: defining the connections between Rab GTPases and motor proteins. *Cur Opin Cell Biol*. **14**(1):69-75

Hanson PI, Roth R, Morisaki H, Jahn R and Heuser JE (1997) Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell*. **90**(3):523-535

Hatsuzawa K, Tamura T, Hashimoto H, Hashimoto H, Yokoya S, Miura M, Nagaya H and Wada I (2006) Involvement of syntaxin 18, an endoplasmic reticulum (ER)-localized SNARE protein, in ER-mediated phagocytosis. *Mol Biol Cell*. **17**(9):3964-3977

Hofmann MW, Honinq S, Rodionov D, Dobberstein B, von Fiquara K and Bakke O (1999) The leucine-based sorting motifs in the cytoplasmic domain of the invariant chain are

recognized by the clathrin adaptors AP1 and AP2 and their medium chains. *J Biol Chem*. **274**(51):36153-36158

Holt M, Varogueaux F, Wiederhold K, Takamori S, Urlaub H, Fasshauer D and Jahn R (2006) Identification of SNAP-47, a novel Qbc-SNARE with ubiquitous expression. *J Biol Chem*. **281**(25):17076-17083

Hou WS and Van Parijs L (2004) A Bcl-2 dependent molecular timer regulates the lifespan and immunogenicity of dendritic cells. *Nat Immunol*. **5**(6):583-589

Hsieh CS, deRoos P, Honey K, Beers C and Rudensky AY (2002) A Role for Cathepsin and Cathepsin S in Peptide Generation for MHC Class II Presentation. *J Immunol*. **168**:2618-2625

Hulpke S and Tampe R (2013) The MHC I loading complex: a multitasking machinery in adaptive immunity. *Trends Biochem Sci*. **38**(8):412-420

Jahn R, Lang T and Sudhof TC (2003) Membrane fusion. *Cell*. **112**(4):519-533

Jensen PE (2007) Recent advances in antigen processing and presentation. *Nat Immunol*. **8**(10):1041-1048

Joffre OP, Segura E, Savina A and Amigorena S (2012) Cross-presentation by dendritic cells. *Nat Rev Immunol*. **12**(8):557-569

Kawai T and Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol*. **11**(5):373-384

Koopmann JO, Albrinq J, Huter E, Bulbuc N, Spee P, Neefjes J, Hammerling GJ and Momburq F (2000) Export of antigenic peptides from the endoplasmic reticulum intersects with retrograde proein translocation through the Sec61p channel. *Immunity*. **13**(1):117-127

Koppelman B, Neefjes JJ, de Vries JE and de Waal Malefyt R (1997) Interleukin-10 down-regulates MHC class II alphabeta peptide complexes at the plasma membrane of monocytes by affecting arrival and recycling. *Immunity*. **7**(6):861-871

Kunte A, Zhang W, Paduraru C, Veerapen N, Cox LR, Besra GS and Cresswell P (2013) Endoplasmic Reticulum Glycoprotein Quality Control Regulates CD1d Assembly and CD1d-mediated Antigen Presentation. *J. Biol. Chem*. **288**: 16391-16402

Kushwah R and Hu J (2011) Complexity of dendritic cell subsets and their function in the host immune system. *Immunology*. **133**: 409-419

Lacy P and Stow JL (2011) cytokine release from innate immune cells: association with diverse membrane trafficking pathways. *Blood*. **118**(1):9-18

Lazar T, Gotte M and Gallwitz D (1997) Vesicular transport: how many Ypt/Rab-GTPases make a eukaryotic cell? *Trends Biochem Sci*. **22**(12):468-472

Letourneur F, Gaynor EC, Hennecke S, Demolliere C, Duden R, Emr SD, Riezman H and Cosson P (1994) Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell*. **79**(7):1199-1207

Levy JA (2001) The important of the innate immune system in controlling HIV infection and disease. *Trends Immunol*. **22**(6):312-316

Lin RC and Scheller RH (2000) Mechanisms of synaptic vesicle exocytosis. *Annu Rev Cell Dev Biol*. **16**:19-49

Liu YJ, Kanzler H, Soumelis V and Gilliet M (2001) Dendritic cell lineage, plasticity and cross-regulation. *Nat Immunol*. **2**(7): 585-589

Lizee G, Basha G, Tionq J, Julien JP, Tian M, Biron KE and Jefferies WA (2003) Control of dendritic cell cross-presentation by the major histocompatibility complex class I cytoplasmic domain. *Nat Immunol*. **4**(11): 1065-1073

Low SH, Li X, Miura M, Kudo N, Quinones B and Weimbs T (2003) Syntaxin 2 and endobrevin are required for the terminal step of cytokinesis in mammalian cells. *Dev Cell*. **4**(5):753-759

Maddaly R, Pai G, Balaji S, Sivaramakrishnan P, Srinivasan L, Sunder SS and Paul SF (2010) Receptors and signaling mechanisms for B-lymphocyte activation, proliferation and differentiation-insights from both in vivo and in vitro approaches. *FEBS Lett*. **584**(24):4883-4894

Mandon B, Chou CL, Nielsen S and Knepper MA (1996) Syntaxin-4 is localized to the apical plasma membrane of rat renal collecting duct cells: possible role in aquaporin-2 trafficking. *J Clin Invest*. **98**(4):906-913

Maric MA, Taylor MD and Blum JS (1994) Endosomal aspartic proteinases are required for invariant-chain processing. *Proc Natl Acad Sci USA*. **91**(6):2171-2175

Martens S and McMahon HT (2008) Mechanisms of membrane fusion: disparate players and common principles. *Nat Rev Mol Cell Biol*. **9**(7):543-556

Matanis T, Akhmanova A, Wulf P, Del Nery E, Weide T, Stepanova T, Galjart N, Grosveld F, Goud B, De Zeeuw CI, Barnekow A and Hoogenraad CC (2002) Bicaudal-D regulates COPI-independent Golgi-ER transport by recruitingthe dynein-dynactin motor complex. *Nat Cell Biol*. **4**(12):986-992

Matheoud D, Moradin N, Bellemare-Pelletier A, Shio MT, Honq WJ, Olivier M, Gaqnon E, Desjardins M and Descoteaux A (2013) Leishmania evades host immunity by inhibiting antigen cross-presentation through direct cleavage of the SNARE VAMP8. *Cell Host Microbe*. **14**(1):15-25

Mbawuike IN and Herscowitz HB (1989) MH-S, a murine alveolar macrophage cell line: morphological, cytochemical, and functional characteristics. *J Leukoc Biol*. **46**(2):119-127

McMahon HT and Mills IG (2004) COP and clathrin-coated vesicle budding: different pathways, common approaches. *Curr Opin Cell Biol*. **16**(4):379-391

McMahon HT, Ushkaryov YA, Edelmann L, Link E, Binz T, Niemann H, Jahn R and Sudoff TC (1993) Cellubrevin is a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein. *Nature*. **364**(6435):346-349

Merzouqui N, Kratzer R, Saveanu L and van Endert P (2011) A proteasome-dependent, TAP-independent pathway for cross-presentation of phagocytosed antigen. *EMBO Rep*. **12**(12): 1257-1264

Misura KM, Scheller RH and Weis WI (2000) Three-dimensional Structure of the neuronal-Sec1-syntaxin 1a complex. *Nature*. **404**(6776):355-362

Mollinedo F and Lazo PA (1997) Identification of two isoforms of the vesicle- membrane fusion protein SNAP-23 in human neutrophils and HL-60 cells. *Biochem Biophys Res Commun*. **231**(3):808-12

Munford RS (2008) Sensing gram-negative bacterial lipopolysaccharides: a human disease determinant? *Infect Immun*. **76**(2):454-465

Munson M and Novick P (2006) The exocyst deforked, a framework of rods revealed. *Nat Struct Mol Biol*. **13**(7): 577-581

Murphy J, Summer R, Wilson AA, Kotton DN and Fine A (2008) The prolonged life-span of alveolar macrophages. *Am J Respir Cell Mol Biol*. **38**(4):380-385

Murray PJ and Wynn TA (2011) Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol*. **11**(11):723-737

Murray RZ, Kay JG, Sangermani DG and Stow JL (2005) A role for the phagosome in cytokine secretion. *Science*. **310**(5753):1492-1495

Neefjes J, Jonqsma ML, Paul P and Bakke O (2011) Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol*. **11**(12):823-836

Neijssen J, Herberts C, Drijfhout JW, Reits E, Janssen L and Neefjes J (2005) Cross-presentation by intercellular peptide transfer through gap junctions. *Nature*. **434**(7029): 83-88

Nickoloff BJ and Turka LA (1994) Immunological functions of non-professional antigen-presenting cells: new insights from studies of T-cell interactions with keratinocytes. *Immunol Today*. **15**(10): 464-469

Olson AL, Knight JB and Pessin JE (1997) Syntaxin4, VAMP2, and/or VAMP3/cellubrevin are functional target membrane and vesicle SNAP receptors for insulin-stimulated GLUT4 translocation in adipocytes. *Mol Cell Biol*. **17**(5):2425-2435

Owen DJ, Collins BM and Evans PR (2004) Adaptors for clathrin coats: structure and function. *Annu Rev Cell Dev Biol*. **20**:153-191

Oyler GA, Higgins GA, Hart RA, Battenberg E, Billingsley M, Bloom FE and Wilson MC (1989) The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpolulations. *J Cell Biol*. **109**(61):3039-3052

Paduraru C and Cresswell P (2010) CD1d antigen presentation-lipids are the key. *Rom. J. Biochem*. **47**(1): 61-78

Pagan JK, Wylie FG, Joseph S, Widberg C, Bryant NJ, James DE and Stow JL (2003) The t-SNARE Syntaxin 4 Is Regulated during Macrophage Activation to Function in Membrane Traffic and Cytokine Secretion *Curr Biol*. **13**(2):156-160

Parihar A, Eubank TD and Doseff AI (2010) Monocytes and Macrophages Regulate Immunity through Dynamic Networks of Survival and Cell Death. *J Innate Immun*. **2**:204-215

Parkin J and Cohen B (2001) An overview of the immune system. *Lancet.* **357**: 1777-178

Patel O, Pellicci DG, Gras S, Sandoval-Romero ML, Uldrich AP, Mallevaey T, Clarke AJ, Le Nours J, Theodossis A, Cardell SL, Gapin L, Godfrey DI and Rossjohn J (2012) Recognition of CD1d-sulfatide mediated by a type II natural killer T cell antigen receptor. *Nat Immunol*. **13**(9):857-863

Paul P, van den Hoom T, Jonqsma ML, Bakker MJ, Henqeveld R, Janssen L, Cresswell P, Eqan DA, van Ham M, Ten Brinke A, Ovaa H, Beijersberqen RL, Kuijl C and Neefjes J (2011) A Genome-wide multidimensional RNAi screen revels pathways controlling MHC class II presentation. *Cell*. **145**(2): 268-283

Pearse BM (1975) Coated vesicles from pig brain: purification and biochemical characterization. *J Mol Biol*. **97**(1):93-98

Perrotta C, Bizzozero L, Cazzato D, Morlacchi S, Simbari F, Zhang Y, Gulbins E, Bassi MT, Rosa P and Clementi E (2010) Syntaxin 4 is required for acid sphingomyelinase activity and apoptotic function. *J Biol Chem*.**285**(51):40240-40251

Pfeffer SR (1999) Transport-vesicle targeting: tethers before SNAREs. *Nat Cell Biol*. **1**(1):17-22

Pfeffer SR (2001) Rab GTPases: specifying and deciphering organelle identity and function. *Trends Cell Biol*. **11**(12):487-491

Potoff JJ, Issa Z, Manke CW Jr and Jena BP (2008) Ca2+-dimethylphosphate complex formation: providing insight into Ca2+-mediated local dehydration and membrane fusion in cells. *Cell Biol Int*. **32**(4):361-366

Prekeris R, Klumperman J, Chen YA and Scheller RH (1998) Syntaxin 13 mediates cycling of plasma membrane proteins via tubulovesicular recycling endosomes. *J Cell Biol*. **143**(4):957-971

Prekeris R, Yang B, Oorschot V, Klumperman J and Scheller RH (1999) Differential roles of syntaxin 7 and syntaxin 8 in endosomal trafficking. *Mol Biol Cell*. **10**(11):3891-3908

Puri N and Roche PA (2006) Ternary SNARE complexes are enriched in lipid rafts during mast cell exocytosis. *Traffic*. **7**:1482-1494

Puri N, Kruhlak MJ, Whiteheart SW and Roche PA (2003) Mast Cell Degranulation Requires N- Ethylmaleimide-Sensitive Factor-Mediated SNARE Disassembly. *J Immunol*. **171**:5345-5352

Puri N, Kruhlak MJ, Whiteheart SW and Roche PA (2003) Mast cell degranulation requires N-ethylmaleimide-sensitive factor-mediated SNARE disassembly. *J Immunol*. 171(10):5345-52

Quinones B, Riento K, Olkkonen VM, Hardy S and Bennett MK (1999) Syntaxin 2 splice variants exhibit differential expression patterns, biochemical properties and subcellular localizations. *J Cell Sci*. **112**(23):4291-4304

Raiborq C and Stenmark H (2009) The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature*. **458**(7237):445-452

Ravichandran V, Chawla A and Roche PA (1996) Identification of a novel syntaxin-and synaptobrevin/VAMP-binding protein, SNAP-23, expressed in non-neural tissues. *J Biol Chem*. **271**(23):13300-3

Reits EA, Vos JC, Gromme M and Neefjes J (2000) The major substrates for TAP in vivo are derived from newly synthesized proteins. *Nature*. **404**(6779):774-778

Rocha N, Kuijl C, van der KantR, Janssen L, Houben D, Janssen H, Zwart W and Neefjes J (2009) Cholesterol sensor ORP1L contacts the ER protein VAP to control Rab7-RILP-p150 Glued and late endosome positioning. *J cell Biol*. **185** (7): 1209-1225

Rock KL and Shen L (2005) Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunological Reviews*. **207**: 166-183

Romieu-Mourez R, Francois M, Boivin MN, Stagg J and Galipeau J (2007) Regulation of MHC class II expression and antigen processing in murine and human mesenchymal stromal cells by IFN- gamma, TGF-beta, and cell density. *J Immunol*. **179**(3):1549-1558

Rothman JE (1994) Mechanisms of intracellular protein transport. *Nature*. **372**:55-63

Rybin V, Rubino M, Alexandrov K, Simon I, Seabra MC, Goody R and Zerial M (1996) GTPase activity of Rab5 acts as a timer for endocytic membrane fusion. *Nature*. **383**(6597):266-269

Sakurai C, Hashimoto H, Nakanishi H, Arai S, Wada Y, Sun-Wada GH, Wada I and Hatsuzawa K (2012) SNAP-23 regulates phagosome formation and maturation in macrophages. *Mol Biol Cell*. **23**(24):4849-4863

Sallusto F, Cella M, Danieli C and Lanzavecchia A (1995) Dendritic Cells Use Macropinocytosis and the Mannose Receptor to Concentrate Macromolecules in the Major Histocompatibility Complex Class II Compartment: Downregulation by cytokines and Bacterial Products. *J. Exp. Med*. **182**: 389-400

Saric T, Beninqa J, Graef CI, Akopian TN, Rock KL and Goldberg AL (2001) Major histocompatibility complex class I-presented antigenic peptides are degraded in cytosolic extracts primarily by thimet oligopeptidase. *J Biol Chem*. **276**(39): 36474-36481

Savina A and Amiqorena S (2007) Phagocytosis and antigen presentation in dendritic cells. *Immunol Rev*. **219**:143-156

Short B, Preisinger C, Schaletzky J, Kopajtich R and Barr FA (2002) The Rab6 GTPase regulates recruitment of the dynactin complex to Golgi membranes. *Curr Biol*. **12**(20):1792-1795

Sollner T, Bennett MK, Whiteheart SW, Scheller RH and Rothman JE (1993) A protein assembly-disassembly pathway *in vitro* that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell*. **75**(3):409-418

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P and Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature*. **362**(6418):318-324

Steegmaier M, Oorschot V, Kluperman J and Scheller RH (2000) Syntaxin 17 is abundant in steroidogenic cells and implicated in smooth endoplasmic reticulum membrane dynamics. *Mol Biol Cell*. **11**(8): 2719-2731

Stow JL, Manderson AP and Murray RZ (2006) SNAREing immunity: the role of SNAREs in immune system. *Nat Rev Immunol*. **6**(12):919-929

Sundstrom JB and Ansari AA (1995) Comparative study of the role of professional versus semiprofessional or nonprofessional antigen presenting cells in the rejection of vascularized organ allografts. *Transplant Immunology*. **3**:273-289

Suqita M, Peters PJ and Brenner MB (2000) Pathways for lipid antigen presentation by CD1 molecules: nowhere for intracellular pathogens to hide. *Traffic*. **1**(4):295-300

Sutton RB, Fasshauer D, Jahn R and Brunger AT (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature*. **395**:347-353

Takeda K and Akira S (2005) Toll-like receptors in innate immunity. *Int Immunol*. **17**(1):1-14

Tang BL, Low DY, Tan AE and Hong W (1998) Syntaxin 10: a member of the syntaxin family localized to the trans-Golgi network. *Biochem Biophys Res commun*. **242**(2):345-350

Tang BL, Tan AE, Lim LK, Lee SS, Low DY and Hong W (1998) Syntaxin 12, a member of the syntaxin family localized to the endosome. *J Biol Chem*. **273**(12):6944-6950

Teng FY, Wang Y and Tang BL (2001) The Syntaxins. Genome Biol. 2(11):reviews3012.1-3012.7

Turvey SE and Broide DH (2010) Innate immunity. *J Allergy Clin Immunol*. **125**:24-32

Tze LE, Horikawa K, Domaschenz H, Howard DR, Roots CM, Riqby RJ, Way DA, Ohmura-Hoshino M, Ishido S, Andoniou CE, Deqli-Esposti MA and Goodnow CC (2011) CD8 increases MHC II and CD86 on dendritic cells by opposing IL-10-driven MARCH1-mediated ubiquitination and degradation. *J Exp Med*. **208** (1): 149-165

Valdez AC, Cabaniols JP, Brown MJ and Roche PA (1999) Syntaxin 11 is associated with SNAP-23 on late endosomes and the trans-Golgi netwrk. *J Cell Sci*. **112**(6):845-854

Valledor AF, Borras FE, Cullell-Young M and Celada A (1998) Transcription factors that regulate monocyte/macrophage differentiation. *J Leukoc Biol*. **63**(4): 405-417

Vardjan N, Gabrijel M, Potokar M, Svajqer U, Kreft M, Jeras M, de Pablo Y, Faiz M, Pekny M and Zorec R (2012) IFN-γ-induced increase in the mobility of MHC class II compartments in astrocytes depends on intermediate filaments. *J Neuroinflammation*. **9**:144

Vyas JM, Van der Veen AG and Ploeqh HL (2008) The known unknowns of antigen processing and presentation. *Nat Rev Immunol*. **8**(8):607-618

Walch-Solimena C, Blasi J, Edelmann L, Chapman ER, von Mollard GF and Jahn R (1995) The t-SNAREs syntaxin 1 and SNAP-25 are present on organelles that participate in synaptic vesicle recycling. *J Cell Biol*. **128**(4):637-645

Waters MG and Hughson FM (2000) Membrane Tethering and Fusion in the Secretory and Endocytic Pathways. *Traffic*. **1**:588-597

Waters MG, Clary DO and Rothman JE (1992) A novel 115-kD peripheral membrane protein is required for intercisternal transport in the Golgi Stack. *J Cell Biol*. **118**(5):1015-26

Waters MG, Hughson FM (2000) Membrane tethering and fusion in the secretory and endocytic pathways. *Traffic*. **1**(8):588-97

Weimbs T, Low SH, Chapin SJ, Mostov KE, Bucher P, Hofmann K (1997) A conserved domain is present in different families of vesicular fusion proteins: a new superfamily. *Proc Natl Acad Sci USA*. **9**(7):3046-51

Wilson DW, Wilcox CA, Flynn GC, Chen E, Kuanq WJ, Henzel WJ, Block MR, Ullrich A and Rothman JE (1989) A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature.* **339**(6223):355-359

Win SJ, Ward VK, Dunbar PR, Young SL and Baird MA (2011) Cross-presentation of epitopes on virus-like particles via the MHC I receptor recycling pathway. *Immunology and Cell Biology*. **89**: 681-688

Wong MH, Chapin OC and Johnson MD (2012) LPS-stimulated cytokine production in type I cells is modulated by the rennin-angiotensin system. *Am J Respir Cell Mol Biol*. **46**(5):641-650

Wong SH,Zhang T, Xu Y, Subramaniam VN, Griffiths G and Hong W (1998) Endobrevin, a novel synaptobrevin/ VAMP-like protein preferentially associated with the early endosome. *Mol Biol Cell.* **9**(6):1549-63

York IA, Chanq SC, Saric T, Keys JA, Favreau JM, Goldberg AL and Rock KL (2002) The ER aminopeptidase ERAP1 enhances or linits antigen presentation by trimming epitopes to 8-9 residues. *Nat Immunol*. **3**(12):1177-1184

Zeng Q, Subramaniam VN, Wong SH, Tang BL, Parton RG, Rea S, James DE and Hong W (1998) A novel synaptobrevin/VAMP homologous protein (VAMP5) is increased during in vitro myogenesis and present in the plasma membrane. *Mol Biol Cell*. **9**(9):2423-37

Zerial M and McBride H (2001) Rab proteins as membrane organizers. *Nat Rev Mol cell Biol*. **2**(2):107-117

APPENDICES

- **5X TBE Buffer**

Tris Base	54g
Boric acid	27.5g
0.5 M EDTA (pH 8.0)	20ml
Make final volume upto one litre final pH 8.	

- **RPMI complete medium (1 litre)**

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

- **6X Gel loading dye**

10 mM Tris pH 8
0.03% bromophenol blue
60% Glycerol
60 mM EDTA

- **5X RNA loading buffer**

Saturated aqueous bromophenol blue solution	16µl
500 mM EDTA, pH8	80µl
37% 12.3 M formaldehyde	720µl
Glycerol	12ml
Formamide	3084µl
10x formaldehyde Agrose gel buffer	4ml
Final volume adjusted to 10 ml by adding RNase free water	

- **Phosphate buffer saline (1 litre)**

Nacl	8g
Na2HPO4.2H2O	1.44g
KCl	0.2g
KH2PO4	0.2g
pH was maintained to 7.3 to 7.4 with HCl	

- **TRIZOL Reagent**

  4M Guanidine thiocyanate Phenol

  0.8 M Sodium citrate

  0.5% N-laurosyl-5-acrosine

  0.1 M B-mercaptoethanol

- **10X formaldehyde Agarose gel buffer**

  200mM MOPS

  50 mM sodium acetate

  10 mM EDTA

  pH adjusted to 7 by NaoH

- **1X formaldehyde Agarose running buffer (1 litre)**

10X formaldehyde Agarose gel buffer	100ml
37% (12.3M) formaldehyde	20ml
Rnase free water	880ml

Western Blot and SDS-PAGE Solutions:

Use autoclaved MQ to prepare all solutions.

- **2X sample Buffer:(**A. take glycerol and water, boil then add SDS and upper gel buffer. B. Prepare 2% PBS in water (0.2g/10ml**)**

  20ml glycerol
  25ml upper gel buffer 4X
  4 gram SDS
  200ul of 2% Bromophenol Blue
  Make upto 100ml with MQ, then add 2% final working conc. β-ME as needed while making 1X.(store at RT)

- **4X lower gel buffer**

  90.8g Tris
  2g SDS
  Make 500ml. pH-8.8 with HCL

- **4X upper Gel buffer**

  30.3g Tris
  2g SDS
  Make 500ml. pH -6.8 with HCL

- **30% acrylamide:**

  29 gram acrylamide
  1g bis-acrylamide

Dissolve un 100ml MQ in dark. Filter with Whatman filter paper, Cover with foil and store at 4c.

- **APS:**

  10% (100mg in 1 ml)
  Aliquet 200ul/vial
  Store at -20c.

- **10X Gel Running Buffer (single use):**

	For 1lit:
0.25M Tris	30.25g Tris
1.92 M Glycine	144g Glycine
1%SDS	10g SDS

pH-8.3

- **Transfer Buffer (Single Use):**

	For 1 liter
10X Gel Running buffer	100ml
Methanol	200ml
MQ	700ml

prepare 1 to 1.5ml for 1 tank a day before use and store in cold room . Store the leftover at 4°c if required .

- **Cell lysis buffer**

50 mM Tris-HCl, pH 7.4	50 ml
150 mM NaCl	8.76 g
1% Triton X-100	10 ml
0.5% Sodium deoxylcholate	5 g
0.1% SDS	1 g
1 mM EDTA	2 ml
10 mM NaF	0.42 g

Add ddH_2O to make final volume of 1000 ml

Add PMSF to a final concentration of 1 mM and any other protease inhibitors immediately before use.