INTERACTION OF MACROPHAGES AND EPITHELIAL CELLS WITH BACTERIA AND BACTERIA DERIVED PAMPs

M.Sc. Thesis

2020

Submitted to:

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Submitted by:

Rachita Gautam

Exam Roll No. 414/073

TU Registration No. 5-3-28-231-2016

Supervisors:

Prof. Rajani Malla, Ph.D.

Date: ……

Recommendation

This is to certify that the research work entitled "**INTERACTION OF MACROPHAGES AND EPITHELIAL CELLS WITH BACTERIA AND BACTERIA DERIVED PAMPs**" has been carried out by Ms. Rachita Gautam under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is her original findings. I, hereby, recommend this thesis for final evaluation.

……..

Prof. Rajani Malla, Ph. D.

(Supervisor)

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Date: …….

Certificate of Evaluation

This is to certify that this thesis entitled "**INTERACTION OF MACROPHAGES AND EPITHELIAL CELLS WITH BACTERIA AND BACETRIA DERIVED PAMPs**" presented to evaluation committee by Ms. Rachita Gautam is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

……

Prof. Krishna Das Manandhar, Ph. D.
Head of Department
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

……..

Dr. Sameer Mani Acharya Dixit
External Examiner
Director of Research for Public Health
Centre for Molecular Dynamics Nepal
Kathmandu, Nepal

……

Dr. Smita Shrestha
Internal Examiner
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

……

Prof. Rajani Malla, Ph. D
Supervisor
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to my supervisor **Dr. Rajani Malla**, Professor, Central Department of Biotechnology, Tribhuvan University for the continuous support, encouragement, strength during my master study and as for her valuable recommendation to carry out my thesis in Jawaharlal Nehru University.

I would like to show my sincere gratitude and thankfulness to my supervisor **Dr. Niti Puri**, Assistant Professor, School of Life Sciences, Jawaharlal Nehru University for providing me an opportunity to join her lab as a trainee where I completed my entire project work successfully. Her guidance helped me in all the time of my dissertation project and writing of the thesis. It was a great privilege to work under her supervision.

I am highly indebted to my senior lab mates **Rahul Srivastava**, **Rangati Didi**, **Deeksha**, **Suchhanda Didi** and **Dirender sir** for their cooperation and valuable suggestions.

I am indeed thankful to our laboratory assistants **Mr. Anwar Ali** and **Sachin Bhaiya** for their day to day work and providing working requirements during the period.

Last but not the least a big thank you to **Anupam Poudel**, **Suleman, Mahima, my family** and **my dearest friends** for continuously supporting and guiding me throughout the period. I feel very lucky to have them by my side during this period.

ACRONYMS

µg	Microgram
µl	Microliter
µM	Micromolar
mg	Milligram
mV	Millivolt
mins	Minutes
Hr	Hour
LPS	Lipopolysaccharide
DMSO	Dimethyl Sulphoxide
EDTA	Ethylenediamine Tetra Acetic Acid
FACS	Fluorescence Activated Cell Sorter
FBS	Fetal Bovine Serum
HEPES	N-2-Hydroxyethylpiperazine-N'-2- ethanesulfonic-acid
Th	T helper cells
IFN	Interferon
IL	Interleukin
MHC	Major Histocompatibility Complex
TLR	Toll-like Receptor
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
RPMI	Roswell Park Memorial Institute
SEM	Standard Errors of the Mean

LIST OF FIGURES

LIST OF TABLES

Table of Contents

ABSTRACT

The innate defense system plays an essential role in protecting organism that is in direct contact with infectious agents. Pulmonary epithelial cells and macrophages are innate immune cells that early recognize the pathogens and their products as they are in direct contact with the ambient environment. These cells recognize bacteria and their products and provide an important first step to initiate a protective immune response mainly by producing various immune effector responses such as cytokines and chemokines (Diamond et al., 2015; Ganz, 2002; P. Zhang, Summer, Bagby, & Nelson, 2000). LPS, a major component of outer membrane of gram negative bacteria is a potent activator of cells of epithelial and macrophage lineage. The induction of LPS to these cells leads to a variety of responses including the synthesis and secretion of the cytokines. Hence, we aimed to characterize the effects of bacteria and bacteria derived PAMPs in two cell lines epithelial LA-4 and macrophage MH-S. The time dependent responses of cytokine expression to LPS stimulation of concentration 100 ng were examined at the transcriptional levels in these cells since cytokine secretion is an early event in the innate host response that alerts the immune system to the presence of a microbial pathogen. In this study, we examined the differences and similarities of both the cell lines on the exposure to bacteria and bacteria derived PAMPs to gain a better understanding of their contributions to immunity.

CHAPTER 1 INTRODUCTION

The innate defense system plays an essential role in protecting organism that is in direct contact with infectious agents. Pulmonary epithelial cells and macrophages are innate immune cells that early recognize the pathogens and their products as they are in direct contact with the ambient environment. These cells recognize bacteria and their products and provide an important first step to initiate a protective immune response mainly producing various immune effector responses such as cytokines and chemokines. LPS, a major component of outer membrane of gram negative bacteria is a potent activator of cells of epithelial and macrophage lineage. The induction of LPS to these cells leads to a variety of responses including the synthesis and secretion of the cytokines. Hence, we aimed to characterize the effects of bacteria and bacteria derived PAMPs in two cell lines epithelial LA-4 and macrophage MH-S. In this study, we examined the differences and similarities of both the cell lines on the exposure to bacteria and bacteria derived PAMPs to gain a better understanding of their contributions to immunity.

1.1 Objectives

- To study the expression of the various cytokines in response to LPS by both the cell lines
- To study the cell recovery of both LA-4 and MH-S cells treated with the bacteria *E coli* as well as the recovery of the bacteria *E coli*
- To visualize the interaction of both the cell lines with the bacteria *Bacillus subtilis*

Chapter 2 LITERATURE REVIEW

2.1 Innate immune system

The immune system is a host defense system that consists of structures and processes within the body which is capable of destroying pathogenic organisms such as bacteria, fungi, viruses, and parasites through a series of effector mechanisms. The system consists of two types of responses: an innate immune response and an adaptive immune response. An innate immunity is a non-specific natural and constitutive host defense mechanism that responds to the invasion and does not require time for the induction. It can be categorized into non- cellular and cellular defenses. Non-cellular defenses include differences in susceptibility to certain pathogens, anatomical defense, tissue bactericides, including complement and microbial antagonism whereas cellular defenses include inflammation and phagocytosis.

The innate immune system is composed mainly of physical barriers, such as skin and mucous membranes, lining of respiratory tract, mouth, stomach and intestinal tract, urogenital tract and eyes, chemical barriers, through the action of antimicrobial peptides and reactive oxygen species, innate immune cells, and soluble mediators such as the complement system, innate antibodies, and associated cytokines (Todar, 2019).

2.2 Function of innate immune system

The main purpose of the innate immune system is: (1) to prevent the entry of pathogens into the body through physical and chemical barriers; (2) to avoid the spread of infections through the complement system and other humoral factors; (3) to remove pathogens through phagocytosis and cytotoxicity mechanisms; and (4) to activate the adaptive immune system through the synthesis of several cytokines and antigen presentation to T and B cells (Carrillo, García, Coronado, García, & Cordero, 2017).

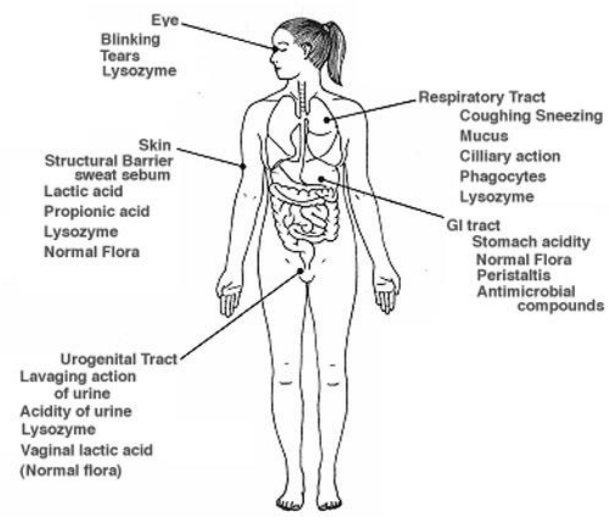


Figure 2.1: Anatomical defenses associated with tissue surfaces (Todar, 2019)

2.3 Innate immune system cells

The cells of the innate immune system include non-myeloid cells, myeloid cells and some lymphoid cells. These cells have several functions that are necessary for defense against the invading pathogens. Some of the cells form physical barriers by expressing various PRRs which recognize PAMPs and DAMPs, in turn, producing inflammatory cytokines that impede infections (Carrillo, García, Coronado, García, & Cordero, 2017).

2.3.1 Non-myeloid cells

The non-myeloid cells of the innate immune system include epithelial cells, fibroblasts, etc. that form a barrier between the external and internal environment (Carrillo, García, Coronado, García, & Cordero, 2017).

2.3.1.1 Epithelial surfaces of the body are the first defenses against infection

Epithelial tissues provide a mechanical barrier to pathogen entry between the outside linings of the organs and outside exposed surfaces of the body. These tissues, therefore, inevitably serve as a first line of defense against the invading pathogens. They play an essential role in host defense as the cells of the epithelia layers are held together by tight junctions to form a seal against the external environment. Epithelia comprise most of the body surfaces such as skin (Stratified epithelia), tubes such as gastrointestional, respiratory and urinogenital tracts, cavities and ducts such as exocrine glands. When the barrier function is breached, as in the case of wounds and burns, entry of pathogens occurs. In absence of wounding, pathogens normally cross the barrier function to establish an infection by adhering to and colonizing to the surfaces of the epithelium (Charles A Janeway, Travers, Walport, & Shlomchik, 2001).

2.3.1.2 Epithelial barriers: why and how?

In multicellular organisms, the endothelia and epithelia form cellular barriers which are necessary for tissue compartmentalization and performance of the specialized functions (Marchiando, Graham, & Turner, 2010). Epithelia serve as effective barriers to the

external environment due to the propensity of the epithelial cells to elaborate a range of cell-cell adhesion complexes called tight junctions. These complexes are formed by tans membrane and intracellular proteins that link with the cytoskeletal to form a robot sheet-like structure which functions as a selective permeability barrier (Shoichiro Tsukita, Furuse, & Itoh, 2001). To control the barrier properties of the epithelium, these junctions are formed around the whole perimeter of each cell forming a continuous belt like structure. Thus, to regulate the para cellular passage of ions, water and various macromolecules across epithelia, tight junction comes into play (D. E. Davies, 2014a). Tight junction proteins differential expression and properties help to maintain this selective permeability function of the epithelial cells (Shoichiro Tsukita et al., 2001). Claudin proteins are the tight junction proteins comprising of a family of at least 24 proteins which actually come into play to facilitate this function (Krause et al., 2008). Moreover, tight junctions also prevent lateral diffusion and intermixing of the molecules in the apical membrane with those in the lateral membrane which maintains the cell polarity and is an important property of the normal epithelial function (D. E. Davies, 2014a)

The adherens junctions and the desmosomes are located below the tight junctions which link to actin cytoskeleton (Ivanov & Naydenov, 2013; Nelson, 2008) and intermediate filaments (Garrod & Chidgey, 2008) respectively. These are essential for the integrity of the cell layer by providing the adhesive force. They, however, do not directly seal the space between the epithelial cells (D. E. Davies, 2014b).

Adherens junctions are the key regulators of cell proliferation and differentiation (Nawijn, Hackett, Postma, van Oosterhout, & Heijink, 2011). They are also the first junctions to form and are essential for the formation of the other junctions (Ivanov & Naydenov, 2013). Hemi desmosomes (Nievers, Schaapveld, & Sonnenberg, 1999) function to facilitate the attachment of intermediate filaments of the epithelial sheet to the basement membrane. And the basement membrane is essential in maintaining epithelial structure, polarization and compartmentalization (D. E. Davies, 2014b).

Tight junction plaque include many signaling molecules which include protein kinase C, Rho proteins, and phosphatidyl inositol 3 kinase (PI3K), as well as transcription factors, lipid phosphatases, and cell cycle regulators (Balda & Matter, 2009; González-Mariscal, Tapia, & Chamorro, 2008). Thus, tight junctions also play a role in regulating cellular responses, in particular suppression of proliferation (S Tsukita, Yamazaki, Katsuno, Tamura, & Tsukita, 2008) to allow epithelial differentiation in a coordinate manner.

Thus, junctional proteins have important role in maintenance and stability of the epithelium and is essential for the permeability barrier (D. E. Davies, 2014b).

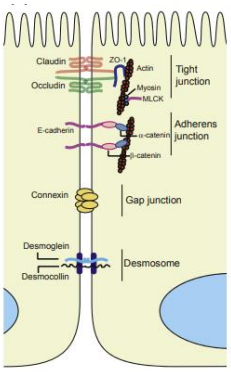


Figure 2.2: Intercellular communication that occurs at various junctional complexes between adjacent epithelial cells. The tight junction is made of several anastomosing strands containing transmembrane proteins that form the functional para cellular barrier. (Edelblum, K. L. et al 2015)

2.3.1.3 Pattern recognition receptors of epithelial cells

Infections are perceived by cells through specific pathogen recognition receptors (PRRs) which detect microbial compounds. The microbial compounds are components of the pathogen surface like lipopolysaccharide (LPS), peptidoglycan, flagellin and also bacterial and viral nucleic acids which are known as pathogen- associated molecular patterns, PAMPs. Other harmful signals may be recognized which are released from stressed or damaged cells known as endogenous danger-associated molecular patterns (DAMPs) (Tang, Kang, Coyne, Zeh, & Lotze, 2012).

Various PAMPs and DAMPs are received by epithelial cells through diverse sets of sensors including Toll like receptors (TLRs), Nucleotide binding oligomerization domain containing proteins(NODs), Dectin-1, Galectins, and retinoic acid-inducible gene1 (RIG-I) (McClure & Massari, 2014).

Toll like receptors (TLRs) are membrane bound PAMPs sensors characterized by an extracellular domain with varying number of leucine rich repeats (LRRs) and a cytoplasmic signaling TIR domain expressed in almost all epithelial cells. TLRs that localize to the plasma membrane are TLR2/1, TLR2/6, TLR4, TLR5 recognize the PAMPs; TLR2/1 and TLR2/6 detects triacetylated lipoproteins from Gram negative bacteria and Gram positive bacteria respectively and TLR4 and TLR5 senses LPS and flagellin respectively. TLRs that localize to endosomes are TLR3, TLR7, TLR8, TLR9 and TLR13 recognize dsRNA; TLR3, ssRNA ; TLR7, TLR 8 , Bacterial and viral CpG motifs; TLR9 and TLR11 recognizes profilin like molecule of *Toxoplasma gondii* (Günther & Seyfert, 2018).

Dectin-1 is found in almost all mucosal epithelial cells. It mediates anti-fungal immunity against the mycobiota. (Dambuza, IM et al, 2015).Nod like receptors (NLRs); NOD1 and NOD2 which are intracellular PRRs are expressed in various epithelial cells. The ligands for NOD1 and NOD2 are γ-D-glutamyl-mesodiaminopimelic acid and muramyl dipeptide respectively (Caruso, R. et al 2014).The family of RIG-I-like receptors (RLRs) in the cytoplasm recognizes Viral RNAs. RIG-1, melanoma-differentiated gene 5 (MDA5), and

DExH-box polypeptide 58 (DHX58; also known as LGP2) which are the three members of this family are all known to be expressed in epithelial cells (Pandey, Kawai, & Akira, 2014).

2.3.1.4 The TLR signaling pathway in general

The engagement of TLRs by the microbial compounds triggers the activation of a number of signal transduction pathways and initiates defensive responses by the host. After binding of the ligand, TLRs dimerize and undergo conformational changes which are required for the recruitment of adaptor molecules containing Toll/interleukin-1 receptor (TIR) domain via the cytoplasmic TIR domain (Akira & Takeda, 2004). There are four adaptor molecules, namely MyD88, TIR-associated protein (TIRAP)/MyD88- adaptor-like (MAL), TIR-domain-containing adaptor protein- inducing IFN-beta (TRIF)/TIR-domain-containing molecule 1 (TICAM1) (Oshiumi, Matsumoto, Funami, Akazawa, & Seya, 2003; Yamamoto et al., 2003, and TRIF-related adaptor molecule (TRAM). The distinct TLR ligands mediate differential responses which explain the specific usage of these adaptor molecules. The adaptor proteins MyD88 and TRIF are responsible for the activation of distinct signaling pathways, leading to the production of pro-inflammatory cytokines and type I IFNs, respectively (Diamond, Khameneh, Brough, & Mortellaro, 2015).

2.3.1.5 Immune response to the entry of a pathogenic microorganism

PRRs located on the surface of and within the epithelial cells come in contact with a pathogenic microorganism as the organism is passing through the epithelial layer. The interaction of PRRs and PAMPs causes the activation of PRRs and leads to the production of chemokines, which, in turn attract immune cells of the innate immunity such as neutrophils and monocytes that are transformed into macrophages, mast cells, etc. to the particular region of the tissue. The recruitment of the immune cells to that site results in the formation of subclinical micro focus of inflammation, where the invading pathogenic microorganism is ex terminated. This results in the end of the immune response.

As the invading microorganisms entering the body are sufficiently numerous in number, the innate immune cells are activated via same PRRs. These cells attract the considerable number of cells which are of the same type into the focus. Thus, this result in the increase of the inflammation micro focus and the clinical signs of inflammation appear.

The involvement of adaptive immunity takes place simultaneously. Professional Antigen presenting cells like Dendritic cells activated via TLRs take up and processes the pathogenic microorganism and present their peptide antigen microorganism and present their peptide antigen to its surface in complex with an MHCII molecule to naïve T and B lymphocytes. This leads to the proliferation and differentiation into the definitive effector cells- cytotoxic T lymphocytes or plasma cells producing antibodies respectively which are specific for the given antigen. The differentiation of lymphocytes depends on the set of cytokines which are produced by activated dendritic cells, which, in turn, activate the

corresponding population of T helper cells Th1 and Th2; stimulation of cellular immune response and humoral immune response (Lebedev & Ponyakina, 2006).

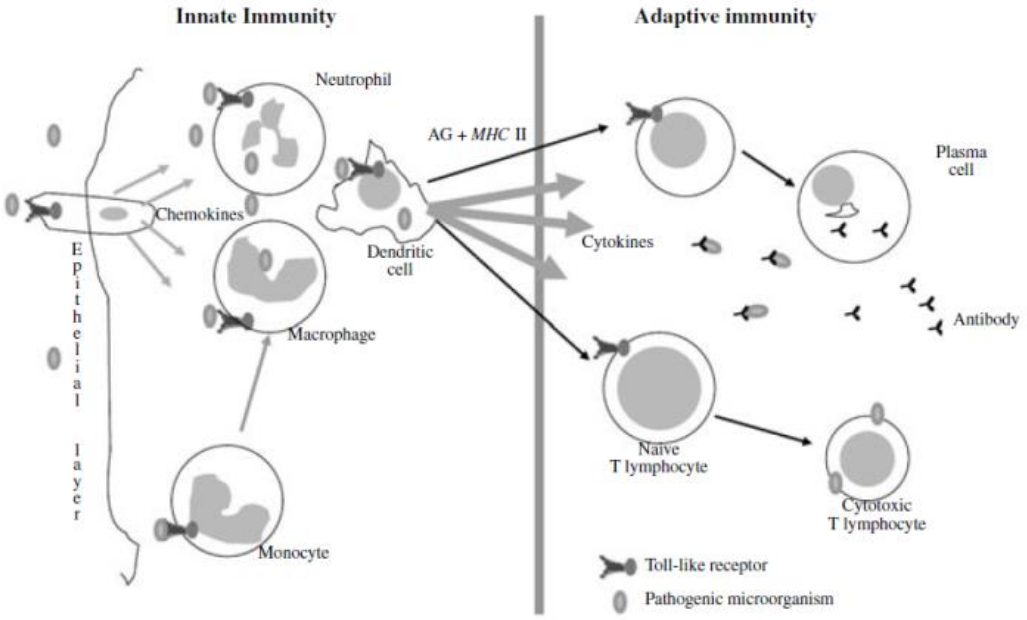


Figure 2.3: Immune response to a pathogenic microorganism entering the body (Lebedev & Ponyakina, 2006)

2.3.1.6 Immunophysiological factors produced by epithelial cells and their functional role

Epithelial tissues formed by large sheets of epithelial cells of different cell populations differ morphologically and functionally from one another (Dwinell, M.B et al 2003). All cell types of the epithelium have PRRs, most of which located in the cytoplasm and Golgi apparatus and some of them present on the cell membrane (Ortega-Cava, C.F et al 2003). Almost all types of membrane-bound PRRs known as TLR are present on different types of cells of the epithelial (Strober, W. et al 2004, Melmed, G. et al 2003, Backhed, F. et al 2003, Hausmann, M. et al 2002).

The specific adhesion of a microbial ligand on a PRR signaling causes conformational changes in the internal portion of the receptor which is followed by the transmission of the signal through the cytoplasmic enzymes. These enzymes lead to the activation of the nuclear transcription factors. Upon activation, the synthesis of array of cytokines and activation of all the effector functions characteristic of the type of the epithelial cells take place (Pierre, K.B et al 2003).

Epithelial cells can synthesize anti-inflammatory cytokines which acts as chemokines. These cytokines such as IL-8, GRO-alpha, GRO-beta, GRO-gamma, ENA78 belong to the C-X-C chemokine family (Eckmann, Kagnoff, & Fierer, 1993; Yang, Eckmann, Panja, &

Kagnoff, 1997). They are characterized by the ability to attract polymorph nuclear leukocytes to the site of the inflammation and activate the emigration of the neutrophil through the epithelial layer (Lebedev & Ponyakina, 2006).

In addition to this, cells of the epithelial also express receptors for some cytokines, including INF-gamma, IL1, TNF-alpha, TGF-beta1, IL-12, IL-4, IL-7, and IL-9 (Reinecker & Podolsky, 1995). This indicates that epithelium can respond to the stimuli of the adjacent cells of the mucosa. Epithelial cells stimulation by anti-inflammatory cytokine IL-1 or TNF-alpha leads to the expression of ICAM-1 adhesion receptor which is necessary for the migration of the neutrophil through the epithelial layer (Kelly et al., 1992; Reinecker & Podolsky, 1995; Savidge et al., 1995). This migration is one of the main anti-microbial factors in human body cavities lined with the epithelium.

Epithelial cells can also produce chemokines of C-C family, including MCP-1, VIP-1, MIP-1, RNATES and IP-10 (Jung et al., 1995; Reinecker et al., 1995). These are responsible for the chemotaxis of monocytes/macrophages, eosinophils, mast cells and basophils, as well as some populations of T cells, mainly natural killer cells. This suggests that the epithelium orchestrates the immune response committing various cell population involved in innate and adaptive immunity, if necessary (Lebedev & Ponyakina, 2006).

Prostaglandins E2 and F2 alpha (Eckmann et al., 1993) and nitrogen oxide (NO) (Ribbons et al., 1995) as well as the secretory component are intensely produced when the cells are activated which binds IgA passing through epithelium (Stefanii, D.V et al 1996).

The epithelial layer contain M cells which are the specialized epithelial cells located at the apices of Peyer's patches in the intestine. They take up a variety of antigens-bacteria, viruses, parasites and proteins from the lumen and transport these antigens in complexes with MHCII activated via TLRs to the underlying immune cells, thereby initiating an immune response (Lebedev & Ponyakina, 2006). Epithelial cells can produce MHC II (Reinecker & Podolsky, 1995)specific for lymphoid cells of their own underlying tissue, including Peyer's patches.

Intraepithelial lymphocytes are the first immune cells that reside within skin epithelial cells and mostly between intestinal epithelial cells. Mainly T lymphocytes are found, most of which are cytotoxic (CD8+ cells)(Macdonald & Monteleone, 2005) within many epithelial cells. They destroy the pathogens as epithelial cells cannot perform this function. The peptide antigen products of their destruction lead to activation of intracellular PRRs. They are the antigen that M cells transport into the lymphoid sub-epithelial tissue (Lebedev & Ponyakina, 2006).

A complex of antibacterial substances is actively synthesized by the epithelial cells which are released into the intestinal lumen to suppress microflora. Most of these substances, including lysozyme, lactoferrin, catelicidin (Ganz, 2002) complement proteins, and the

antimicrobial polypeptides defensins are produced by Paneth's granular cells located at the bases of intestinal crypts.

The most important antimicrobial substances produced by the epithelial cells are Defensins. The activation of their production is related to the activation of PRRs on the producing cells. A high concentration of defensins, however, may increase the number of TLRs on the cell and activate them (Bals & Hiemstra, 2004; Kao, Chen, Zhao, & Wu, 2003). Thus, defensins not only suppress microorganism growth and cause their death in lumens lined with the epithelium, but are more likely to activate the immune responses non-specifically via the activation of TLRs on cells involved in innate and adaptive immunity (Lebedev & Ponyakina, 2006).

2.3.1.7 Epithelial cell response induced by LPS

TLR4 is the member of TLR family that recognizes and activates by LPS which is the integral component of the outer membrane in Gram negative bacteria exposed on the cell surface of non-capsulated strains (O'Neill, Golenbock, & Bowie, 2013).

The adaptor protein; myeloid differentiation factor 2 (MD2) (Kuzmich et al., 2017)recognizes the lipophilic part of LPS (Lipid A) and binds non-covalently to TLR4 which form the activated heterodimer (T. L. Gioannini, Teghanemt, Zhang, Levis, & Weiss, 2005; Theresa L. Gioannini et al., 2004; Park et al., 2009). The formation of activated TLR4/MD-2 leads to the dimerization of TLR4 forming heterodimer on the cell surface.

The activation of TLR4 occurs through a series of sequential steps in which LPS is bound by LPS binding proteins (LBP) and transferred to MD-2/TLR4 (Kim & Kim, 2017). The LBP binds a LPS monomer and transfers this molecule to cluster of differentiation 14 (CD14) and are presented to the TLR4-MD2 complex (Ryu et al., 2017). The formation of activated TLR4/MD-2 leads to the dimerization of TLR4 forming heterodimer on the cell surface. The signal pathway activated by TLR4 involve different adaptor proteins which couple to downstream protein kinases that ultimately lead to the activation of transcription factors such as nuclear factor-κB (NF-κB) and members of the interferon (IFN)-regulatory factor (IRF) family (O'Neill et al., 2013). Upon activation, two types of pathway that involve myeloid differentiation primary response protein 88 (MYD88) and TIR domain-containing adaptor protein inducing IFNβ (TRIF) are activated and the subsequent production of the pro-inflammatory cytokines takes place (Lu, Yeh, & Ohashi, 2008).

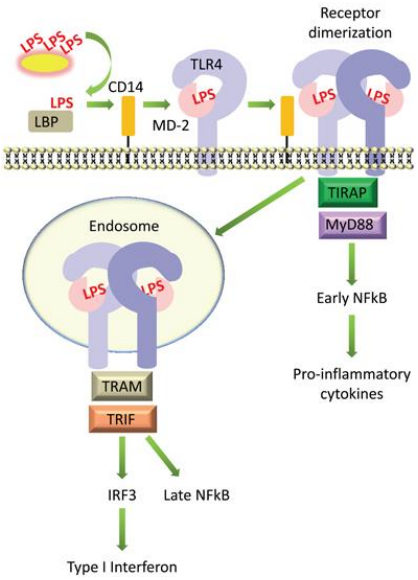


Figure 2.4: TLR4 signaling pathway in response to LPS involves two different pathways MyD88 dependent and independent resulting in the release of pro inflammatory cytokines and Type I Interferon respectively (Diamond, Khameneh, Brough, & Mortellaro, 2015).

2.3.2 Myeloid cells

Myeloid cells of the innate immune system include monocytes, macrophages, dendritic cells (DCs), neutrophils, eosinophils, basophils, mast cells, and platelets. All of these cells have specialized functions that impede infections (Carrillo, García, Coronado, García, & Cordero, 2017).

2.3.2.1 Macrophage

Macrophages also called as mononuclear phagocytes arise from the bone marrow stem cells, which are mobilized into the bloodstream and are differentiated into macrophages in the local tissues mostly at the site of inflammation (Gordon, 1976).Macrophages, however, derived from embryonic yolk sacs maintain in peripheral tissues by self-renewal (Takahashi, Yamamura, & Naito, 1989). Macrophages are considered as an effector cell of the innate immune system where they serve as sentinel cells for the pathogens and orchestrate an appropriate host response.

System of mononuclear phagocytes is referred as the total pool of macrophages. This system is scattered throughout the connective tissue, basement membranes of small blood vessels, liver sinusoids, spleen, lung, bone marrow and lymph nodes. Monocytes from the blood stream migrate into every organ in the body where they mature into fixed macrophages. Macrophages function as scavengers, in the lymph nodes, to remove foreign material from the circulation (Todar, 2019).

Macrophages can be divided into two populations: Resident macrophages and inflammatory macrophages (Raggatt et al., 2014). Resident macrophages are found in almost all tissues and essential for the development, immunological surveillance, homeostasis and tissue repair(L. C. Davies, Jenkins, Allen, & Taylor, 2013; Wynn & Vannella, 2016). Inflammatory macrophages derived from circulatory monocytes and

necessary to rapidly infiltrate tissues which are compromised by injury. Macrophages can be activated and adopt different functions in response to several signals from the microenvironment, and they are classically activated macrophages M1 macrophages and alternatively activated macrophages M2 macrophages(Wynn, TA et al 2013, XQ, W. et al 2016).

Table 2.1: The location and function of some different macrophage populations- (Adapted from (Todar, 2019).

Type of Macrophage	**Location**	**Function**
Alveolar Macrophage	Lung alveoli	Phagocytosis of small particles, dead cells or bacteria. Initiation and control of immunity to respiratory pathogens.
Kupffer cells	Liver	Initiate immune responses, hepatic tissue re modelling.
Microglia	Central Nervous System	Elimination of old or dead neurons and control of immunity in the brain.
Splenic macrophages (marginal zone, metallophilic and red pulp macrophages)	Spleen marginal zone, red pulp and white pulp	Elimination of dysfunctional or old red blood cells.

2.3.2.2 Receptors in macrophage

Macrophages detect the pathogen via a plethora of receptors, engulf them and impede an appropriate host immune response (Aderem, 2003). They constitutively express a variety of scavenger receptors; C-type lectin receptor that include the mannose receptor, dectin-1, DC-SIGN and the soluble receptors mannose binding lectin which facilitates the removal of aged cells, necrosis, or toxic material to maintain tissue homeostasis by recognizing the endogenous ligands (For example: Apoptotic cells) (Gordon & Taylor, 2005).Once they receive a variety of stimuli from tissue injury or infections, they express a large repertoire of different classes of pathogen associated receptors (PRRs), including TLRs, RIG-like helicase (RLH) receptors and Biosensor Nod-like receptors (NLR) that recognize pathogen associated molecular patterns (PAMPs) (For example: microbial products)(Geissmann et al., 2010; Gordon & Taylor, 2005).

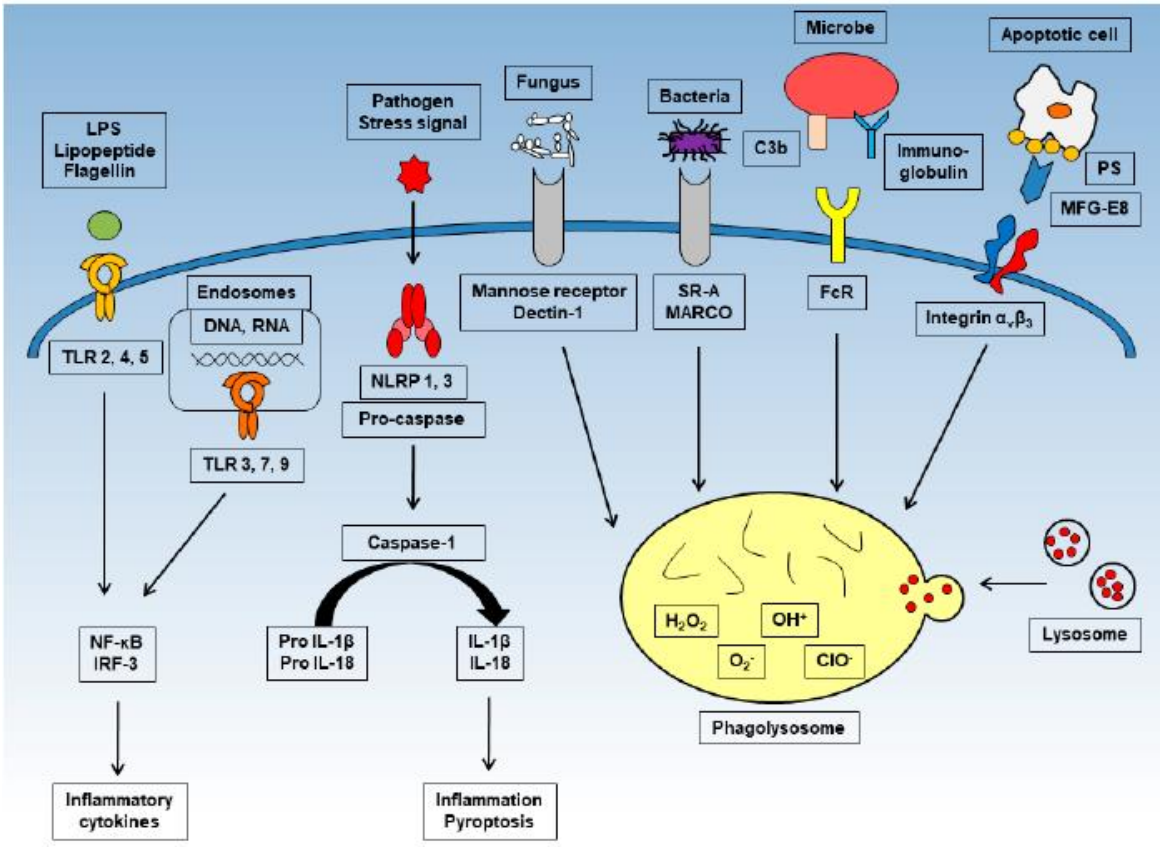


Figure 2.5: Receptors present in the macrophage and inflammation and phagocytic mechanism of Macrophage Adapted from: (Hirayama, Iida, & Nakase, 2017)

2.3.2.3 Phagocytosis

The clearance of the most pathogenic microbes involves the phagocytic cells. Inflammation is initiated to impede the infection once the microorganism penetrates the physical barriers. The neutrophils are dominated in the early (acute) responses within 30 minutes at the sites of the local infection. Macrophages, however, take over in longstanding condition (chronic) condition which is generally observed within 48 hours (begins in 6 hours) (Todar, n.d.-a).

Normally, macrophages phagocytose the material present in their surroundings without being activated. However, some microbial products activate these cells and start an inflammatory process. The local tissue inflammation occurs from the secretory products of the macrophages such as cell differentiation factors, colony stimulation factors, cytotoxic factors, tumor necrosis factor alpha, cachectin, hydrolytic enzymes: collagenase, lipase, phosphatase, endogenous pyrogen, interleukin-1, complement components C1 to C5, properdin factors B, D, I, H, Interferon alpha, plasma proteins, coagulation factors, oxygen metabolites, hydrogen peroxide, superoxide anion, arachidonic acid metabolites, prostaglandins, thromboxanes, leukotrienes, etc.

The released cytokines also in turn activate T cells through antigen presentation. Thus, macrophages play an essential role in the innate immunity by forming a bridge between innate resistant and specific immunity (Todar, 2019).

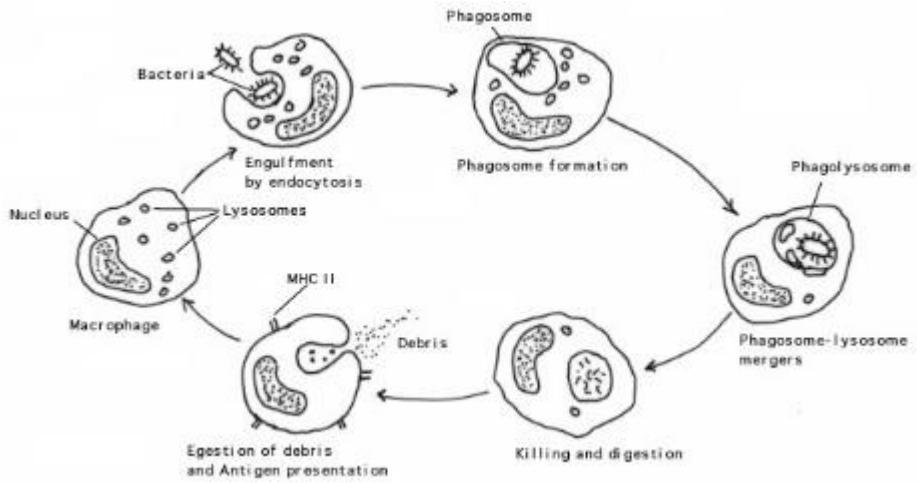


Figure 2.6: The mechanism of the process of phagocytosis by a macrophage (Todar, 2019).

2.3.2.4 Mechanism of phagocytosis

Phagocytosis occurs in the macrophage through actin based mechanism which involves the engagement of the various receptors (Mannose, Ig G, Complement, Fc γ receptors) on the cell surface with the ligand. The binding of the ligand initiate the arrangement of cytoskeleton and membrane trafficking which include the signals that are generated to induce the actin polymerization under the membrane at the site of the contact. The extensions of the actin around the particle caused the membranes fuse behind the particle, in turn pulling the particle toward the center of the cell called the phagosome. Once the particle is internalized, the phagosome matures via a series of membrane fusion and fission events to become phagolysosome, where the foreign particles are enzymatically degraded for antigen presentation through the action of hydrolases. The peptide ligands bind to MHCII molecule and these complexes are presented on the cell surface where it cognate T cell receptor in the process called antigen presentation which results in the activation of specific T cell (Aderem, 2003).

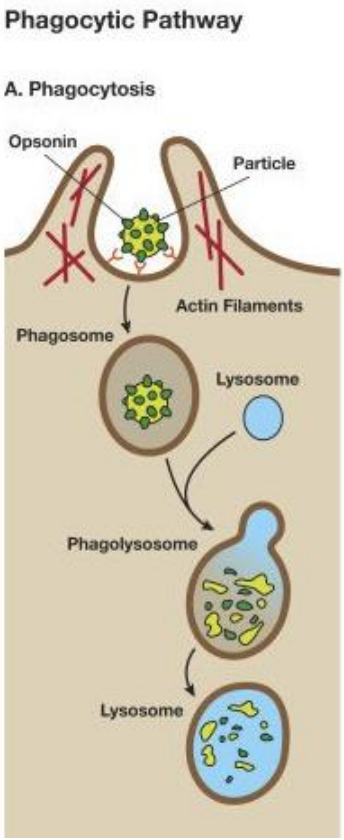


Figure 2.7: The actin dependent mechanism of phagocytosis (Hillaireau & Couvreur, 2009)

Table 2.2: Role of macrophages in the immune system- Adapted from (Janeway, C.A. et al 2002)

Effect	**Functioning mechanism**
Anti-microbial activity	Natural mechanism: Phagocytic killing via oxygen-dependent free radical or oxygen-independent hydrolases Adaptive mechanism: inflammatory reaction following antigen presentation and cytokine (IL-1, IL-6, IL-8, IL-12, THNF alpha) secretion
Lymphocyte activation	Antigen presenting cell function, cytokine secretion
Immune response modulation	Th-1 response: interleukin-12 secretion Th-2 response: interleukin-10 secretion
Tumor immunity	Tumor cell breakdown by toxic factors, free radicals, hydrolases, and TNF alpha secretion
Tissue reorganization	Elastases, collagenases, fibroblast growth factors, and angiogenesis factors secretion

2.3.2.5 Role of macrophage in LPS elimination

Classically activated macrophage differentiation requires the priming signal from IFN gamma. The primed macrophage when encounters a stimulus such as Bacterial LPS, it

becomes classically activated. LPS binds to soluble LBP and then to either soluble or membrane CD14.TlR4 along with MD2 recognize the complex that is delivered by CD14 (C. A. Janeway & Medzhitov, 2002). Pathogens and its components are taken up by the process of phagocytosis (Greenberg & Grinstein, 2002) and are transported to lysosomes to form phagolysosome where they are exposed to variety of degradation enzymes including cathepsin cysteine proteases (Honey & Rudensky, 2003). The peptide antigens are processed and loaded onto MHC II molecule as well as co stimulatory B7 family members are presented to T cells (Harding, Ramachandra, & Wick, 2003). All of these events lead to a significant change in cellular morphology and alternation in the secretory profile of the cell. Several pro-inflammatory cytokines are released including IL-1 beta/IL-1F2, IL-6, and TNF-alpha/TNFSF1A (Duffield, 2003; Gordon & Martinez, 2010; Ma et al., 2003; Mosser, 2003) and a variety of chemokines including IL-8/CXCL8, IP-10/CXCL10, MIP-1 alpha/CCL3, MIP-1 beta/CCL4, and RANTES/CCL5 (Luster, 2002). The release of these molecules is important for the host defense and in the adaptive immune system.

2.4 Epithelial cells barrier dysfunction related diseases

2.4.1 Atopic dermatitis

The cross linkage of different epidermal barrier proteins, including Filaggrin (FLG), transglutaminases (TGs), keratins, loricrin and intracellular proteins form an impermeable skin barrier (Candi, Schmidt, & Melino, 2005; Kalinin, Marekov, & Steinert, 2001). In Atopic Dermatitis, the skin barrier dysfunction is attributed by altering protein and lipid content by the overexpression of cytokines. Also, the dysfunction of the barrier induced by the disruption of stratum corneum, a dense protein-lipid matrix that functions as a barrier to a water loss and allergens and high deficiency of Filaggrin (FLG) which is filament associated epidermal differentiation complex protein that regulate the epidermal homeostasis is found in most of the patients of AD (Candi et al., 2005).

2.4.2 Asthma

In order to maintain the airway homeostasis, an intact functional mucosal barrier plays a crucial role (Schleimer & Berdnikovs, 2017). In most forms of Asthma, epithelium exhibits a dysregulated epithelial barrier. This altered epithelium is characterized by an increase in mucin gene expression and the increase in basal and goblet cells (Ordoñez et al., 2001)and a decrease in terminally differentiated ciliated cells which are frequently accompanied by basement membrane thickening (Hoňková, Uhlík, Beránková, Svobodová, & Pohunek, 2014; Zhou, Yin, Liu, Liu, & Zhao, 2011) which is characterized by increase in the deposition of provisional matrix compoenents such as the glycoproteisn fibronectin, periostin, tenascin-C, hyaluronan, and versican. The disruption of epithelial tight junction and adherens with marked loss of E cadherin(Heijink et al., 2007) and claudin 18 (Sweerus et al., 2017) is typical for asthma.

2.4.3 INFLAMMATORY BOWEL DISEASE

The characteristic feature of Inflammatory Bowel Disease is the loss of the barrier integrity (Chelakkot, Ghim, & Ryu, 2018). IBD including Crohn's disease (CD) and Ulcerative colitis (UC) are characterized by chronic relapsing inflammation (Zhang & Li, 2014)of the distal ileum and/ or colon due to leaky gut in the initial event of pathogenesis allowing bacteria derived molecules into the mucosa and flaring up uncontrolled inflammatory signal cascades (Gerova V. A.2016).

2.4.4 Ulcerative colitis

The barrier of epithelial cells in Ulcerative colitis is impaired by erosion/ulcer-type lesions. The apoptosis of epithelial cells cause the local leaks and the generalized alternation of tight junctions increase the basal permeability (Gitter, Wullstein, Fromm, & Schulzke, 2001).

2.4.5 Crohn's disease

The disruption of epithelial barrier function that is characterized by the alteration in the intestinal permeability allows increase mucosal penetration by toxic luminal antigens. The penetration of antigens triggers immunologic and inflammatory response. These responses produce epithelial injury and further damage the intestinal epithelial barrier function (Gerova V. A.2016).

2.5 Immuno-deficiencies in macrophage function

2.5.1 Defects in macrophage activation

The defects in Macrophage activation interfere with the ability of the immune system to recognize a large repertoire of pathogens which respond to them by becoming activated and initiating an inflammatory response and processing and presenting the antigens to the naïve T cells.

Inherited IFN Gamma receptor deficiency is one of the diseases which have been better studied in at least three families with a history of this defect. It is autosomal recessive and rare. The mutation in *IFNGR1* gene which encodes one of the components of the IFN gamma receptor heterodimer; IFN gamma R1 was identified (Lee, Yin, Verschoor, & Bowdish, 2013)

2.5.2 Defects in PRRs and signaling

Macrophages are able to recognize a broad range of pathogens by binding the PAMPs of these pathogens to PRRs such as TLRs. This binding cause the activation of TLR signaling pathway leading to the recruitment of a number of adaptor and signaling proteins and ultimately the translocation of NF-kB to the nucleus which , in turn, elicit a strong immune response.

Cold infections or the infections that lack inflammation and the resulting fever response are one of the deficiencies in TLR signaling pathways. Primary immune-deficiencies that

affect the signaling components include two proteins- MyD88, IRAK4 which play a role in the initial propagation and amplification of TLR signaling (Lee et al., 2013)

2.5.3 Defects in phagocytosis and bacterial killing

Macrophages are able to engulf the invading pathogens through the process of phagocytosis upon the recognition of the pathogen. They destroy them through the production of ROS and the activation of proteases and other microbial compounds, the process which is known as Respiratory brust. This process is necessary to control the infection.

Defect in the uptake of the bacteria such as defect in actin polymerization that impairs phagocytic vesicle formation is known. Similarly, deficiencies in the respiratory burst that kills the engulfed pathogens is known and tend to result in severely compromised immune function. Chronic Granulomatous disease (CGD) and Chediak-Higashi syndrome are the two common relatively studied respiratory burst defeciencies.

CGD results in the defect of one of the four components of nicotiamide adenine dinucleotide phosphate (gp91phox, p22phox, p47phox and p67phox). A defect in any of these four peptides mostly mutation in gp91phox which is encoded by an X linked gene is the most common form (Lee et al., 2013).

2.5.4 Chronic disease

Recently, a number of diseases including Osteopetrosis, Crohn's disease and some types of cancer are thought to be related to the primary macrophage immunodeficiency. This depicts the role of macrophage in the control and in the maintenance of the critical body functions (Lee et al., 2013).

2.5.4.1 Osteopetrosis

Osteopetrosis is a hereditary disease that results from the dysfunction of the osteoclasts. Osteoclasts are the cells derived from the same precursors as monocytes/ macrophages that resorb cartilage during the remodeling events. A primary immunodeficiency would therefore affect both the cell types (Lee et al., 2013). However, the recent finding showed that monocyte function is unaffected which led to the hypothesis that osteopetrosis should be categorized as a macrophage primary immunodeficiency (Atkins & Findlay, 2012).

2.5.4.2 Crohn's disease

Crohn's is an inflammatory disorder of the bowel which is commonly attributed to the chronic inflammation mediated by the T-lymphocyte (Lee et al., 2013).The primary immunodeficiency of macrophages is considered to be the candidate for the causative agent of CD, recently. The defects in the production of chemotactic cytokines results in the impaired neutrophil recruitment and clearance of the bacteria in response to the tissue infection site. This accumulation of the pathogens results in the phenotype of the chronic inflammation that is clinically associated with CD patients (Korzenik, 2007).

2.5.4.3 Cancer

The studies of single nucleotide polymorphism linkage have identified the association between mutations in *MSR1* which encodes CD204, a member of the class A scavenger receptors of the macrophage and prostate cancer. With a family history of prostate cancer, seven single amino acid substitutions have been identified. Among them, two of these mutations are predicted to result in non-functional copies of Arg293X and Asp174Tyr of CD204. Arg293X results in the truncation of the most of the collagenous domain and the cysteine rich domain and Asp174Tyr result in the inability of CD2014 to form homo-trimer conformation. These suggest that macrophage scavenger receptor deficiency might have a role in the aetiology of the prostate cancer (Xiang et al., 2013).

2.6 *E coli* and its pathogenesis

E coli first isolated from the newborns of feces were described by Theodor Escherich in 1885 as Bacterium coli commune. It was later named as *Escherichia coli* as the bacterium was known to be a commensal organism of the large intestine.

Enterobacteriaceae, the large bacterial family includes enteric bacteria called *E coli*. They are facultative anaerobic Gram negative rods that can grow in the presence or absence of oxygen while under anaerobic conditions they grow by fermentation producing characteristics mixed acids and gas as end products. They can, however, grow by means of anaerobic respiration as it is able to utilize NO_3, NO_2 or fumarate as final electron acceptors for respiratory electron transport processes. Wild type *E coli* have no any growth factor requirements and can metabolically transform glucose into all of the macromolecular components that make up the cells. They are generally well adapted to its characteristics habitats and usually live in the intestinal tracts (anaerobic) of healthy and disease animal. In human gastrointestinal tract, *E coli*are a consistent inhabitant and are predominant facultative organism.

Over 700 antigenic types (serotypes) of *E. coli* are categorized based on O, H, and K antigens. The O antigen is the part of lipopolysaccharide layer, K antigen is the capsular polysaccharide and H antigen is the major component of flagella called flagellin that can elicit an immune response.

The pathogenic strains of *E coli* are responsible for infections in human mainly urinary tract infections (UTI), Neonatal meningitis, and the Intestinal diseases (Gastroenteritis). The distribution and expression of an array of virulence determinants, including adhesins, invasins, toxins, and abilities to withstand host defenses determines whether a particular strain of *E coli* cause any disease or not (Todar, 2019)

CHAPTER 3 MATERIALS AND METHODS

3.1 Reagents

RPMI-1640 Medium, HEPES were purchased from Sigma Aldrich (MO, USA), Essential Medium Eagle with Earle's salts, Iscove's Modified Dulbecco's Medium, Dulbecco's Modified Essential Medium, Trypsin, Chloroform, Isopropanol, Ethanol, Ethylene Diamine Tetra Acetic Acid (EDTA), Trizol (TRI reagent), Diethyl Pyro Carbonate (DEPC), Dimethyl sulfoxide (DMSO), Agarose, Primers were purchase from Sigma Aldrich(India), Fetal Bovine Serum (FBS) was purchased from Gibco, Life Technologies (Grand Island, NY, USA). Reagents such as RNase inhibitor, Oligo(dt)18, dNTPs mix, Taq Polymerase, Taq Buffer, M-MuLV reverse transcriptase enzyme and its buffer and Nuclease free water (GeNei) were purchased from NEB (UK). 100 bp DNA ladder was purchased from NEB (UK) and GeneDireX (Taiwan). All disposable plastic culture wares were supplied from Costar (NY, USA).

3.2 Maintenance of cell lines

LA-4, a mouse lung adenoma cell line and MH-S, a murine alveolar macrophage cell line were purchased from respectively. They were maintained in RPMI media containing Minimum Essential Medium Eagle with Earle's salts and Iscove's Modified Dulbecco's Medium supplemented with HEPES, Gentamicin sulfate, and 20% heat-inactivated Fetal Bovine Serum (FBS) in a humidified atmosphere containing 5% CO_2 at 37^0C at Forma SERIES II WATER JACKET, Thermo Scientific incubator. These cell lines were maintained as adherent and sub cultured by typsinization. Viability of cells was determined by using trypan blue exclusion method using formula:

Percent (%) Viability = (Live cells /Total number of cells) x 100.

3.3 Thawing of the cell line

Firstly, the media was warmed to 37^0C. Then the cell line vial was taken out from the liquid nitrogen. It was then transferred to 10 ml pre warmed media in the falcon under aseptic conditions. It was centrifuged at 1200 rpm at 4^0C for 5 minutes. The supernatant was removed and the cells were again resuspended in 10ml media. Again, the centrifugation was done at 1200 rpm at 4^0C for 5 minutes. The supernatant was removed and 10ml fresh media was added to resuspended the cells. Cell counting was done to know about the live and dead cells. After cell counting, 5 million cells were transferred to the culture flask and incubated in CO_2 incubator.

3.4 Growth curve of LA-4 cell line

In 24 well plates, we seeded $5x10^4$ cells in each well in 1 ml media. For 22 wells, $11x10^5$ cells were suspended in 22 ml media dispensing 1 ml to each well. The cells were incubated at 37^0C supplied with 5% CO2. Cells were harvested after each time point (12,

24, 36, 48, 60, 72, 84, 96, 108, 120 and 132 hours) and counted by tryphan blue staining under hemocytometer using Nikon ECLIPSE TS100 light microscope.

1ml of the media was collected from each well in 1.5ml vial. Each well was washed twice with 500µl PBS. Trypsin with PBS was added to each well and incubated at 37^0C for 3 minutes. The cells were harvested with repeated flushing and collected in the vial and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the pellet was dissolved in 500µl media. 10µl from the vial was taken and 10µl tryphan blue was added and the cells were counted in haemocytometer.

Method of calculation:

Total volume of 25 squares = 1x1x0.1 = $0.1mm^3$

Number of cells in $0.1mm^3$ or 10^{-4}ml= 'x'

Number of cells in volume of sample = x/ 10^{-4} X volume of sample X dilution factor

Doubling time of cells = $\frac{\text{Duration} * \log 2}{\log(\text{Final concentration}) - \log(\text{Initial concentration})}$

3.5 Isolation of RNA

The extraction of RNA is complicated by the presence of RNases that are abundant in the environment including on hands and surfaces. It is also difficult to remove RNases completely. RNA isolation is therefore carried out by using RNase free solutions treated with Di-ethyl pyro carbonate (DEPC) as well as RNase free pipette and glassware treated with Di-ethyl pyro carbonate (DEPC).

RNA from the cell line was isolated from resting and activated cells. 500 ml TRIzol was used for 1.5 million cells. It was mixed properly and kept at room temperature for 5 minutes. This procedure was followed by the addition of 200 µl chloroform. It was mixed properly by inverting and incubated at room temperature for 15 minutes. It was then centrifuged at 12,000g for 15 minutes at 4^0C at Eppendorf centrifuge 580R. After centrifugation, three layers were obtained upper aqueous phase, middle phase and lower phase containing phenol-chloroform layer containing RNA, DNA and protein respectively. The upper aqueous phase containing RNA was transferred to a fresh DEPC treated Eppendorf tube. 500µl isopropanol was added and mixed to the precipitate RNA and incubated at room temperature for 15 minutes. After incubation, it was centrifuged at 12,000g for 15 minutes at 4^0C. RNA which was in white gel like pellet form was washed twice with 75% ethanol by centrifugation at 7500g for 5 minutes at 4^0C. After centrifugation, it was air dried. After complete dry, pellet was mixed with DEPC treated water and stored at -80^0C.

3.5.1 Determination of yield and quality of RNA

The yield and the integrity (quality) of the RNA were determined by measuring absorbance at 260nm using Nano drop ND 2000 Spectrophotometer. At first reference baseline was set by a using TBE buffer and ratio of the readings at A260/280 and A260/230 was

measured. A260/280 ratio around 2.0 and A260/230 ratio greater than 1.8 denotes the purity of RNA. Absorbance values below 2 indicate phenol, protein or other contaminants. The integrity of the isolated RNA was measured by running 1µg of RNA on 1.2% agarose gel.

After confirming its purity and integrity, RNA was used for cDNA synthesis.

Table 3.1: RT-PCR primers used for studying cytokine release after treatment with the antigen.

Gene	**Forward Primer**	**Reverse Primer**	**Product Length**
GAPDH	5'-GTCGGTGTGAACGGATTTGG-3'	5'-CTAAGCAGTTGGTGGTGCAG-3'	475 bp
Caspase-3	5'-CAGTGGACTCTGGGATCTATCT-3'	5'-TTCAGGCCCATGAATGTCTC-3'	170 bp
Cox-2	5'-GTCATTGGTGGAGAGGTGTATC-3'	5'-GATGCTCCTGCTTGAGTATGT-3'	230 bp
IL-4	5'-CTCCATGCACCGAGATGTT-3'	5'-ACTGCAAGTATTTCCCTCGTAG-3'	291 bp
IL-6	5'- CCGTTTCTACCTGGAGTTTGT-3'	5'-GTTTGCCGAGTAGACCTCATAG-3'	234 bp
IL-13	5'-CTGGAATCCCTGACCTCATAG-3'	5'-TGAGGTCCACAGCTGAGAT-3'	224 bp
TNF-alpha	5'- CGTGTTCATCCGTTCTCTACC-3'	5'-GCAATCCAGGCCACTACTT-3'	232 bp

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

RT-PCR is a commonly used technique for the detection and comparison of RNA expression levels. It qualitatively detects the expression of gene through complementary DNA (cDNA) transcripts from RNA.

Table 3.2: Reaction mixture for RT-PCR

Components	**20µl Reaction**
Nuclease Free Water	Calculated according to 20µl
RNA template	Variable
Oligo(dt)18	1.0 µl
dNTPs mix	2 µl
Heated for 5 minutes at 65^0C and kept at room temperature for 2 minutes and then to the ice	
5XM-MuLVRT Buffer	2 µl
M-MuLV RT Enzyme	1 µl
Total	20 µl

The first strand cDNA for isolated RNA was synthesized by using M-MuLV reverse transcriptase enzyme (200U/µl) and oligo(dT)18(50µM) primer as described by the manufacturer in a reaction mixture of 10µL.The products obtained were used further for PCR amplification. RT-PCR was synthesized using 1µg of RNA per reaction mixture.

Synthesis of cDNA from RNA template was done in Eppendorf master cycler pro PCR thermocycler started with heating reaction mixture to 42^0C for 60 min, followed by heating at 70^0 C for 15 mins.in order to terminate the reaction. Finally the reaction product was held at 4^0C.

3.6.1 Agarose gel electrophoresis for cDNA

1.2% of agarose gel was prepared in 0.5X running buffer (5X TBE buffer+ Milli Q Water) by heating and after cooling to 40-50^0C, 0.5µg/ml of EtBr was added and casted on tray. After gel was set it was transferred to gel tank containing 0.5 X in TBE running buffer. cDNA samples(1 Volume of 6X DNA loading dye + 5 Volume of cDNA) were loaded along with DNA ladder (100bp).The gel electrophoresis was carried out at 90 V, for 2 hours and visualized under ALPHAIMAGE gel document system.

3.7 Semi-qualitative reverse transcriptase PCR

The cDNA obtained was amplified by conventional PCR by using different gene specific primers (designed at standardized annealing temperature and number of thermos cycles for individual molecule) for cytokines in a total reaction mixture of 10 µl. The amplification started with heating the sample to 95^0C for 2 mins, followed by number of cycles 35 cycles consisting of denaturation for 15 sec at 95^0C, annealing at 56^0C for 15 sec and extension

for 68^0C for 45 sec. The last cycle was followed by an additional extension for 5 mins at 68^0C and final hold at 4^0C.

Table 3.3: Reaction mixture for semi-quantitative PCR

Components	**25µl Reaction**	**Stock Concentration**
Nuclease Free Water	Calculated accordingly to 25 µl	-
Taq Buffer (20mM $MgCl_2$)	4 µl	10X
dNTPs Mix	2 µl	800µM
Forward Primer	1 µl	100µM
Reverse Primer	1 µl	100µM
Template (cDNA)	1 µl	-
Taq Polymerase	0.125 µl	5U/µl

3.7.1 Agarose gel electrophoresis of amplified cDNA

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

3.7.2 Quantitation of band intensity

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

3.8 Seeding of the MH-S and LA-4 cells

MH-S and LA-4 cells were collected and harvested and cell counting was done by tryphan blue staining using hemocytometer. In 35mm dish, two cover slips were added for each time point 4 hours, 8 hours respectively.0.5and 0.5 million cells were added for each time point for MH-S and LA-4 respectively and the media volume was maintained up to 1500µl. The dishes were kept in incubator at 37^0C for an overnight for the adherence of the cells.

3.8.1 Maintenance of log phase of *Bacillus subtilis* subsps RG

All culture and experiments with *Bacillus subtilis* were carried out in the Biosafety Lab (BSL) level 1 and were used in the experiments. The bacteria from the primary culture were transferred with the inoculating loop in the LB broth and kept for an overnight. The optical density at 600nm of the broth was measured next day.

3.8.2 CFSE labelling of *Bacillus subtilis* subsps RG and FACS analysis

The bacteria were cultured, harvested and washed with Phosphate Buffer Saline (PBS) buffer. The bacteria thus cultured were measured in the spectrophotometer at a wavelength of 600nm. The bacteria in late log phase were labelled with 10µM CFSE (Sigma, MO, USA) for 1 hour at 37^0C. After two washes with PBS at 4000 rpm for 10 minutes in order to remove excess unlabeled bacteria, the cells were suspended in 200µl PBS and 200µl of 4% PFA for fixing of the cells. The staining intensity data was subsequently acquired using BD FACS caliber for 1000 events per sample and was analyzed by using Cell Quest Pro software to determine the percentage of stained cells. Unstained bacteria were taken as negative control for the experiment.

3.8.3 In vitro co-culture of LA4 cells and *Bacillus subtilis*

To the cells incubated for an overnight on glass coverslips in 35mm disk**,** we incubated late log CFSE labelled bacteria in RPMI medium (without gentamicin) at MOI 1:10 for 4 hours, 8 hours at 37^0C in CO_2 incubator.

3.8.4 Slide preparation for microscopy

The wells containing the coverslips were then washed with PBS and fixed with 4% PFA for 20 minutes at room temperature. After that fixative was removed and reaction was quenched by adding quencher for 5 minutes at room temperature. The quenching step was followed two times to quench PFA completely. Next clean slide was taken and to that the coverslips were mounted onto the slides using 10 µl fluoro mount. The coverslips were then sealed with nail polish and stored at 4^0C in dark until acquiring the images by Microscopy (Olympus FluoView FV1000).

3.9 MTT assay

To determine the cell viability of MH-S and LA-4, the colorimetric MTT metabolic activity assay was used. 0.1 million MH-S and LA-4 cells were culture in 96-well plate at 37^0C for an overnight. The co-culture was done with the bacteria *Escherichia coli* for different time points 2 hour, 4 hour, 8 hour and 12 hour respectively. Cells with medium were used as a negative control. The supernatant of each well was removed and transferred to another 96-well plate. 20µl of MTT solution (Sigma, MO, USA) (5mg/ml in PBS) were added to the same well plate for different time points. After the incubation for another 4 hour, the resulting formazan crystals were dissolved in 100µl Dimethyl sulfoxide (DMSO). The absorbance intensity was measured by a microplate reader (Spectramax M2, USA) at 595 nm.

Similarly, to the supernatant collected in another well 20µl of MTT solution (5mg/ml in PBS) were added it each well for different time points. After the incubation for another 4 hour, the resulting formazan crystals were dissolved in 100µl Dimethyl sulfoxide (DMSO). The absorbance intensity was measured by a microplate reader at 595 nm to determine the viability of the bacteria.

CHAPTER 4 RESULTS

4.1 Growth kinetics of LA-4 cell line and MH-S cell line in RPMI media

LA-4 is a mouse lung adenoma cell line isolated from a urethane-induced lung adenoma of a 28 week old A/He strain mouse. The cells are routinely grown in RPMI-1640 medium containing 10% fetal bovine serum.

MH-S is a murine alveolar macrophage cell line which has been established following transformation of cells obtained by broncho alveolar lavage from Balb/cJ mice with simian virus 40 (SV40). These cells are also grown in RPMI-1640 medium containing 10% fetal bovine serum.

To determine the growth kinetics of these two cells in laboratory condition, cells were cultured in RPMI-1640 media and growth curves were generated. Two different parameters; cell morphology and doubling time were calculated. LA-4 cells are adherent and have epithelial like morphology whereas MH-S cells are also adherent and exhibit typical macrophage morphology. Growth curve was plotted on the basis of the viable cell count by Trypan blue staining. The doubling time of LA-4 cells and MH-S cells were determined to be 34 and 17 hours in RPMI complete medium respectively by using the formula described in materials and methods.

A) **B)**

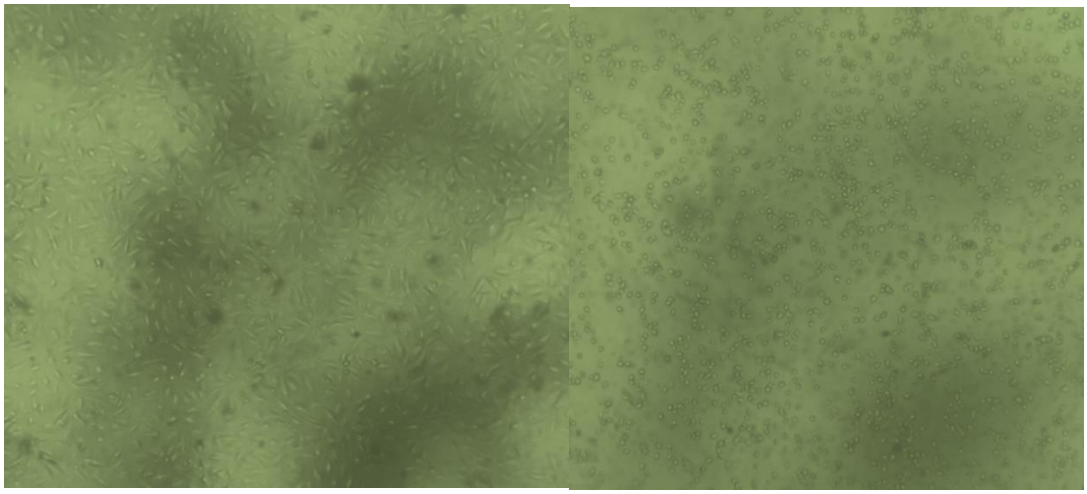
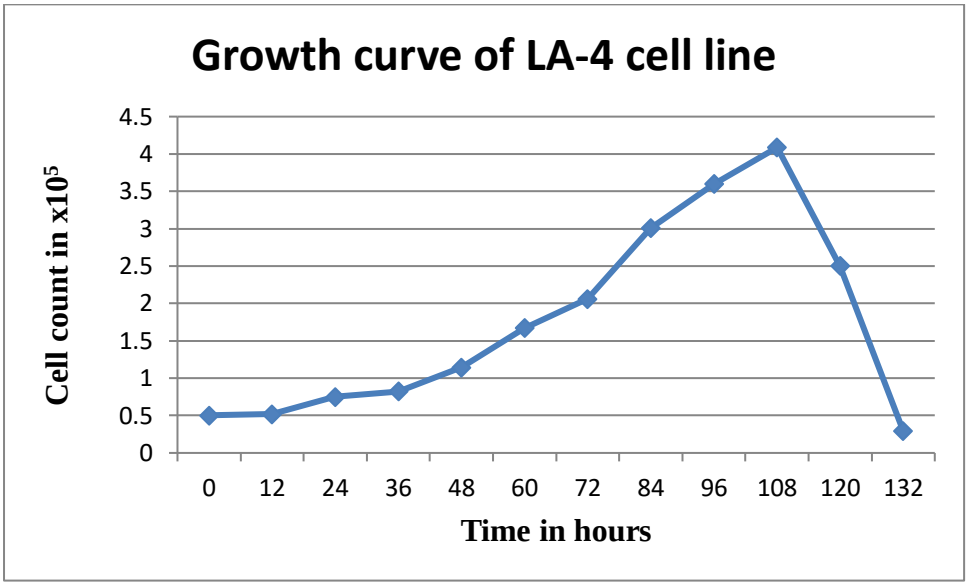


Figure 4.1: Morphology of A) LA-4 cells and B) MH-S cells cultured in RPMI 1640 media with $0.5*10^6$ cells in 35 mm dish and observed under the microscope under 10X magnifications.

A)



B)

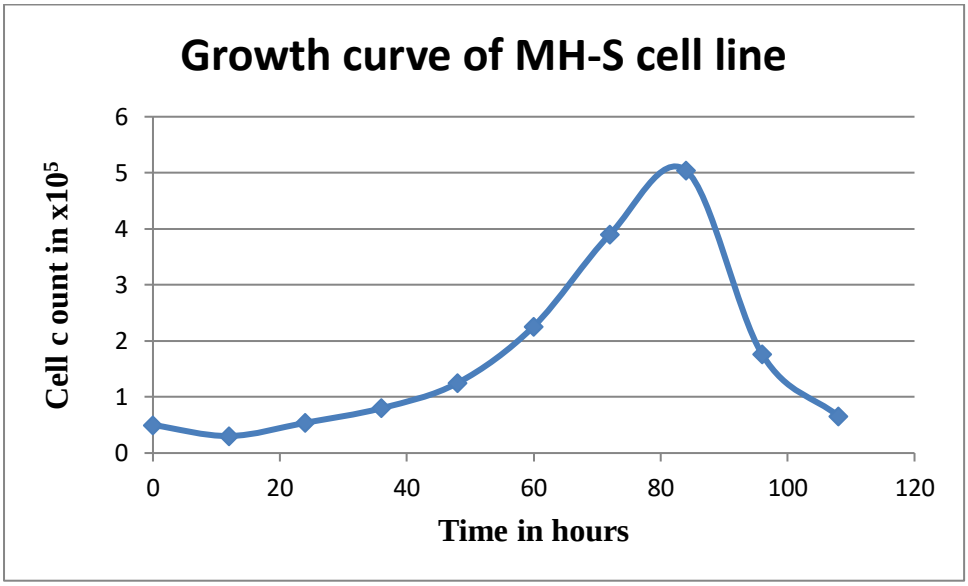


Figure 4.2: Growth kinetics of A) LA-4 cells and B) MH-S cells in RPMI media plotted on the basis of viable cell recoveries.

4.2 Isolation of RNA from resting and activated LA-4 cells by the treatment of lipopolysaccharide (LPS) with 20µg/ml for different time periods - 12 hours and 24 hours

RNA was isolated from resting and LPS activated with higher dose of LPS for different time periods 12 and 24 hours respectively by using Trizol method.

4.2.1 Isolation of RNA from MH-S cells and LA-4 cells by the treatment of lipopolysaccharide (LPS) with 100ng/ml for different time periods -2, 4, 6, 8, 12, 16, 18 and 24 hours with higher dose of LPS

RNA was isolated from LPS activated MH-S and LA-4 cells with lower dose for different time periods 2, 4, 6, 8, 12, 16, 18 and 24 hours respectively by using Trizol method.

4.2.2 Quantitation of RNA

The amount and purity of RNA were determined by measuring the absorbance at 260nm using Nanodrop (ND 2000) spectrophotometer. Amount of isolated RNA from resting and activated LA-4 cells after the treatment with LPS for different time periods were variable. The purity ratio with respect to contaminants, such as protein and organic content were found within the range.

Table 4.1: Yield and purity of RNA isolated from LA-4 cells using Nano drop (ND-2000) spectrophotometer.

Cell line	**Different time point by the treatment with LPS (20µg/ml)**	**RNA concentration (ng/µl)**	**Purity ratio (260/280)**	**Purity ratio (260/230)**
LA-4	Resting	1392.2	2.23	2.09
LA-4	Activated for 12 hour	2028.8	2.25	1.96
LA-4	Activated for 24 hour	2224.1	1.88	2.00

Cell line	**Different time point by the treatment with LPS (100ng/ml)**	**RNA concentration (ng/µl)**	**Purity ratio (260/280)**	**Purity ratio (260/230)**
MH-S	Activated for 2 hour	1005.09	2.0	1.94
MH-S	Activated for 4 hour	1556.75	2.18	1.95
MH-S	Activated for 6 hour	1366.2	1.83	1.94
MH-S	Activated for 8 hour	1107.05	2.0	1.92
MH-S	Activated for 12 hour	1005.1	2.14	1.93
MH-S	Activated for 16 hour	1130.35	1.90	1.92
MH-S	Activated for 18 hour	1187.3	2.0	1.94
MH-S	Activated for 24 hour	1135	1.84	1.94
LA-4	Activated for 2 hour	1145	2.0	1.81
LA-4	Activated for 12 hour	1221.5	2.02	1.95

LA-4	Activated for 18 hour	1286.02	2.06	1.96
LA-4	Activated for 24 hour	1333.6	1.99	1.96

4.2.3 Agarose gel electrophoresis of RNA for determining the quality and integrity of RNA

The quality of RNA isolated from LA-4 cells resting and LA-4 cells after treatment with the concentration of LPS (20µg/ml) for different time periods 12 and 24 hours were determined by using 1.2% agarose gel. Sharp 28S and 18S rRNA band along with 5S smear was observed. The band intensity of 28S and 18S rRNA band is nearly 2:1 indicating that the isolated RNA is intact.

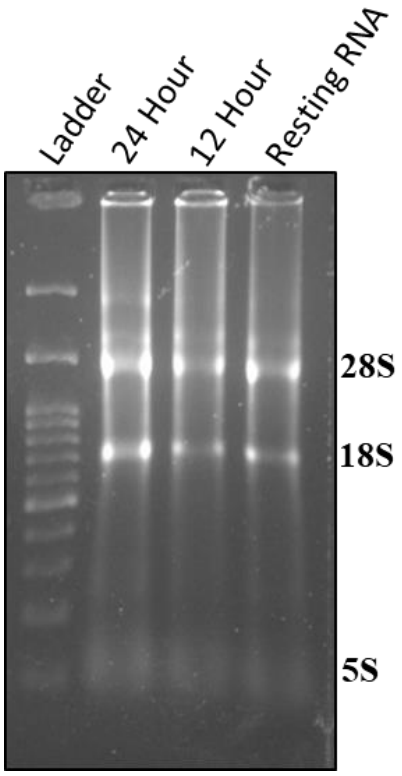


Figure 4.3: Agarose gel electrophoresis of RNA. Total RNA was isolated from resting and activated LA-4 cells for 12 and 24 hour and quality of isolated RNA was determined using 1.2% agarose gel. Presence of sharp 28S and 18S rRNA band along with 5S smear indicates isolated RNA is of good quality for further analysis.

The quality of RNA isolated from MH-S and LA-4 cells after treatment with the concentration of LPS (100ng/ml) for different time periods 2, 4, 6, 8, 12, 16, 18 and 24 hours and 2, 12, 18 and 24 hours respectively were determined by using 1.2% agarose gel. Sharp 28S and 18S rRNA band along with 5S smear was observed. The band intensity of 28S and 18S rRNA band is nearly 2:1 indicating that the isolated RNA is intact.

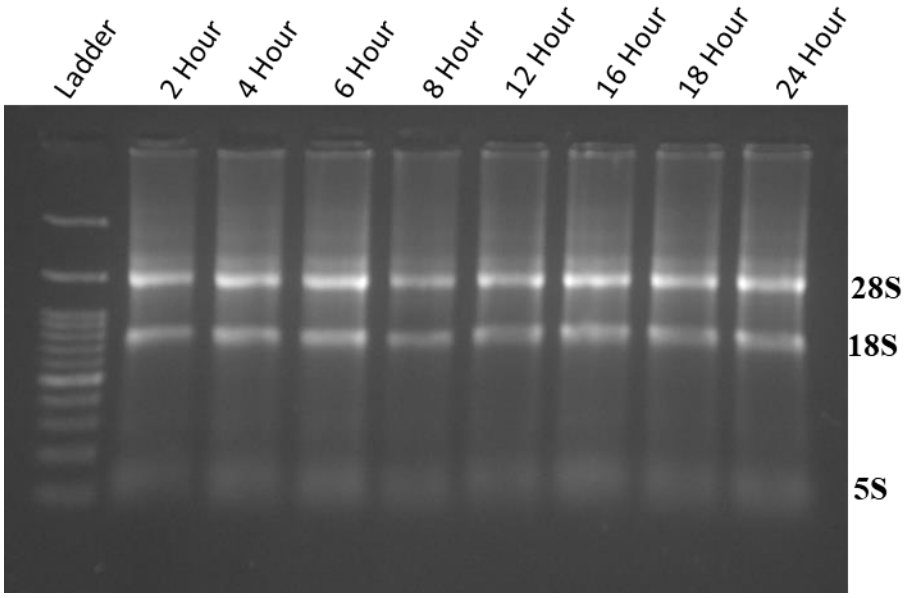


Figure 4.4: Agarose gel electrophoresis of RNA. Total RNA was isolated from activated MH-S cells for different time points 2, 4, 6, 8, 12, 16, 18 and 24 hours and quality of isolated RNA was determined using 1.2% agarose gel. Presence of sharp 28S and 18S rRNA band along with 5S smear indicates isolated RNA is of good quality for further analysis.

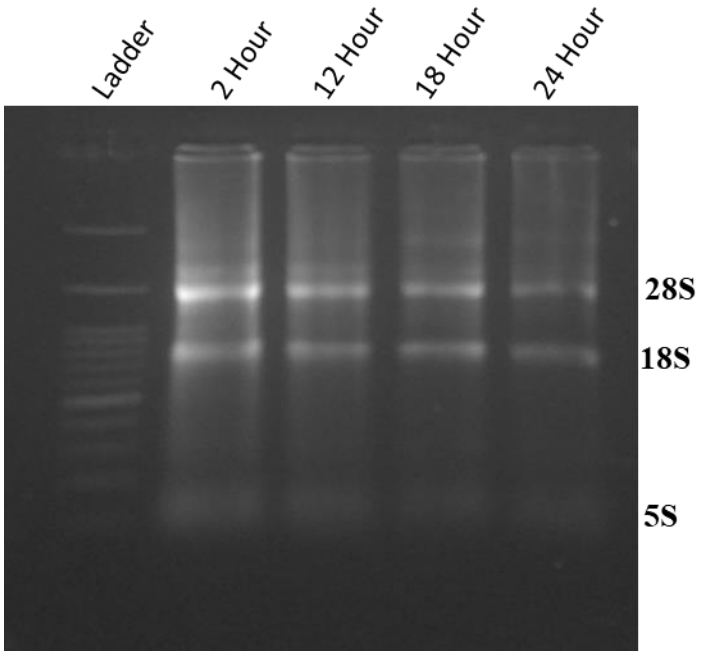


Figure 4.5: Agarose gel electrophoresis of RNA. Total RNA was isolated from activated LA-4 cells for different time points 2, 12, 18 and 24 hours and quality of isolated RNA was determined using 1.2% agarose gel. Presence of sharp 28S and 18S rRNA band along with 5S smear indicates isolated RNA is of good quality for further analysis.

4.2.4 Gel electrophoresis of cDNA isolated from MH-S and LA-4 cell after treatment with LPS for different time periods

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

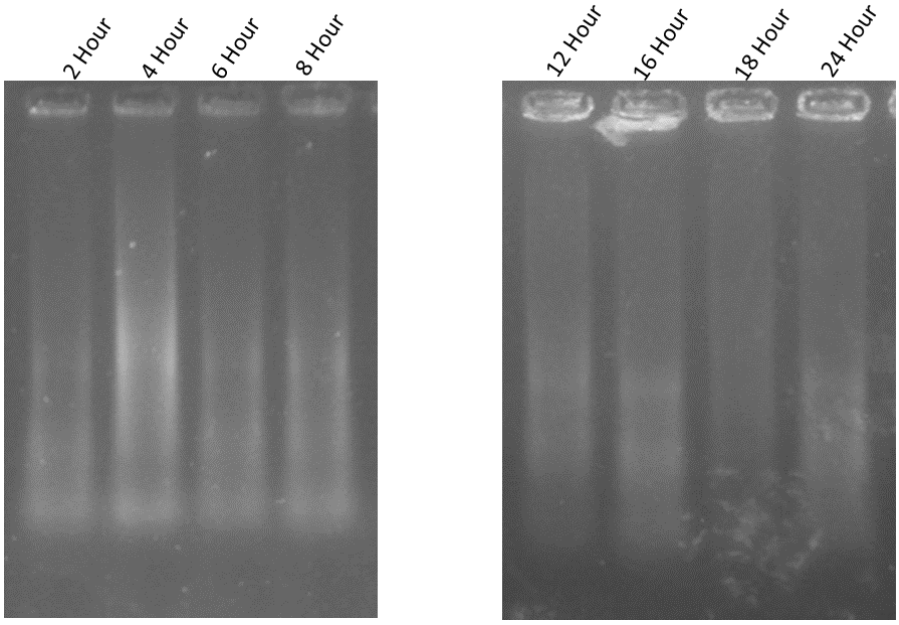


Figure 4.6: 1.2% agarose gel electrophoresis of cDNA of MH-S cells for different time points 2, 4, 6, 8, 12, 16, 18 and 24 hours obtained by reverse transcription of total RNA using oligo dT primer along with 100 bp ladder. Presence of smearing indicates RNA is converted to DNA.

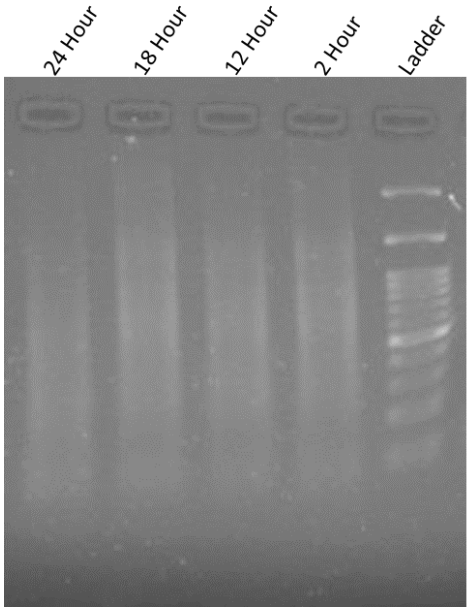


Figure 4.7: 1.2% agarose gel electrophoresis of cDNA of LA-4 cells for different time points 2, 12, 18 and 24 hours obtained by reverse transcription of total RNA using oligo dT primer along with 100 bp ladder. Presence of smearing indicates RNA is converted to DNA

4.3 Time kinetics of IL-4 expression in MH-S cells in response to the LPS antigen

PCR reaction was performed with RNA isolated from different time points with the concentration of LPS 100 ng/ml from MH-S cell line. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION (ALPHA IMAGER).

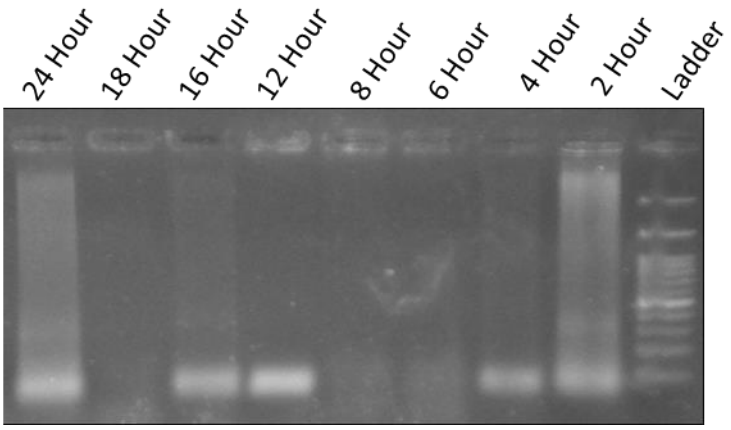


Figure 4.8: Time kinetics of IL-4 expression in MH-S cells in response to the LPS antigen.

There was no any band for IL-4 expression for different time points in MH-S cells in response to the LPS antigen.

4.3 Time kinetics of IL-6 expression in MH-S cells in response to the LPS antigen

PCR reaction was performed with RNA isolated from different time points with the concentration of LPS 100 ng/ml from MH-S cell line. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION (ALPHA IMAGER).

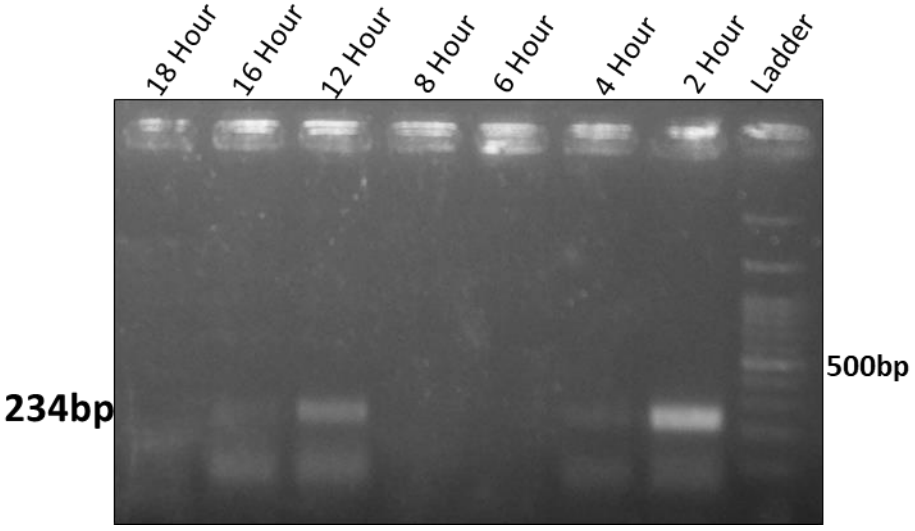


Figure 4.9: Time kinetics of IL-6 expression in MH-S cells in response to the LPS antigen.

For IL-6 expression, there was an intense band for 2 hour. And there was also band for 4 hour, 12 and 18 hour in MH-S cells in response to LPS antigen.

4.4 Time kinetics of IL-13 expression in MH-S cells in response to the LPS antigen

PCR reaction was performed with RNA isolated from different time points with the concentration of LPS 100 ng/ml from MH-S cell line. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION (ALPHA IMAGER).

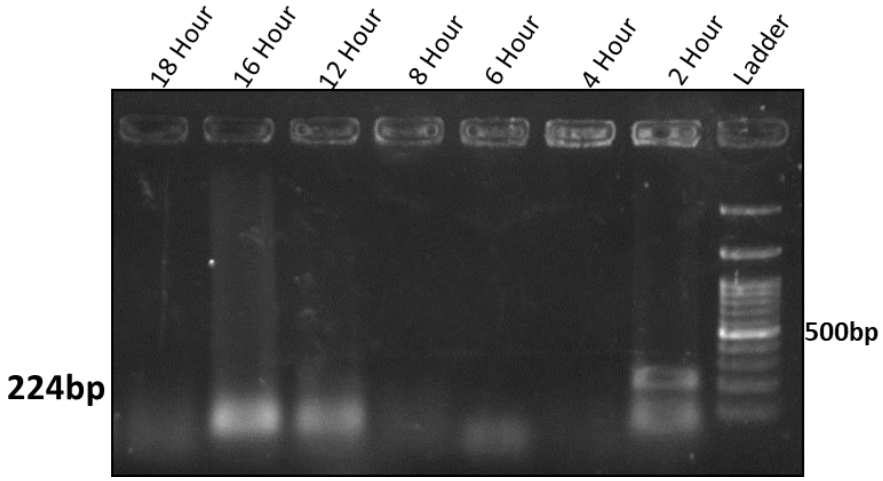


Figure 4.10: Time kinetics of IL-13 expression in MH-S cells in response to the LPS antigen.
For IL-13 expression, there was band for 2 hour in MH-S cells in response to LPS antigen.

4.5 Time kinetics of TNF- alpha expression in MH-S cells in response to the LPS antigen

PCR reaction was performed with RNA isolated from different time points with the concentration of LPS 100 ng/ml from MH-S cell line. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION (ALPHA IMAGER).

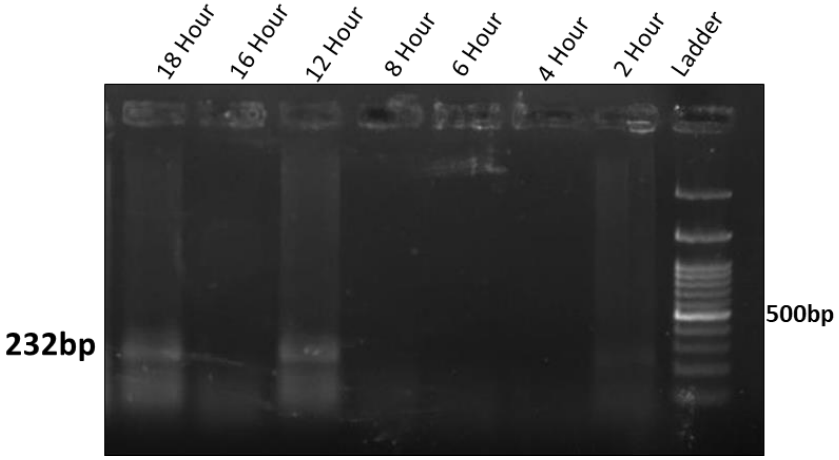


Figure 4.11: Time kinetics of TNF- Alpha expression in MH-S cells in response to the LPS antigen

For TNF-alpha, there was band for 2, 12 and 24 hour in MH-S cells in response to the LPS antigen.

4.6 Time kinetics of COX-2 expression in MH-S cells in response to the LPS antigen

PCR reaction was performed with RNA isolated from different time points with the concentration of LPS 100 ng/ml from MH-S cell line. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION (ALPHA IMAGER).

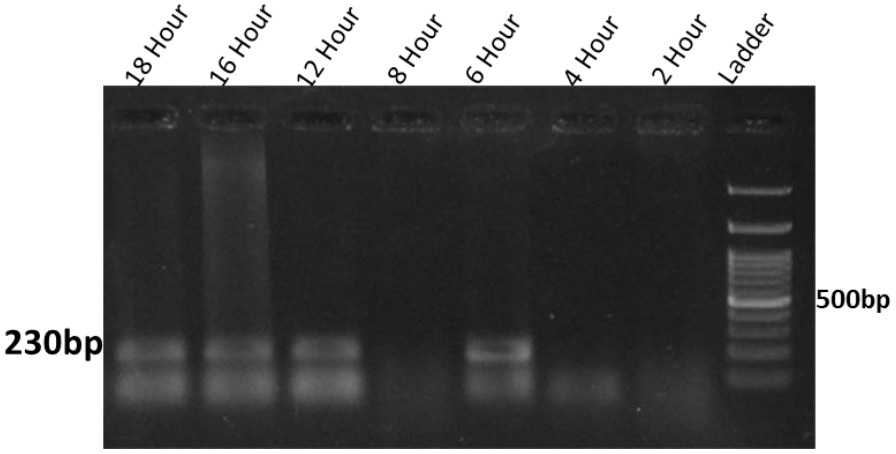


Figure 4.12: Time kinetics of Cox-2expression in MH-S cells in response to the LPS antigen

For Cox-2, there was band for 2, 6, 12, 18 and 24 hour in MH-S cells in response to the LPS antigen.

4.7 Time kinetics of Caspase-3 expression in MHS cells in response to the LPS antigen

PCR reaction was performed with RNA isolated from different time points with the concentration of LPS 100 ng/ml from MH-S cell line. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION (ALPHA IMAGER).

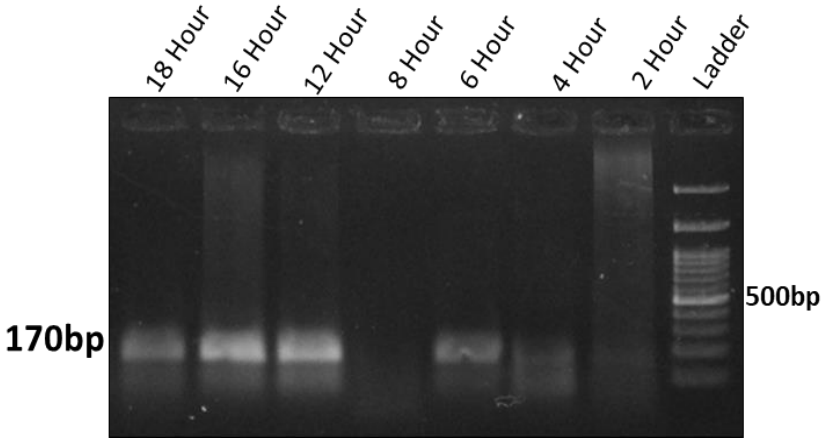


Figure 4.13: Time kinetics of Caspase-3expression in MH-S cells in response to the LPS antigen

For Caspase-3, there was band for 6, 12, 18, 24 hour in MH-S cells in response to the LPS antigen.

4.8 Comparison of the release of cytokine IL-6 from control MH-S and experimental LA-4 cell line in response to the treatment with LPS

For LPS (100ng/ml) activated MHS, IL-6 expression was observed at 12, 18 and 24 hours. However, no band was observed for the same concentration of LPS activated LA-4 cells.

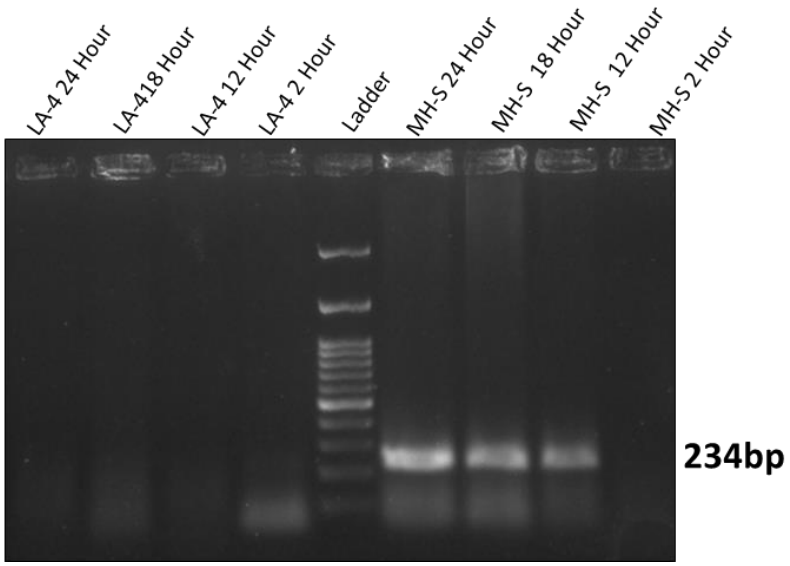


Figure 4.14: IL-6 expression profile by LA-4 and MH-S cells after treatment with LPS of concentration 100 ng. PCR was perfomed using 1000 ng of RNA from different time points at 56^0C for 30 cycles. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION (ALPHA IMAGER).

4.9 Comparison of the release of cytokine IL-13 from control MH-S and experimental LA-4 cell line in response to the treatment with LPS

For LPS (100ng/ml) activated MHS cells, IL-13 expression was observed at 2, 12, 18 and 24 hours. The band was less intense for 18 and 24 hours. However, no band was observed for the same concentration of LPS activated LA-4 cells.

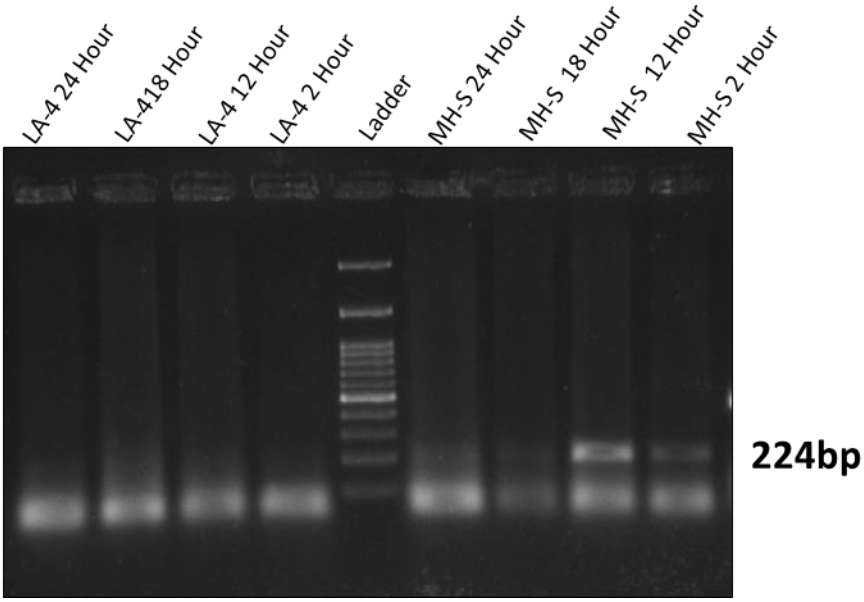


Figure 4.15: IL-13 expression profile by LA-4 and MH-S cells after treatment with LPS of concentration 100 ng. PCR was perfomed using 1000 ng of RNA from different time points at 56^0C for 30 cycles. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION (ALPHA IMAGER).

4.10 Comparison of the release of cytokine TNF- alpha from control MH-S and experimental LA-4 cell line in response to the treatment with LPS

For LPS (100ng/ml) activated MHS cells, TNF-alpha expression is observed at 2, 12, 18 and 24 hours. However, no band is observed for the same concentration of LPS activated LA-4 cells.

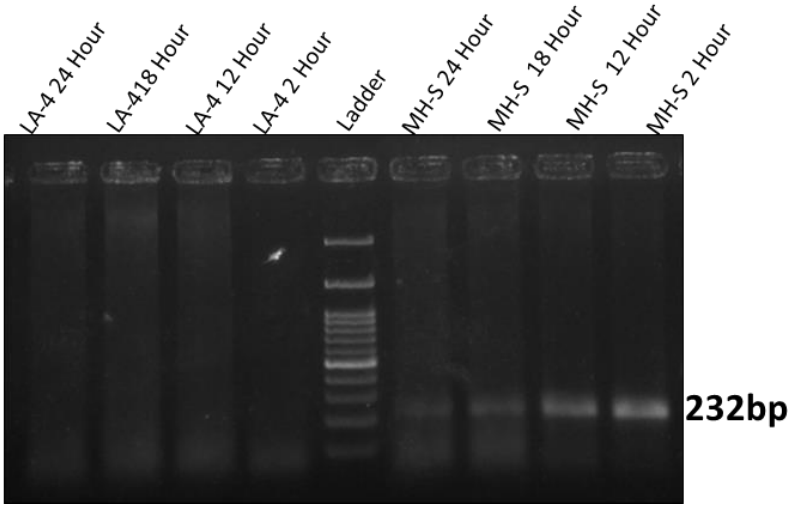


Figure 4.16: TNF-alpha expression profile by LA-4 and MH-S cells after treatment with LPS of concentration 100 ng. PCR was perfomed using 1000 ng of RNA from different time points at 56^0C for 30 cycles. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION (ALPHA IMAGER).

4.11 Comparison of the release of cytokine COX-2 from control MH-S and experimental LA-4 cell line in response to the treatment with LPS

For LPS (100ng/ml) activated MHS cells, Cox-2 expression was observed at 2, 12, 18 and 24 hours. However, no band was observed for the same concentration of LPS activated LA-4 cells.

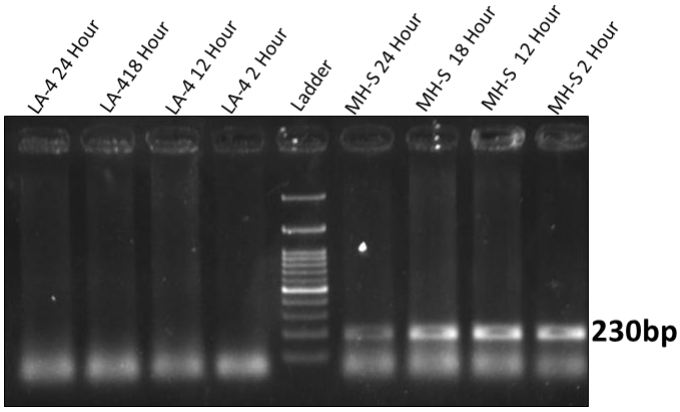


Figure 4.17: Cox-2 expression profile by LA-4 and MH-S cells after treatment with LPS of concentration 100 ng. PCR was perfomed using 1000 ng of RNA from different time points at 56^0C for 30 cycles. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION (ALPHA IMAGER).

4.12 Comparison of the release of cytokine Caspase-3 from control MH-S and experimental LA-4 cell line in response to the treatment with LPS

For LPS (100ng/ml) activated MHS, Caspase-3 expression was observed at 12, 18 and 24 hours. However, no band was observed for the same concentration of LPS activated LA-4.

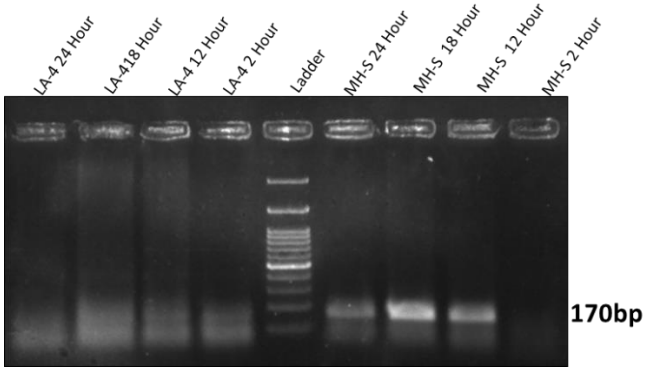


Figure 4.18: Caspase-3 expression profile by LA-4 and MH-S cells after treatment with LPS of concentration 100 ng. PCR was performed using 1000 ng of RNA from different time points at 560C for 30 cycles. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION (ALPHA IMAGER).

4.13 Comparison of the release of cytokine GAPDH from control MH-S and experimental LA-4 cell line in response to the treatment with LPS

For LPS (100ng/ml) activated MHS cells, GAPDH expression is observed at 2, 12, 18 and 24 hours. Similarly, the band is observed for the LPS activated LA-4 cells at 2, 12, 18 and 24 hour.

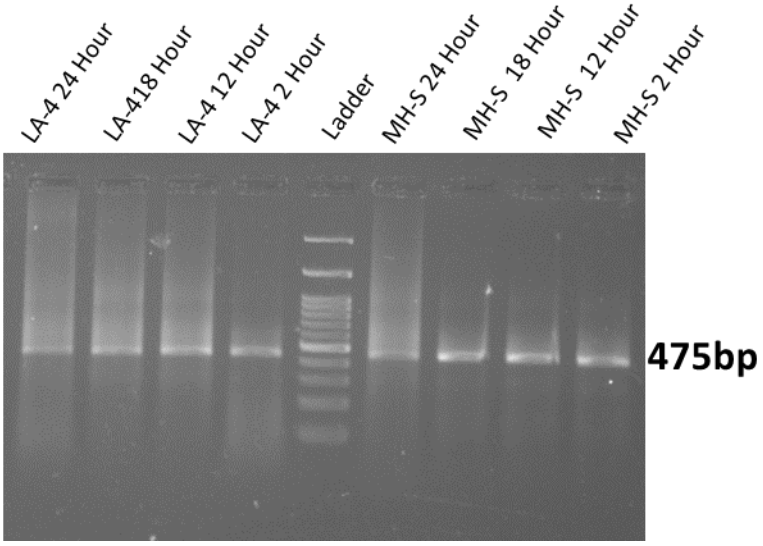


Figure 4.19: GAPDH expression profile by LA-4 and MH-S cells after treatment with LPS of concentration 100 ng. PCR was performed using 1000 ng of RNA from different time points at 56^0C for 30 cycles. The PCR products were run on 1.2% agarose gel and visualized under ALPHA IMAGE GEL DOCUMENTATION (ALPHA IMAGER).

4.14 Effect of *Escherichia Coli* 0104:H21 in LA-4 and MH-S

To evaluate the cytotoxic activity of *Escherichia coli* against LA-4 and MH-S cell lines, the cells were incubated with *E coli* with MOI 1:10 for different time points 2, 4, 8 and 12 hours. After each hour, cell viability was determined by using MTT assay.

The LA-4 and MH-S cell co-cultured with *E coli* has resulted in the increased viability of these cells for each time point. The viability of these cells has doubled as compared to the cells without treatment. However, the recovery of viable *E coli* when cultured with LA-4 and MH-S has reduced after each time point.

A)

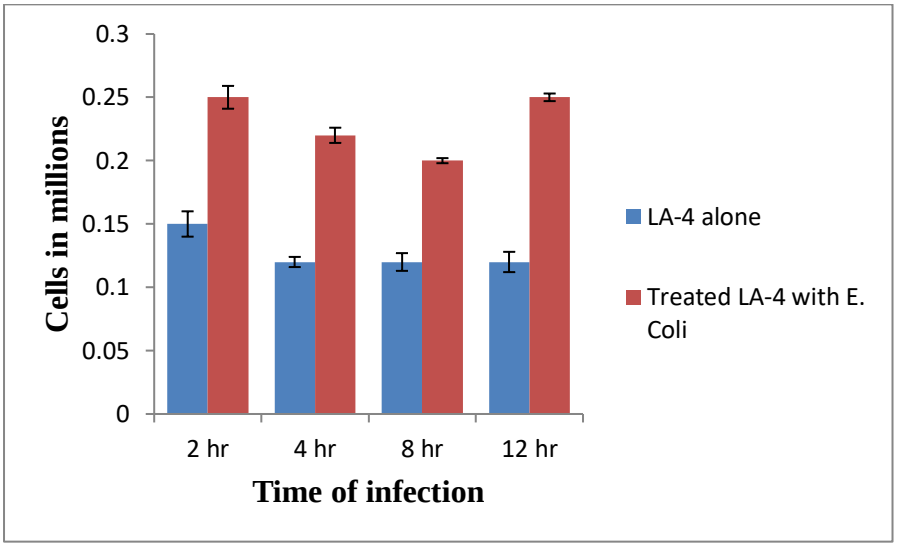


Figure 4.20: Effect of *E. coli* on LA-4 cell lines. 0.01 Million cells were cultured in 24mmdish. After overnight culture, cells were treated with *E.coli* (MOI 10: 1) for 2, 4, 8 12 hrs. Cell viability was determined by MTT assay. Results are represented as Mean± SEM of 3 observations. *p< 0.05 effect of *E coli*.

The cells treated with *E.coli* resulted in marked boosting of the number of LA-4 cells. In contrast, while LA-4 cells alone did not show much difference. Thus, the result indicates that the number of LA-4 cells could be marginally augmented by E coli in a time dependent manner.

B)

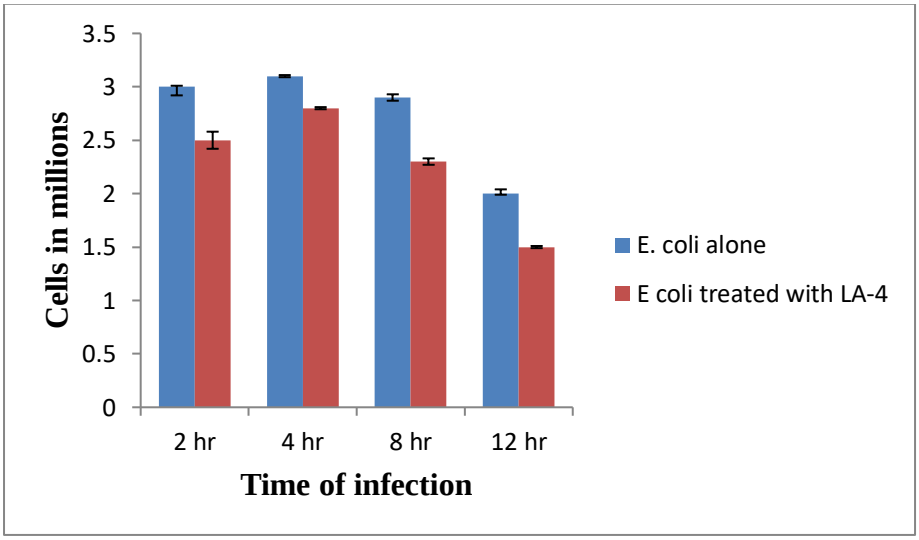


Figure 4.21: Effect on E coli with co-culture on LA-4 cells.0.01 Million cells were cultured in 24mm dish. After overnight culture, cells were treated with *E.coli* (MOI 10: 1) for 2, 4, 8 12 hrs. Cell viability was determined by MTT assay. Results are represented as Mean± SEM of 3 observations. *p< 0.05 effect of *E coli*.

The number of *E coli* cells treated with LA-4 decreased as compared to the *E coli* alone.

C)

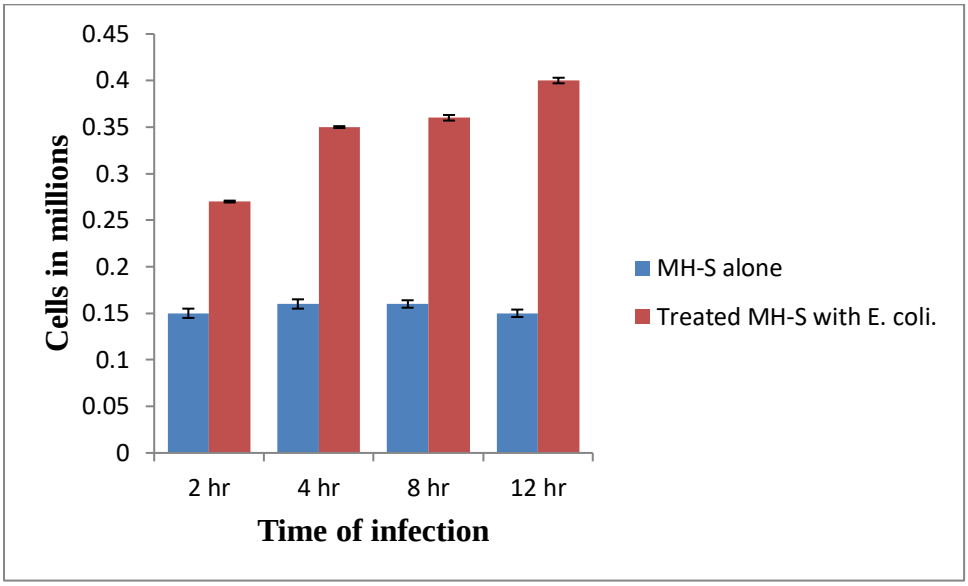


Figure 4.22: Effect of *E. coli* on MH-S cell lines. 0.01 Million cells were cultured in 24mm dish. After overnight culture, cells were treated with *E.coli* (MOI 10: 1) for 2, 4, 8 12 hrs. Cell viability was determined by MTT assay. Results are represented as Mean± SEM of 3 observations. *p < 0.05 effect of *E coli*.

Effects of *E. coli* were examined on MH-S cells. The cells treated with *E.coli* resulted in marked boosting of the number of MH-S cells. In contrast, while MH-S cells alone did not show much difference. Thus, the result indicates that the number of MH-S cells could be marginally augmented by E coli in a time dependent manner.

D)

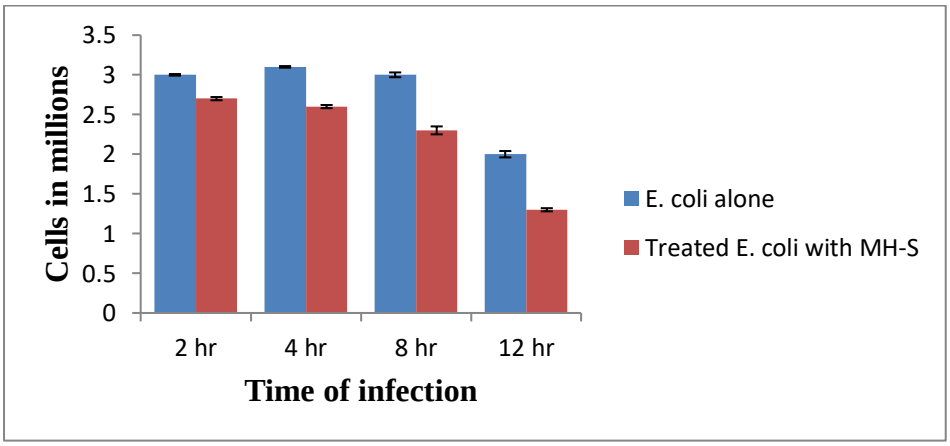


Figure 4.23: Effect on E coli with co-culture on MH-S cells. 0.01 Million cells were cultured in 24mm dish. After overnight culture, cells were treated with *E.coli* (MOI 10: 1) for 2, 4, 8 12 hrs. Cell viability was determined by MTT assay. Results are represented as Mean± SEM of 3 observations. *p< 0.05 effect of *E coli*.

The number of *E coli* cells treated with MH-S decreased as compared to the *E coli* alone.

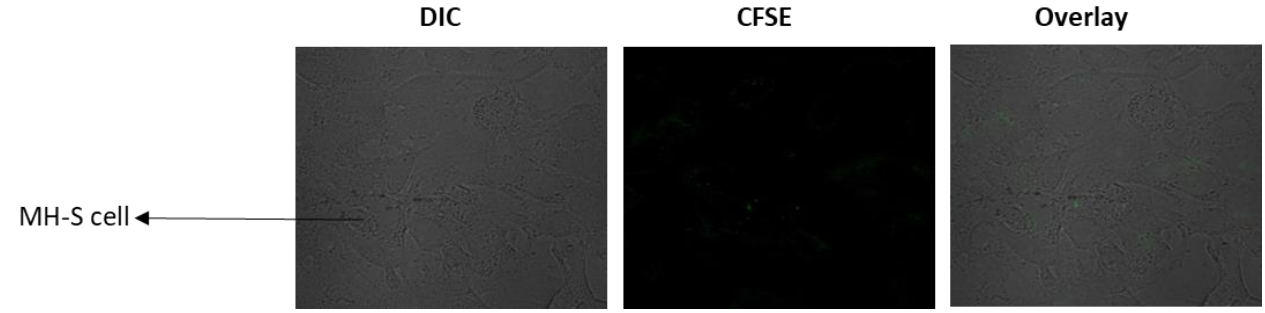
4.15 Interaction of MH-S and LA-4 with CFSE labelled *Bacillus subtilis* strain subspecies RG

As we know from the previous experiment, on infection with log phase of *E coli* to LA-4 and MH-S cells, the number of these cells gets increased. Thus, in order to visualize the interaction of these cells with the bacteria by microscopy, the bacteria were labelled by CFSE and co-cultured with LA-4 and MH-S respectively for two different time points 4 hour and 8 hour.

In this study, we found that the number of MH-S cells is more in 8 hour time of infection than in 4 hour. Similarly, in case of LA-4, the cells number has increased in 8 hour as compared to 4 hour time

However, the number of bacterial interaction with the cells is less for 8 hour time of infection than in 4 hour for both MH-S and LA-4 cells.

A)



B)

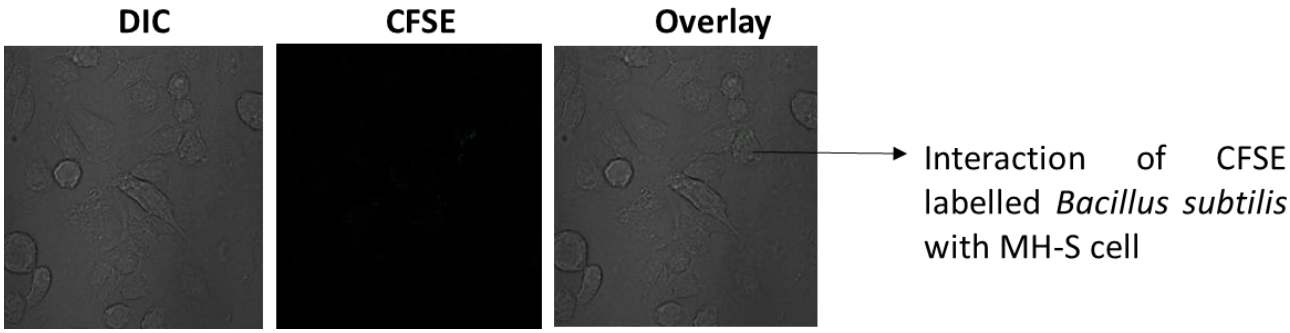
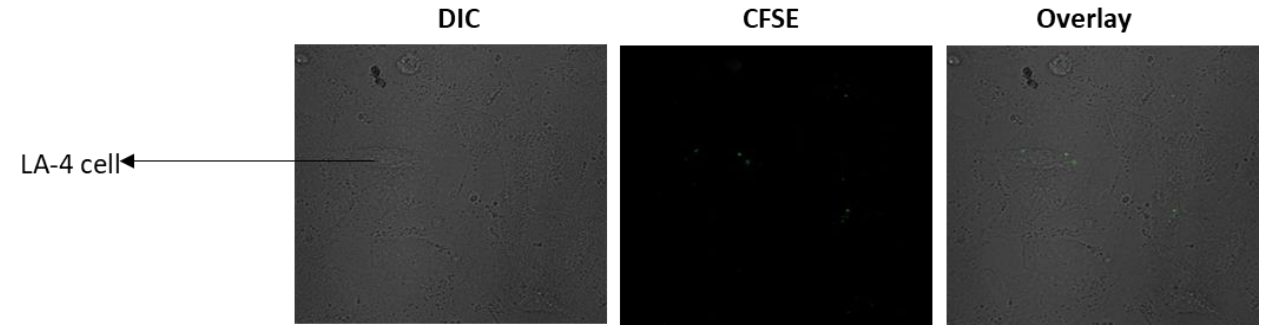


Figure 4.24: Visualization of the interaction of *Bacillus subtilis* with MH-S for two different time points A) 4 hour and B) 8 hour. MH-S cultured on a cover glass slip for overnight incubated with *Bacillus subtilis* for 4 and 8 hour at an MOI of 1:10 in RPMI without gentamicin. Cells were washed with PBS thrice, fixed with PFA and visualized under confocal laser scanning microscope as shown in Fig. Scale bar is 10 µm and magnification is 100X, n = 2.

C)



D)

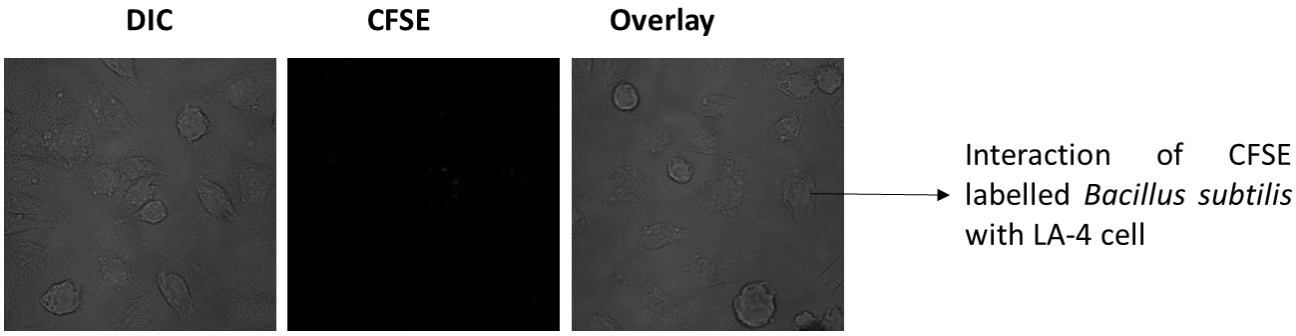


Figure 4.25: Visualization of the interaction of *Bacillus subtilis* with LA-4 for two different time points C) 4 hour and D) 8 hour. LA-4 cultured on a cover glass slip for overnight incubated with *Bacillus subtilis* for 4 and 8 hour at an MOI of 1:10 in RPMI without gentamicin. Cells were washed with PBS thrice, fixed with PFA and visualized under confocal laser scanning microscope as shown in Fig. Scale bar is 10 µm and magnification is 100X, n = 2.

Chapter 5 DISCUSSION

In this study, we used epithelial and macrophage cells to demonstrate how these two types of critical immune cells respond to bacteria and bacterial derived PAMPs. The present study examined the role and responses of epithelial and macrophage cells upon induction using LA-4 and MH-S which are mouse lung adenoma epithelial cell line and murine alveolar macrophage cell line respectively. First of all, we studied the growth kinetics of both LA-4 and MH-S cells in RPMI1640 media. The doubling time of cells was calculated and cells time was found to be 34 hour and 17 hour respectively.

We demonstrated the differential immune responses of LPS stimulated epithelial and macrophage cells by the production of pro inflammatory cytokines using RT-PCR. The qualitative RT-PCR based method relies on co-amplification of the cDNA of interest, with housekeeping genes as control. Housekeeping genes such as glyceraldehyde-3 phosphate dehydrogenase (GAPDH) are commonly used as a reference gene as they are constitutively expressed in the cells. RT-PCR technique is an extensively used approach as it offers the advantage of being superior, sensitive, cost efficient, simple, highly specific and versatile. The protein-based methodologies are often time consuming, not adapted to low number of cells, and antibodies of newly characterized proteins are not always available. Conversely, the development of molecular biology techniques enables detection of gene expression at the RNA level with their quantification. So we decided to use quantitative RT-PCR as our method of choice to study expression analysis of pro inflammatory cytokines and chemokine secreted by the epithelial and macrophage cells. We selected inflammatory cytokines and chemokines including IL-4, IL-6, IL-13, TNF alpha, Cox-2 and Caspase-3 from the literature survey.

The PCR conditions for IL-4, IL-6, IL-13, TNF-alpha, Cox-2 and Caspase-3 and GAPDH were already standardized in the lab. We looked for the expression of these cytokine's expression by activated epithelial and macrophage cells for different time periods by LPS. For this, the cells were treated with LPS of concentration 100ng. After treatment, RNA was isolated, and cDNA was synthesized followed by PCR amplification of cytokines and chemokines. GAPDH expression was similar in all the cells after treatment with different time periods. Its expression was not altered during the treatment and in the resting cells.

In this study, the induction of MH-S was done in the time dependent manner 2, 4, 6, 8, 12, 14, 16, 18 and 24 hours of LPS activation with the concentration of 100 ng and LA-4 induction was done for time periods 2, 12, 18 and 24 hours. MH-S cells responded earlier to LPS by synthesizing pro-inflammatory cytokines. In general, the LPS induced mRNA response in MH-S cells was initiated at 2 hour, but the LA-4 cell response did not appear at 2 hour. In MH-S cells, IL-6, IL-13, TNF-alpha, Cox-2, and Caspase-3 exhibited a band for

2, 12, 18 and 24 hour over the LPS concentration of 100 ng. LPS dependent macrophage activation revealed the expression of various cytokines IL-6, IL-13, TNF-alpha, Cox-2, and Caspase-3 mainly after 8 hours. However, LPS induced IL-4 production in macrophages was not observed as the production of IL-4 is usually delayed (Mukherjee et al., 2009). Moreover, after 8 hour induction of LPS, Cox-2 mRNA level and Caspase-3 mRNA expression of LPS remained elevated for all time points. In contrast, the LPS response in LA-4 cells differed. LPS-induced cytokine production in these two cell types was time dependent. Thus, 100ng/ml was sufficient to induce all these cytokines in MH-S cell type. But LPS alone of concentration of 100ng/ml was not sufficient to activate LA-4 cells.

This study showed the effects of LPS induced immune response in both the cells; epithelial and macrophage. Under the same experimental conditions, macrophages produce number of cytokines and chemokines whereas epithelial cells did not respond to LPS. LPS alone was not sufficient to activate the epithelial cells. These results implicate that the activated macrophage is an important innate immune source of IL6, IL13, TNF-alpha, Cox2, Caspase3 and they may play an important role in shaping the adaptive immune responses against the given antigen.

We also demonstrated the cytotoxic effects of *E coli* on LA-4 and MH-S. These cells co-cultured with *E coli* have increased in their viabilities as observed by MTT assay. These suggest that *E coli* is a potent activator of these cells. However, the reduction in the viability of *E coli* reveals a significant role of these cells in infection. We, therefore, decided to study the direct interaction of these cells with the bacteria *Bacillus subtilis* being gram positive which is effectively labelled by CFSE as compared to *E coli* (Vander Top, Perry, & Gentry-Nielsen, 2006). The number of organism interacting with these cells was decreased in a time dependent manner. However, the cells number has increased in the time periods. Thus, we can conclude that the innate immune cells- epithelial and macrophage cells are activated by recognizing the bacteria through the PAMPs.

CHAPTER 6 CONCLUSION

Our study shows that macrophage cell line MH-S induce pro inflammatory responses to LPS challenge, while the epithelial cell line LA-4 was not induced to produce the cytokines with the same concentration of LPS. However, the cells number has increased in both the cell lines upon co culture with the bacteria *E coli.*

Hence, macrophages are considered to be the major defenses system against the invasion of the host by the bacteria and bacterial derived PAMPs as they released the significant amount of cytokines in response to LPS stimulation contributing to the immunity. Epithelial cells also being the first cells along with macrophage cells to be challenged by LPS, however, did not show effect response in host defense in our study.

REFERENCES

Aderem, A. (2003). Phagocytosis and the Inflammatory Response. *The Journal of Infectious Diseases*, *187*(s2), S340–S345. https://doi.org/10.1086/374747

Akira, S., & Takeda, K. (2004). Toll-like receptor signalling. *Nature Reviews Immunology*, *4*(7), 499–511. https://doi.org/10.1038/nri1391

Atkins, G. J., & Findlay, D. M. (2012). Osteocyte regulation of bone mineral: A little give and take. *Osteoporosis International*, *23*(8), 2067–2079. https://doi.org/10.1007/s00198-012-1915-z

Balda, M. S., & Matter, K. (2009). Tight junctions and the regulation of gene expression. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1788*(4), 761–767. https://doi.org/10.1016/j.bbamem.2008.11.024

Bals, R., & Hiemstra, P. S. (2004). Innate immunity in the lung: How epithelial cells fight against respiratory pathogens. *The European Respiratory Journal*, *23*(2), 327–333. https://doi.org/10.1183/09031936.03.00098803

Candi, E., Schmidt, R., & Melino, G. (2005). The cornified envelope: A model of cell death in the skin. *Nature Reviews. Molecular Cell Biology*, *6*(4), 328–340. https://doi.org/10.1038/nrm1619

Carrillo, J. L. M., García, F. P. C., Coronado, O. G., García, M. A. M., & Cordero, J. F. C. (2017). Physiology and Pathology of Innate Immune Response Against Pathogens. *Physiology and Pathology of Immunology*. https://doi.org/10.5772/intechopen.70556

Carrillo, J. L. M., Rodríguez, F. P. C., Coronado, O. G., García, M. A. M., & Cordero, J. F. C. (2017). Physiology and Pathology of Innate Immune Response Against Pathogens. In N. Rezaei (Ed.), *Physiology and Pathology of Immunology*. https://doi.org/10.5772/intechopen.70556

Charles A Janeway, J., Travers, P., Walport, M., & Shlomchik, M. J. (2001). The front line of host defense. *Immunobiology: The Immune System in Health and Disease. 5th Edition*. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK27105/

Chelakkot, C., Ghim, J., & Ryu, S. H. (2018). Mechanisms regulating intestinal barrier integrity and its pathological implications. *Experimental & Molecular Medicine*, *50*(8), 103. https://doi.org/10.1038/s12276-018-0126-x

Davies, D. E. (2014a). Epithelial barrier function and immunity in asthma. *Annals of the American Thoracic Society*, *11 Suppl 5*, S244-251. https://doi.org/10.1513/AnnalsATS.201407-304AW

Davies, D. E. (2014b). Epithelial Barrier Function and Immunity in Asthma. *Annals of the American Thoracic Society*, *11*(Supplement 5), S244–S251. https://doi.org/10.1513/AnnalsATS.201407-304AW

Davies, L. C., Jenkins, S. J., Allen, J. E., & Taylor, P. R. (2013). Tissue-resident macrophages. *Nature Immunology*, *14*(10), 986–995. https://doi.org/10.1038/ni.2705

Diamond, C. E., Khameneh, H. J., Brough, D., & Mortellaro, A. (2015). Novel perspectives on non-canonical inflammasome activation. *Immunotargets and Therapy*, *4*, 131. https://doi.org/10.2147/ITT.S57976

Eckmann, L., Kagnoff, M. F., & Fierer, J. (1993). Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. *Infection and Immunity*, *61*(11), 4569–4574.

Ganz, T. (2002). Antimicrobial polypeptides in host defense of the respiratory tract. *The Journal of Clinical Investigation*, *109*(6), 693–697. https://doi.org/10.1172/JCI15218

Garrod, D., & Chidgey, M. (2008). Desmosome structure, composition and function. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1778*(3), 572–587. https://doi.org/10.1016/j.bbamem.2007.07.014

Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M., & Ley, K. (2010). Development of monocytes, macrophages, and dendritic cells. *Science (New York, N.Y.)*, *327*(5966), 656–661. https://doi.org/10.1126/science.1178331

Gioannini, T. L., Teghanemt, A., Zhang, D., Levis, E. N., & Weiss, J. P. (2005). Monomeric endotoxin:protein complexes are essential for TLR4-dependent cell activation. *Journal of Endotoxin Research*, *11*(2), 117–123. https://doi.org/10.1179/096805105X35198

Gioannini, Theresa L., Teghanemt, A., Zhang, D., Coussens, N. P., Dockstader, W., Ramaswamy, S., & Weiss, J. P. (2004). Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(12), 4186–4191. https://doi.org/10.1073/pnas.0306906101

Gitter, A. H., Wullstein, F., Fromm, M., & Schulzke, J. D. (2001). Epithelial barrier defects in ulcerative colitis: Characterization and quantification by electrophysiological imaging. *Gastroenterology*, *121*(6), 1320–1328. https://doi.org/10.1053/gast.2001.29694

González-Mariscal, L., Tapia, R., & Chamorro, D. (2008). Crosstalk of tight junction components with signaling pathways. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1778*(3), 729–756. https://doi.org/10.1016/j.bbamem.2007.08.018

Gordon, S. (1976). MACROPHAGE NEUTRAL PROTEINASES AND CHRONIC INFLAMMATION. *Annals of the New York Academy of Sciences*, *278*(1 Seventh Inter), 176–189. https://doi.org/10.1111/j.1749-6632.1976.tb47028.x

Gordon, S., & Taylor, P. R. (2005). Monocyte and macrophage heterogeneity. *Nature Reviews Immunology*, *5*(12), 953–964. https://doi.org/10.1038/nri1733

Greenberg, S., & Grinstein, S. (2002). Phagocytosis and innate immunity. *Current Opinion in Immunology*, *14*(1), 136–145. https://doi.org/10.1016/s0952-7915(01)00309-0

Günther, J., & Seyfert, H.-M. (2018). The first line of defence: Insights into mechanisms and relevance of phagocytosis in epithelial cells. *Seminars in Immunopathology*, *40*(6), 555–565. https://doi.org/10.1007/s00281-018-0701-1

Harding, C. V., Ramachandra, L., & Wick, M. J. (2003). Interaction of bacteria with antigen presenting cells: Influences on antigen presentation and antibacterial immunity. *Current Opinion in Immunology*, *15*(1), 112–119. https://doi.org/10.1016/s0952-7915(02)00008-0

Heijink, I. H., Kies, P. M., Kauffman, H. F., Postma, D. S., van Oosterhout, A. J. M., & Vellenga, E. (2007). Down-regulation of E-cadherin in human bronchial epithelial cells leads to epidermal growth factor receptor-dependent Th2 cell-promoting activity. *Journal*

of Immunology (Baltimore, Md.: 1950), *178*(12), 7678–7685. https://doi.org/10.4049/jimmunol.178.12.7678

Hillaireau, H., & Couvreur, P. (2009). Nanocarriers' entry into the cell: Relevance to drug delivery. *Cellular and Molecular Life Sciences*, *66*(17), 2873–2896. https://doi.org/10.1007/s00018-009-0053-z

Hirayama, D., Iida, T., & Nakase, H. (2017). The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. *International Journal of Molecular Sciences*, *19*(1). https://doi.org/10.3390/ijms19010092

Honey, K., & Rudensky, A. Y. (2003). Lysosomal cysteine proteases regulate antigen presentation. *Nature Reviews. Immunology*, *3*(6), 472–482. https://doi.org/10.1038/nri1110

Hoňková, L., Uhlík, J., Beránková, K., Svobodová, T., & Pohunek, P. (2014). Epithelial basement membrane thickening is related to TGF-Beta 1 expression in children with chronic respiratory diseases. *Pediatric Allergy and Immunology: Official Publication of the European Society of Pediatric Allergy and Immunology*, *25*(6), 593–599. https://doi.org/10.1111/pai.12275

Ivanov, A. I., & Naydenov, N. G. (2013). Dynamics and Regulation of Epithelial Adherens Junctions. In *International Review of Cell and Molecular Biology* (Vol. 303, pp. 27–99). https://doi.org/10.1016/B978-0-12-407697-6.00002-7

Janeway, C. A., & Medzhitov, R. (2002). Innate Immune Recognition. *Annual Review of Immunology*, *20*(1), 197–216. https://doi.org/10.1146/annurev.immunol.20.083001.084359

Jung, H. C., Eckmann, L., Yang, S. K., Panja, A., Fierer, J., Morzycka-Wroblewska, E., & Kagnoff, M. F. (1995). A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *The Journal of Clinical Investigation*, *95*(1), 55–65. https://doi.org/10.1172/JCI117676

Kalinin, A., Marekov, L. N., & Steinert, P. M. (2001). Assembly of the epidermal cornified cell envelope. *Journal of Cell Science*, *114*(Pt 17), 3069–3070.

Kao, C. Y., Chen, Y., Zhao, Y. H., & Wu, R. (2003). ORFeome-based search of airway epithelial cell-specific novel human [beta]-defensin genes. *American Journal of Respiratory Cell and Molecular Biology*, *29*(1), 71–80. https://doi.org/10.1165/rcmb.2002-0205OC

Kelly, C. P., O'Keane, J. C., Orellana, J., Schroy, P. C., Yang, S., LaMont, J. T., & Brady, H. R. (1992). Human colon cancer cells express ICAM-1 in vivo and support LFA-1-dependent lymphocyte adhesion in vitro. *The American Journal of Physiology*, *263*(6 Pt 1), G864-870. https://doi.org/10.1152/ajpgi.1992.263.6.G864

Kim, S. J., & Kim, H. M. (2017). Dynamic lipopolysaccharide transfer cascade to TLR4/MD2 complex via LBP and CD14. *BMB Reports*, *50*(2), 55–57. https://doi.org/10.5483/bmbrep.2017.50.2.011

Korzenik, J. R. (2007). Is Crohn's disease due to defective immunity? *Gut*, *56*(1), 2–5. https://doi.org/10.1136/gut.2006.095588

Krause, G., Winkler, L., Mueller, S. L., Haseloff, R. F., Piontek, J., & Blasig, I. E. (2008). Structure and function of claudins. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1778*(3), 631–645. https://doi.org/10.1016/j.bbamem.2007.10.018

Kuzmich, N. N., Sivak, K. V., Chubarev, V. N., Porozov, Y. B., Savateeva-Lyubimova, T. N., & Peri, F. (2017). TLR4 Signaling Pathway Modulators as Potential Therapeutics in Inflammation and Sepsis. *Vaccines*, *5*(4). https://doi.org/10.3390/vaccines5040034

Lebedev, K. A., & Ponyakina, I. D. (2006). Immunophysiology of epithelial cells and pattern-recognition receptors. *Human Physiology*, *32*(2), 224–234. https://doi.org/10.1134/S0362119706020198

Lee, K. M., Yin, C., Verschoor, C. P., & Bowdish, D. M. (2013). Macrophage Function Disorders. In John Wiley & Sons Ltd (Ed.), *ELS* (p. a0002174.pub3). https://doi.org/10.1002/9780470015902.a0002174.pub3

Luster, A. D. (2002). The role of chemokines in linking innate and adaptive immunity. *Current Opinion in Immunology*, *14*(1), 129–135. https://doi.org/10.1016/s0952-7915(01)00308-9

Macdonald, T. T., & Monteleone, G. (2005). Immunity, inflammation, and allergy in the gut. *Science (New York, N.Y.)*, *307*(5717), 1920–1925. https://doi.org/10.1126/science.1106442

Marchiando, A. M., Graham, W. V., & Turner, J. R. (2010). Epithelial Barriers in Homeostasis and Disease. *Annual Review of Pathology: Mechanisms of Disease*, *5*(1), 119–144. https://doi.org/10.1146/annurev.pathol.4.110807.092135

McClure, R., & Massari, P. (2014). TLR-Dependent Human Mucosal Epithelial Cell Responses to Microbial Pathogens. *Frontiers in Immunology*, *5*, 386. https://doi.org/10.3389/fimmu.2014.00386

Mukherjee, S., Chen, L.-Y., Papadimos, T. J., Huang, S., Zuraw, B. L., & Pan, Z. K. (2009). Lipopolysaccharide-driven Th2 cytokine production in macrophages is regulated by both MyD88 and TRAM. *The Journal of Biological Chemistry*, *284*(43), 29391–29398. https://doi.org/10.1074/jbc.M109.005272

Nawijn, M. C., Hackett, T. L., Postma, D. S., van Oosterhout, A. J. M., & Heijink, I. H. (2011). E-cadherin: Gatekeeper of airway mucosa and allergic sensitization. *Trends in Immunology*, *32*(6), 248–255. https://doi.org/10.1016/j.it.2011.03.004

Nelson, W. J. (2008). Regulation of cell–cell adhesion by the cadherin–catenin complex. *Biochemical Society Transactions*, *36*(2), 149–155. https://doi.org/10.1042/BST0360149

Nievers, M. G., Schaapveld, R. Q. J., & Sonnenberg, A. (1999). Biology and function of hemidesmosomes. *Matrix Biology*, *18*(1), 5–17. https://doi.org/10.1016/S0945-053X(98)00003-1

O'Neill, L. A. J., Golenbock, D., & Bowie, A. G. (2013). The history of Toll-like receptors—Redefining innate immunity. *Nature Reviews. Immunology*, *13*(6), 453–460. https://doi.org/10.1038/nri3446

Ordoñez, C. L., Khashayar, R., Wong, H. H., Ferrando, R., Wu, R., Hyde, D. M., … Fahy, J. V. (2001). Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. *American Journal of Respiratory and Critical Care Medicine*, *163*(2), 517–523. https://doi.org/10.1164/ajrccm.163.2.2004039

Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T., & Seya, T. (2003). TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nature Immunology*, *4*(2), 161–167. https://doi.org/10.1038/ni886

Pandey, S., Kawai, T., & Akira, S. (2014). Microbial sensing by Toll-like receptors and intracellular nucleic acid sensors. *Cold Spring Harbor Perspectives in Biology*, *7*(1), a016246. https://doi.org/10.1101/cshperspect.a016246

Park, B. S., Song, D. H., Kim, H. M., Choi, B.-S., Lee, H., & Lee, J.-O. (2009). The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature*, *458*(7242), 1191–1195. https://doi.org/10.1038/nature07830

Raggatt, L. J., Wullschleger, M. E., Alexander, K. A., Wu, A. C. K., Millard, S. M., Kaur, S., … Pettit, A. R. (2014). Fracture healing via periosteal callus formation requires macrophages for both initiation and progression of early endochondral ossification. *The American Journal of Pathology*, *184*(12), 3192–3204. https://doi.org/10.1016/j.ajpath.2014.08.017

Reinecker, H. C., Loh, E. Y., Ringler, D. J., Mehta, A., Rombeau, J. L., & MacDermott, R. P. (1995). Monocyte-chemoattractant protein 1 gene expression in intestinal epithelial cells and inflammatory bowel disease mucosa. *Gastroenterology*, *108*(1), 40–50. https://doi.org/10.1016/0016-5085(95)90006-3

Reinecker, H. C., & Podolsky, D. K. (1995). Human intestinal epithelial cells express functional cytokine receptors sharing the common gamma c chain of the interleukin 2 receptor. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(18), 8353–8357. https://doi.org/10.1073/pnas.92.18.8353

Ribbons, K. A., Zhang, X. J., Thompson, J. H., Greenberg, S. S., Moore, W. M., Kornmeier, C. M., … Clark, D. A. (1995). Potential role of nitric oxide in a model of chronic colitis in rhesus macaques. *Gastroenterology*, *108*(3), 705–711. https://doi.org/10.1016/0016-5085(95)90442-5

Ryu, J.-K., Kim, S. J., Rah, S.-H., Kang, J. I., Jung, H. E., Lee, D., … Kim, H. M. (2017). Reconstruction of LPS Transfer Cascade Reveals Structural Determinants within LBP,

CD14, and TLR4-MD2 for Efficient LPS Recognition and Transfer. *Immunity*, *46*(1), 38–50. https://doi.org/10.1016/j.immuni.2016.11.007

Savidge, T. C., Morey, A. L., Ferguson, D. J., Fleming, K. A., Shmakov, A. N., & Phillips, A. D. (1995). Human intestinal development in a severe-combined immunodeficient xenograft model. *Differentiation; Research in Biological Diversity*, *58*(5), 361–371. https://doi.org/10.1046/j.1432-0436.1995.5850361.x

Schleimer, R. P., & Berdnikovs, S. (2017). Etiology of epithelial barrier dysfunction in patients with type 2 inflammatory diseases. *The Journal of Allergy and Clinical Immunology*, *139*(6), 1752–1761. https://doi.org/10.1016/j.jaci.2017.04.010

Sweerus, K., Lachowicz-Scroggins, M., Gordon, E., LaFemina, M., Huang, X., Parikh, M., … Frank, J. A. (2017). Claudin-18 deficiency is associated with airway epithelial barrier dysfunction and asthma. *The Journal of Allergy and Clinical Immunology*, *139*(1), 72-81.e1. https://doi.org/10.1016/j.jaci.2016.02.035

Takahashi, K., Yamamura, F., & Naito, M. (1989). Differentiation, maturation, and proliferation of macrophages in the mouse yolk sac: A light-microscopic, enzyme-cytochemical, immunohistochemical, and ultrastructural study. *Journal of Leukocyte Biology*, *45*(2), 87–96. https://doi.org/10.1002/jlb.45.2.87

Tang, D., Kang, R., Coyne, C. B., Zeh, H. J., & Lotze, M. T. (2012). PAMPs and DAMPs: Signal 0s that spur autophagy and immunity. *Immunological Reviews*, *249*(1), 158–175. https://doi.org/10.1111/j.1600-065X.2012.01146.x

Todar, K. (2019). *Online Textbook of Bacteriology*. Retrieved from http://textbookofbacteriology.net/innate.html

Tsukita, S, Yamazaki, Y., Katsuno, T., Tamura, A., & Tsukita, S. (2008). Tight junction-based epithelial microenvironment and cell proliferation. *Oncogene*, *27*(55), 6930–6938. https://doi.org/10.1038/onc.2008.344

Tsukita, Shoichiro, Furuse, M., & Itoh, M. (2001). Multifunctional strands in tight junctions. *Nature Reviews Molecular Cell Biology*, *2*(4), 285–293. https://doi.org/10.1038/35067088

Vander Top, E. A., Perry, G. A., & Gentry-Nielsen, M. J. (2006). A novel flow cytometric assay for measurement of in vivo pulmonary neutrophil phagocytosis. *BMC Microbiology*, *6*, 61. https://doi.org/10.1186/1471-2180-6-61

Wynn, T. A., & Vannella, K. M. (2016). Macrophages in Tissue Repair, Regeneration, and Fibrosis. *Immunity*, *44*(3), 450–462. https://doi.org/10.1016/j.immuni.2016.02.015

Xiang, Y., Qiu, Q., Jiang, M., Jin, R., Lehmann, B. D., Strand, D. W., … Yi, Y. (2013). SPARCL1 suppresses metastasis in prostate cancer. *Molecular Oncology*, *7*(6), 1019–1030. https://doi.org/10.1016/j.molonc.2013.07.008

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., … Akira, S. (2003). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science (New York, N.Y.)*, *301*(5633), 640–643. https://doi.org/10.1126/science.1087262

Yang, S., Eckmann, L., Panja, A., & Kagnoff, M. (1997). Differential and regulated expression of C-X-C, C-C, and C-chemokines by human colon epithelial cells. *Gastroenterology*, *113*(4), 1214–1223. https://doi.org/10.1053/gast.1997.v113.pm9322516

Zhang, P., Summer, W. R., Bagby, G. J., & Nelson, S. (2000). Innate immunity and pulmonary host defense. *Immunological Reviews*, *173*(1), 39–51. https://doi.org/10.1034/j.1600-065X.2000.917306.x

Zhang, Y.-Z., & Li, Y.-Y. (2014). Inflammatory bowel disease: Pathogenesis. *World Journal of Gastroenterology*, *20*(1), 91–99. https://doi.org/10.3748/wjg.v20.i1.91

Zhou, C., Yin, G., Liu, J., Liu, X., & Zhao, S. (2011). Epithelial apoptosis and loss in airways of children with asthma. *The Journal of Asthma: Official Journal of the Association for the Care of Asthma*, *48*(4), 358–365. https://doi.org/10.3109/02770903.2011.565848

APPENDICES

5XTBE Buffer

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

Make the final volume upto one litre and final pH 8.

6XGel loading dye

10 mMTris pH8

0.03% Bromophenol blue

60% Glyerol

60 mM EDTA

5XRNA Loading Buffer

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

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

TRIZOL Reagent

4M Guanidine thiocyanate Phenol

0.8 M Sodium Citrate

0.5% N-laurosyl-Sarcosine

0.1 M β-mercaptoethanol

Phosphate Buffer Saline (1 litre)

NaCl	8g
$Na_2HPO_4.2H_2O$	1.44g
KCl	0.2g
KH_2PO4	0.2g

pH was maintained to 7.3-7.4 with HCl

Trypan Blue (2% Stock solution)

Tryphan Blue	2g

Sodium azide	0.2g
MilliQ water	100ml

Kept at 37^0C for 10 minutes and stored at 4^0C.

Trypan Blue (0.2% Working solution)

2% stock solution 3ml

PBS 27ml

Final volume 30ml kept at 37^0C for 10 minutes and stored at 4^0C.

DEPC Treated Water:

MilliQ water	2L
Di-ethyl pyrocarbonate	2ml