TOXICOLOGY: INVESTIGATION OF INTERLEUKIN-1β FROM POOLED HUMAN BLOOD STIMULATED WITH TOXICANTS AND ASSOCIATED MOLECULAR TOXICITY

A THESIS PRESENTED BY

PRAJITHA N

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE AWARD OF

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CERTIFICATE

I, Prajitha N, hereby certify that I had personally carried out the work depicted in the thesis entitled "***Toxicology: Investigation of Interleukin-1β from Pooled Human Blood Stimulated with Toxicants and Associated Molecular Toxicity***". No part of the thesis contains any matter previously published or written by another person, nor any material that has been submitted for the award of any other degree or diploma of any University or Institute of higher learning, prior to this date except where due acknowledgment has been made in the text.

Place: Thiruvananthapuram
Date: 7th April 2021

Signature:
Name: Prajitha N.
Reg. No: 2017/PhD/13

Dr. P.V. Mohanan

Toxicology Division

This is to certify that Ms. Prajitha N, in the Toxicology Division of this institute has fulfilled the requirements prescribed for the Ph.D. degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The thesis entitled "***Toxicology: Investigation of Interleukin-1β from Pooled Human Blood Stimulated with Toxicants and Associated Molecular Toxicity***" was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

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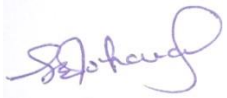

Signature:
Date: 7th April 2021

Dr. P. V. Mohanan
Scientist & Head, Toxicology Division
Sree Chitra Tirunal Institute for Medical-
Sciences and Technology (Govt. of India),
Biomedical Technology Wing, Poojappura,
Thiruvananthapuram-695 012, Kerala, India.

The thesis entitled

'Toxicology: Investigation of Interleukin-1β from pooled human blood stimulated with toxicants and associated molecular toxicity'

Submitted by

Prajitha N

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Thiruvananthapuram

Is evaluated and approved by

Name and signature of the guide

Name and signature of thesis examiner

Kannan Pakshirajan

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ABBREVIATIONS

AB/AM	Antibiotic/ Antimycotic
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AO	Acridine orange
AST	Aspartate transaminase
BBB	Blood Brain Barrier
BSA	Bovine serum albumin
cAMP	cyclic AMP
CARD	Caspase activation and recruitment domain
CD	Cluster of differentiation
cDNA	complementary DNA
cm	Centimetre
CNS	Central nervous system
COX	Cycloxygenase
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
CRP	C-reactive protein
DAMP	Death associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DCFH-DA	Dichloro-dihydro-fluorescein diacetate

dH2O	Deionised water
DilC1 (5)	1, 1′,3,3,3′,3′-hexamethylindodicarbo - cyanine iodide
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbant assay
EP	Endogenous pyrogens
ESR	Erythrocyte sedimentation rate
EU	Endotoxin unit
FACS	Fluorescence Activated Cell Sorting
FBS	Foetal Bovine Serum
FDA	Food and drug administration
FSC	Forward scatter
g	Gram
GALT	Gut associated lymphoid tissue
GGT	Gamma glutamyl transferase
GPx	Glutathione Peroxidise
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
h	Hour

HCT	Haematocrit
HGB	Haemoglobin
HMGB1	High mobility group box1
HRP	Horse radish peroxidase
i.c.v.	Intracerebroventricular
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
IAEC	Institutional Animal Ethics Committee
IFN	Interferon
Ig	Immunoglobulins
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor associated kinase
IRF	Interferon regulatory factor
IVPT	*In vitro* pyrogen test
JAK	Janus kinase
JC1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanineiodide
kDa	Kilodalton
kg	Kilogram

LAL	Limulus amoebocyte lysate
LDH	Lactate dehydrogenase
LMP	Lysosomal membrane permeabilisation
LPO	Lipid peroxidation
LPS	Lipopolysaccharides
LTA	Lipoteichoic acid
M	Molar
MALT	Mucosa associated lymphoid tissue
MAT	Monocyte activation test
MCHC	Mean corpuscular hemoglobin concentration
MD	Myeloid differentiation
MDA	Malonedialdehyde
MDM	Monocyte derived macrophages
mg	Milligram
min	Minutes
MIP	Macrophage inflammatory protein
ml	Millilitre
mm	Millimetre
mM	Millimolar
MMP	Mitochondrial membrane potential

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (Reduced)
NFκB	Nuclear Factor κB
NK	Natural killer cells
nm	Nanometre
nM	nanomolar
NO	Nitric Oxide
NOX	NADPH oxidase
NRU	Neutral red uptake
oC	Celsius
OD	Optical density
OVLT	Organum vasculosum of the lamina terminalis
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PG	Prostaglandin
PHA	Phytohemagglutinin

PI	Propidium Iodide
PLT	Platelets
PMA	Phorbol 12-myristate 13-acetate
POA	Pre-optic area of anterior hypothalamus
PRR	Pattern recognition receptor
PS	Phosphatidylserine
RBC	Red blood cell
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive Oxygen Species
rpm	Revolutions per minutes
RPMI	Roswell Park Memorial Institute medium
RPT	Rabbit pyrogen test
RT-PCR	Reverse transcriptase PCR
s	Second
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEM	Scanning electron microscopy
SOD	Superoxide dismutase
SSC	Sideward scatter
sVCAM	Soluble vascular cellular adhesion molecule

TBS	Tris buffered saline
TCR	T cell receptor
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
TLR	Toll like receptor
TLR	Toll like receptor
TNF	Tumor necrosis factor
TNP	2,4,6-Trinitrophenol
TRAF	TNF receptor associated factor
USP	United states pharmacopeia
UV	Ultraviolet
v/v	Volume/Volume
w/v	Weight/Volume
WBC	White blood cells
WHO	World health organization
µg	Microgram
µl	Microliter
µM	Micromolar

SYNOPSIS

Toxicology: Investigation of Interleukin-1β from Pooled Human Blood Stimulated with Toxicants and Associated Molecular Toxicity

The human immune system is maintained in a homeostatic condition to defend against foreign invaders and respond primarily as an inflammatory networking system. Fever is one of the cardinal symptoms of inflammatory response. Pyrogens are fever-inducing substances that are broadly classified into exogenous and endogenous. All the external agents that can cause fever and febrile reaction in human beings are collectively called exogenous pyrogens, whereas cytokines or inflammatory mediators secreted from immune cells on exposure with exogenous pyrogens are called endogenous pyrogens or pyrogenic cytokines. All the regulatory agencies strictly adhere to the safety aspects of individuals, who tend to get exposed to medical products as part of their diagnostic and therapeutic purposes. Considering these safety measures, it is inevitable that all medical and implantable materials should be free from any pyrogenic contamination.

The most possible pyrogenic contamination in medical products is the lipopolysaccharides (LPS) from Gram-negative bacteria. Lipoteichoic acid (LTA) from Gram-positive bacteria, Phytohemagglutinin (PHA) from red kidney beans and the chemical 2,4,6, trinitrophenol (TNP) was also found to be pyrogenic to human. Over the past years, pyrogenic contamination in medical products is mainly assessed by the Rabbit pyrogen test (RPT). According to reports, large numbers of animals are consumed for pyrogen assay worldwide. An *in vitro* alternative Limulus Amebocyte Lysate (LAL) test developed in the early 1980s to test LPS contamination in medical products is still employed as one of the leading test systems for endotoxin detection. RPT and LAL tests are not suitable for different classes of pyrogens and also rely on ethical issues with animal consumption and species specificity.

Replacement of RPT and LAL is necessary to minimize animal use, ensure species specificity, detect various classes of pyrogens, ease of assay and cost-effectiveness. Later in the 1990s, research focused on developing *in vitro* pyrogen test (IVPT) utilizing human whole blood and monocyte-macrophage cell lines to detect pyrogens. In IVPT, the release of pyrogenic cytokines

interleukin-1β (IL-1β), interleukin-6 (IL-6) and Tumor necrosis factor-α (TNF-α) by immune cells on the challenge with exogenous pyrogens are evaluated using enzyme-linked immunosorbent assay (ELISA). IVPT method fulfils the criteria including species specificity, cost-effectiveness, detection of various classes of pyrogens and replacement of animals. The use of human whole blood or human-derived monocyte-macrophage cell lines precisely mimic the immune response of human against exogenous pyrogens with the production of pyrogenic cytokines and activation of inflammatory signals. Based on the available literature, the present study focused on investigating IL-1β release from pooled human blood stimulated with LPS, LTA, PHA, TNP and associated molecular toxicity. The study hypothesized that detection of IL-1β release from pooled human blood on challenge with toxicants and associated molecular toxicity using THP-1 cells. This leads to an alternative to animal experimentation. Following objectives are planned to achieve the hypothesis.

- **Objective 1**: Evaluation of Inflammatory cytokine release from pooled human blood on challenge with toxicants (LPS, LTA, PHA and TNP) using an indigenously developed ELISA kit.
    - ❖ Phase I: Induction of cytokine release from pooled human blood
    - ❖ Phase II: Detection of IL-1β using ELISA
- **Objective 2**: Molecular toxicity of toxicants (LPS, LTA, PHA and TNP) using THP-1 cells
- **Objective 3**: Administration of endotoxin to rabbits to determine pyrogenicity

The thesis consists of **six chapters**. The **first chapter** is the introduction to the background and motivation of the study. This chapter has briefed about toxicology, immunotoxicology, immune system, inflammation, pro-inflammatory markers and the importance of IL-1β. It gives an idea about the functioning of the immune system to fight against foreign invaders and the significance of inflammatory mediators on immune defence execution. The chapter also mentions pyrogens and their role in fever induction.

The second chapter emphasizes the review of the literature. This chapter explained the existing literature on pyrogens and their classification. Different classes of exogenous and endogenous pyrogens are reviewed in detail. The chapter also directs to the pyrogen detection methods,

limitations and advantages. The chapter reviewed immunotoxicology and methods of estimation of pro-inflammatory cytokines.

Third chapter details the materials and methods adapted to fulfill the objectives. This chapter is divided into three major objectives. The first objective describes the evaluation of IL-1β release from pooled human blood on challenge with LPS, LTA, PHA and TNP. Here, whole blood is collected from healthy human volunteers and evaluated for biochemical and haematological parameters. Blood was pooled together and used to induce cytokine release on challenge with LPS, LTA, PHA and TNP. The supernatant was collected after incubation and stored at -20^{o}C to analyze IL-1β using indigenously developed ELISA. The development of ELISA is detailed in this section.

The second objective includes molecular interactions of toxicants with THP-1 cells. The THP-1 cells were first differentiated into macrophages using Phorbol Myristic acid and were characterized by phase-contrast microscopy, Immuno-cytochemistry and flow cytometric analysis. The cellular morphology and actin organization was studied after exposure with LPS, LTA, PHA and TNP using Giemsa and Rhodamine phalloidine staining, respectively. Cellular organelle function was analyzed using MTT and NRU assay. Free radical generation was assessed by DCFH-DA and Griess assay. Mitochondrial membrane potential (MMP) and lysosomal membrane integrity was studied using JC1, DilC1 (5) and Acridine orange (AO) staining, respectively. The extend of cell membrane damage and loss of viability were analyzed by LDH release and Calcein AM-PI staining. The mechanism of cell death was assessed by Annexin V-PI FACS analysis. Immunotoxicity was analyzed by NFκB and COX2 activation using flow cytometry. Molecular toxicity evaluated by qRT-PCR for analyzing the expression of inflammation-associated genes including NFκB, COX2, IL-1β and TNF-α.

The third objective of the study explains the pyrogenic response of rabbits against intravenously administrated LPS. The rise in rectal temperature for 180 min was measured and recorded. Blood was collected from rabbits to check the haematological and biochemical changes in rabbit blood before and after administration of LPS. Immune activation was analyzed by investigating cytokine release in rabbit blood and expression of inflammation-associated genes in PBMC isolated from rabbits.

The **fourth** and **fifth chapter** detail results and discussion respectively. The result emphasized the investigation of IL-1β released from pooled human blood using indigenously developed ELISA kit. The rabbit was administered with recombinant human IL-1β to induce the production of anti-human IL-1β antibodies. The IgG fraction from rabbit serum was purified using affinity chromatography. The protein concentration was estimated using micro Bradford assay and these proteins were used for coating the ELISA plate. Blood from healthy human volunteers was collected and pooled together. The result of the haematological and biochemical analysis indicates the health status of the pooled blood. The selected toxicants allowed interacting with pooled human blood for 0 to 8h and induction of cytokine release was assessed by ELISA. The result showed dose-dependent release of IL-1β from pooled human blood on challenge with LPS, LTA, PHA and TNP.

This chapter also discuss about the molecular toxicity of LPS, LTA, PHA and TNP using THP-1/ MDM cells. The PMA induced differentiation of THP-1 cells was confirmed using phase-contrast imaging, Immuno-cytochemistry with macrophage marker CD68 and granularity changes with flow cytometric analysis. It was found that LPS do not induce any change in cellular morphology and actin organization. The highest concentration of LTA, PHA and TNP showed a significant difference in morphology and actin organization in MDM cells. MTT and neutral red assays were carried out to check the organelle functions in terms of mitochondrial reduction potential and dye uptake by lysosomes. LPS did not affect the cellular organelle function even after 72h of exposure. However, LTA, PHA and TNP caused dose and time-dependent reduction in organelle function and associated loss of viability. Reactive oxygen and nitrogen radical production after LTA and TNP exposure indicates potential oxidative stress development inside the cells. Loss of mitochondrial and lysosomal membrane integrity observed with JC1 and AO fluorescent staining after LTA, PHA and TNP exposure. Loss of mitochondrial membrane potential (MMP) was again confirmed by FACS analysis using DilC1 (5) fluorescent probe. Shift in DilC1 (5) fluorescence compared to control in LTA, PHA and TNP treated cells was observed in a dose-dependent manner. LPS up to 5EU/ml is not capable of inducing mitochondrial and lysosomal membrane destabilization.

The mechanism of cell death in terms of apoptotic or necrotic stages was assessed by AnnexinV-PI flow cytometric analysis. It was found that LPS do not induce apoptotic or necrotic cell death even at 5EU/ml concentration. PHA mediated cell death is seemed to be via necrosis, where 45.6% of cells were stained with PI at 1000µg/ml treatment. After exposure with LTA and TNP, the major part of cells was in the early and late stages of apoptosis. The results support Calcein AM/PI and LDH release assay that LTA, PHA and TNP induced damage to the cell membrane in a dose and time-dependent manner. The cytoplasmic enzyme LDH was released from the cells to the external media through the membrane pores created by the toxic interaction of LTA, PHA and TNP with the cell membrane. Loss of cell viability after LTA, PHA and TNP exposure indicate the direct cytotoxic potential of these toxicants to monocytes and macrophages.

Expression of inflammatory-associated genes such as NFκB, COX2, IL-1β and TNF-α on LPS, LTA, PHA and TNP exposure were analyzed using qRT-PCR. The increased expression of genes to dose dependant exposure of toxicants indicates their potential immune activation in THP-1 cells. The immunotoxic response of LPS, LTA, PHA, and TNP was again confirmed by analyzing the activation of inflammation-related transcription factor NFκB and inflammatory enzyme COX2 in THP-1 cells after 24h exposure. FACS analysis established that LPS, LTA, PHA and TNP induce dose-dependent activation of NFκB and COX2.

This chapter also explains the results of the pyrogenic response of rabbits to endotoxin administration. All three animals showed pyrogenic response to LPS with a significant rise in body temperature within 30 min following intravenous administration. The leucopenia observed in LPS administrated rabbits indicates potential effects on white blood cells. However, red blood cells parameters are not affected by LPS and were comparable to that of control. The immunotoxic potential of LPS was observed after intravenous administration with a significant rise in inflammatory cytokines IL-1α, IL-1β and TNF-α release in rabbit serum. An increased expression of inflammatory-related genes IL-1β, IL-8 and NFκB in PBMC isolated from rabbit blood were also confirmed.

The summary and conclusion of the study was explained in the **sixth chapter.** The work was summarized with major findings of the study that, pooled human blood acts as an alternative test system for pyrogen analysis with high specificity and sensitivity. The *in vitro* pyrogen test

system is suitable for detecting all classes of pyrogens either in solid or liquid form. The indigenously developed ELISA kit is efficient for the detection of IL-1β at the picogram level. Molecular toxicity of LPS, LTA, PHA and TNP with monocyte model THP-1 depicts that LPS from 0.1EU/ml to 5EU/ml could induce an immune response in monocytes but is not cytotoxic even after 72h of exposure. All the selected toxicants amplified NFκB and COX2 expression and activation in THP-1 cells. A dose-dependent increase in IL-1β and TNF-α gene expression confirmed the immunotoxic potential of LPS, LTA, PHA and TNP. LTA, PHA and TNP showed dose and time-dependent cytotoxicity to THP-1cells with a significant reduction in cell membrane integrity, organelle function, morphology, actin organization, mitochondrial and lysosomal integrity *etc.* LTA, PHA and TNP induced oxidative stress and associated apoptotic or necrotic cell death. The pyrogenic and immunotoxic potential of LPS was confirmed after intravenous administration to rabbits with a significant rise in rectal temperature, cytokine release and inflammatory gene expression. Based on the data obtained from the study it was concluded that pooled blood served as an alternate medium for detection of various classes of pyrogens with high sensitivity.

CHAPTER 1: INTRODUCTION

1. INTRODUCTION

The basic science of poison, toxicology, is a multidisciplinary science, which deals with the study of harmful effects of chemicals or physical agents on living organisms. Toxicology mainly focuses on understanding the mechanisms and reactions taking place in a biological system. German-Swiss physician and alchemist Paracelsus defined the importance of the dose-response relationship as 'All substances are poisons; it is the dose that differentiates a poison and a remedy'. Rather than the dose, route of exposure, duration of interaction, nature of the substance and physical condition of an individual also determines the magnitude of toxicity (Michaleas *et al.,* 2021). Spanish toxicologist Mathieu Joseph Bonaventure Orfila (24 April 1787 – 12 March 1853) was considered the founder of toxicology (Myers, 1961). He first defined the systematic correlation between the chemical and biological attributes of poisons. Generally, in a living system, toxicity exerted by a substance will be systemic or organ-specific. Several sub-disciplines exist, including environmental toxicology, occupational toxicology, regulatory toxicology, food toxicology, clinical toxicology, forensic toxicology, analytical toxicology, mechanistic toxicology, *etc.,* on the concerned area of toxicology (Hayes *et al*., 1996).

Biomaterials, medical devices, injectable and pharmaceuticals intended to use in living subjects should be non-toxic and biocompatible. Contamination of medical products with chemical or biological agents is the major challenge facing clinicians, medical device manufacturers, pharmaceutical manufacturers and regulators. Therefore, it is inevitable that all the medical products should be free from contamination and biological risk (fever and febrile reactions, inflammatory response *etc.*) allied with their use.

The medical products should be screened for an inflammatory response such as fever and febrile reaction and is a mandatory regulatory requirement. The term 'fever/febrile reaction' represents elevated body temperature above normal physiological range (36.5–37.5°C) and is the cardinal symptom of the onset of an infection, injury or tissue destruction. Basically, in every organism, an internal homeostatic mechanism is maintained by adjusting various physiological processes. In human, this homeostatic mechanism is controlled by the pre-optic area of the anterior hypothalamus (POA). POA acts as the principal thermoregulatory centre of the brain. Thermo receptors present in the hypothalamus receive signals of temperature fluctuation from the internal

environment, whereas receptors present in the skin receive signals of temperature fluctuation from external domains. The coordinated action of the central nervous system (CNS) and endocrine hormones is active to achieve constant thermal balance (Anochie, 2013).

Pyrogens are substances that can induce fever and febrile reactions in human beings and animals. The term pyrogen is derived from the Greek word 'Pyr', meaning fever. Fever and febrile reactions are beneficial to the host to fight against infection or injury. Uncontrolled immune responses associated with fever will result in adverse effects like cellular toxicity, hyper- activation of cytokine networks and systemic inflammation. Pyrogenic fever is the most common response present after administering contaminated pharmaceuticals (Das *et al*., 2004), implanted devices and biotechnological products (Garrana *et al*., 2016). Pyrexia is the word commonly used to denote pyrogen mediated fever. Complications associated with pyrexia depend on the type and complexity of pyrogens, immunity of individuals' affected, genotypic and phenotypic difference of the individuals to tolerate heat. Hyperthermic reactions may lead to heatstroke, multiple organ failure and even death if it worsens (Walter *et al*., 2016).

Most biotechnological products are based on recombinant proteins, antibodies or plasmids synthesized or isolated from bacterial species, especially *Escherichia coli* (*E. coli*). There are chances of contamination of bacterial cell components along with these preparations. Introduction of bacterial cell components (remnants/pyrogenic contamination) may result in inflammation or toxic shock. The removal of pyrogenic contaminations from biotechnological preparations is the method of depyrogenation. Elimination of pyrogens is achieved through techniques including chromatography, diffusion, ultra-filtration, heat sterilization, distillation, reverse osmosis, chemical, gas, or radiation sterilization (Salama and Mobarez, 2015). As per the US Food and Drug Administration (FDA), it is mandatory to evaluate the presence of pyrogenic contaminations in biotechnological/biomedical products before their clinical applications. The most commonly used and approved tests are the rabbit pyrogen test (RPT) and Limulus amoebocyte lysate (LAL) assay. Owing to increased animal usage and several other ethical considerations, the RPT have limitations. Monocyte activation tests (MAT) and *in vitro* pyrogen assay (IVPT) is under consideration as the new generation alternative test system. However,

these are still under validation and comprehensive information is not yet fully released (European Pharmacopoeia, 1997, European Pharmacopoeia, 2010).

1.1. IMMUNE SYSTEM

The immune system is a complex network of cells and organs that help human to fight against foreign invaders like bacteria, fungi, viruses and parasites (**Figure 1.1**). Specific self and non-self discriminations characterize the immune system and thereby, the immune defence mechanism is maintained in a homeostatic condition. However, in some situations, these homeostatic mechanisms will fail and that results in autoimmune reactions. In this condition body's own cells or tissues are attacked by the immune cells or antibodies produced by these cells.

A human possesses immunity to a wide variety of biotic and abiotic invaders. This specific or non-specific resistance offered by the immune system is called natural immunity. Innate immune barriers including skin and mucous membrane prevent the entry of microbes into the body and are the central line of primary defence. In some cases, adaptive or acquired immunity is required to fight against infection. Adaptive immunity develops after birth through active infection or passive immunization via maternal or monoclonal antibodies. Immune cells, which participate in natural and adaptive immunity, are closely linked at the time of their action (McCullough and Summerfield, 2005).

1.2. ORGANS OF IMMUNE SYSTEM

Lymphoid organs and immune cells are the two central pillars of the immune system. Lymphoid organs are distributed throughout the body and involve the bone marrow, lymph node, thymus, spleen, gut-associated lymphoid tissue (GALT), mucosa-associated lymphoid tissue (MALT) *etc*.

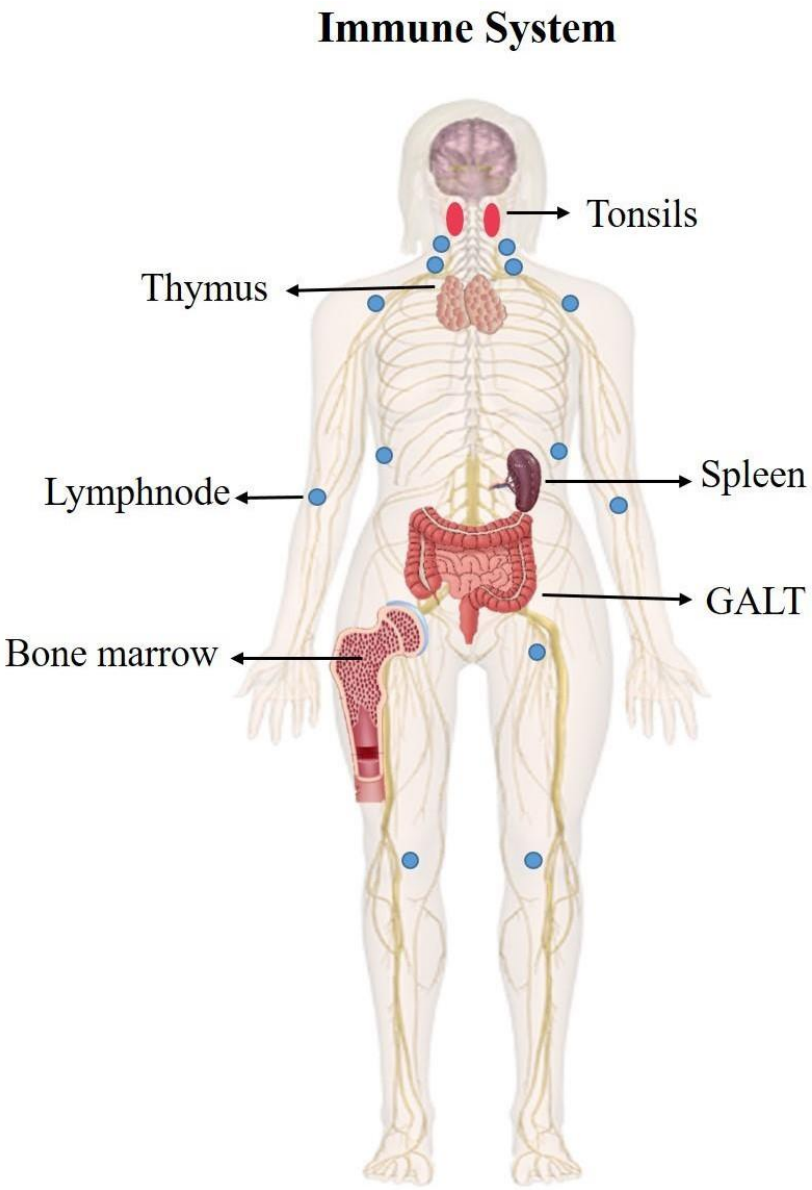

Figure 1.1: Immune system

1.2.1. BONE MARROW

Bone marrow is the primary lymphoid organ where haematopoiesis, or the production of all blood cells, takes place (**Figure 1.2**). Bone marrow present in ribs, vertebrae, sternum and hips is the primary sites for the production of blood cells (approximately 500 billion). The development of Red Blood cells (RBCs) from haematopoietic stem cells present in bone marrow is called

erythropoiesis. The process of lymphopoiesis and myelopoiesis respectively produces principal immune cells like lymphocytes and phagocytes. The lymphoid lineage composed of T-cells, B-cells and natural killer cells (NK) is derived from common lymphoid progenitors. Granulocytes, megakaryocytes and macrophages are derived from common myeloid progenitors. All the above cells in their immature form migrate from bone marrow and enter into blood circulation. Once it enters into the blood, they reach secondary lymphoid organs where they mature and functionally active to defend against foreign invaders (Le Douarin *et al.*, 1984).

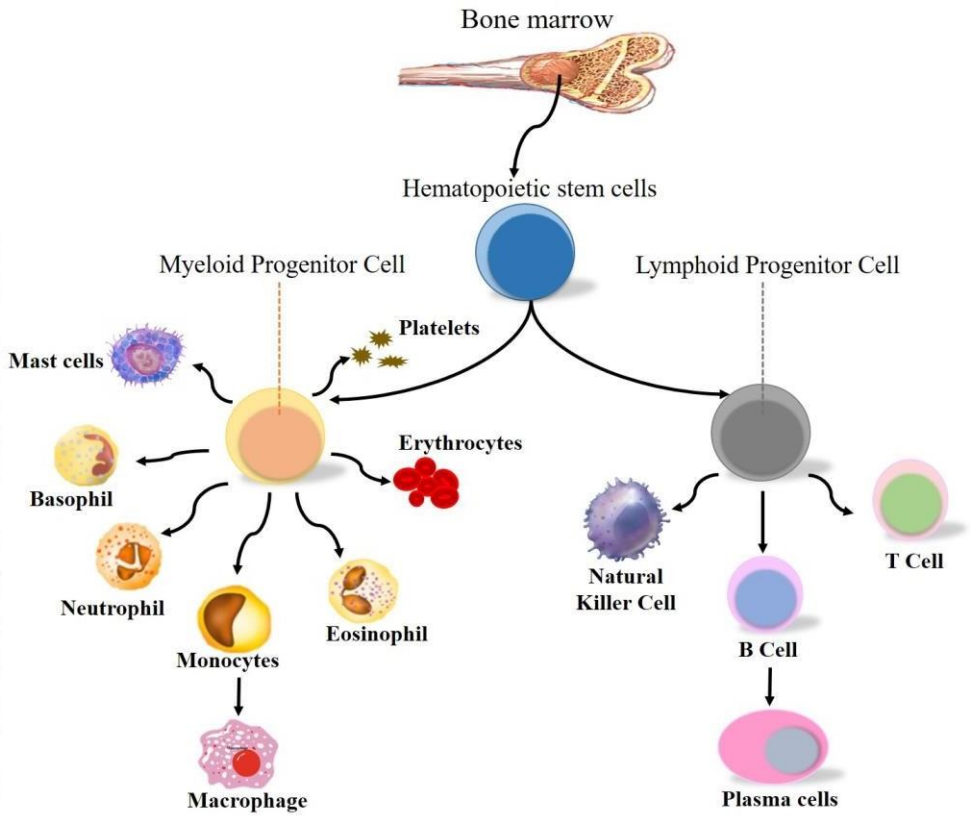

Figure 1.2: Hematopoiesis from bone marrow

1.2.2. THYMUS

Thymus is a primary lymphoid organ situated behind the sternum. Thymus is the site where the production and maturation of thymus-derived T-cells occurs. Thymus derived T-cells protect against foreign antigens, mainly at the time of birth to puberty. Thymus plays a vital role in

immune defence until the adolescence stage of life. After attaining puberty, the size of the thymus reduces drastically and the production of T cells ceases accordingly.

1.2.3. LYMPH NODE

Lymph nodes are the secondary lymphoid organ present in almost all part of the body. They are interconnected through lymphatic vessels in which lymph fluid carry foreign antigens. Lymph node, a bean-like structure divided into a number of lobes or nodules. The lymph node has regions called the cortex, paracortex and medulla, which possess specific immune cells. B-cells produced from bone marrow reach the lymph node and forms clusters in the cortex region are called lymphoid follicles. Naive B-cells mature in the lymphoid follicles and produce antibodies. Paracortex is rich in T-cells where they are in contact with dendritic cells and macrophages (antigen-presenting cells). Foreign antigen reaches the lymph node through the drainage of lymph fluid via afferent blood vessels. After encountering antigens, B-cells get activated and produce specific antibodies. B-cells differentiate to form plasma cells and are active in the production of multiple copies of antibodies. Rather than producing antibodies, B-cells process the antigens engulfed into the cytoplasm and present antigenic determinants to T-cells via major histocompatibility complex Class II (Class II MHC). MHC presentation of antigens activates T-cells to defend against specific antigens through the direct cytotoxic effect or through helping activation of other immune cells (macrophages). The antibodies, mature T-cells, macrophages and plasma cells from lymph node exit into the blood stream through efferent vessels. These antigen-specific antibodies and other immune cells can effectively destroy similar antigens present in other parts of the body (Von Andrian and Mempel, 2003).

1.2.4. SPLEEN

Spleen is principally considered as the graveyard of red blood cells where it filters the blood. Defective blood cells are get trapped by macrophages and release their heme and globin part inside the organ. Similar to the lymph node, the spleen also works as a secondary lymphoid organ. Structurally spleen is divided into two compartments named red pulp and white pulp. Red pulp is the area where degradation of RBC’s occurs. White pulp is the region contributing role in the immune response. B and T-cells actively participate in encountering foreign antigens entering into the organ through the blood. There is a marginal zone between red and white pulp,

where blood circulates freely. Macrophages and other antigen-presenting cells present in the blood interact with the antigens. However, the entry of macrophages into white pulp requires activation of specific G protein-coupled receptors. B-cells and antigen-presenting cells enter the white pulp through these receptor interactions and contact with T-cells. Activation of T-cells further initializes an adaptive immune response against specific antigens (Mebius and Kraal, 2005).

1.2.5. GUT-ASSOCIATED AND MUCOSA-ASSOCIATED LYMPHOID TISSUE (GALT)

Gut associated lymphoid tissue (GALT) are the diffused patches of lymphoid tissues distributed throughout the sub-mucosal membrane lining of the body including nasopharynx, elementary tract, thyroid, breast, lung, salivary glands, eye, and skin. Tonsils, payer's patch of the small intestine and vermiform appendix, participate in the defence against foreign bodies by initiating inflammatory responses. These specialized structures possess lymphoid follicles similar to the lymph node, where B-cells get activated and release antibodies. IgA are the major antibodies participating in defence against antigens entering through these mucosal surfaces (Brandtzaeg, 2003)

1.3. CELLS OF IMMUNE SYSTEM

Immune cells emerging from bone marrow travel throughout the body via blood vessels and enter into other lymphoid organs. Within the lymph node and thymus, immune cells encounter antigenic substances and differentiate into their active form. The preliminary stage of immune detection begins with the detection of foreign invaders by phagocytes and dendritic cells. The recognition of antigenic determinants is done by specialized receptors present on the membrane surface of immune cells collectively called pattern recognition receptors (PRR). Cytoplasmic, endosomal as well as receptors in secreted form, also participate in such recognition. They specifically recognize pathogen-associated and cell death associated molecular patterns (PAMP and DAMP). Different classes and types of such receptors present on cells include toll- like, nod like, retinoic acid-inducible gene 1 like and the C-type lectin receptors. Interaction of these receptors with antigens activates various signalling pathways and ultimately results in

inflammation, phagocytosis, antibody production, apoptosis *etc.*, (Amarante-Mendes *et al.*, 2018).

Monocytes, macrophage, granulocytes (neutrophils, eosinophils, basophiles), mast cells *etc.*, engulf and process antigens within their cytoplasm and present specific antigenic determinants toB and T-cells. B and T-cells are the primary lymphocytes responding to antigenic signals. B-cells produce specific antibodies against foreign antigens and T-cells directly (Cytotoxic T-cells) or indirectly (helper T-cells) attack infected or injured cells. Eosinophils, basophils and mast cells possess granules within their cytoplasm rich in proteolytic enzymes and allergic mediators. Enzymes like peroxidases, ribonucleases and lipases released from eosinophils are highly reactive against parasites and allergens. Histamines and tryptase enzyme released from mast cells also activate anaphylactic reactions in the host. Eosinophils and mast cells are the primary cells contributing to hypersensitivity reactions (Guermonprez *et al*., 2002). Cellular response to foreign invaders was depicted in **Figure 1.3**.

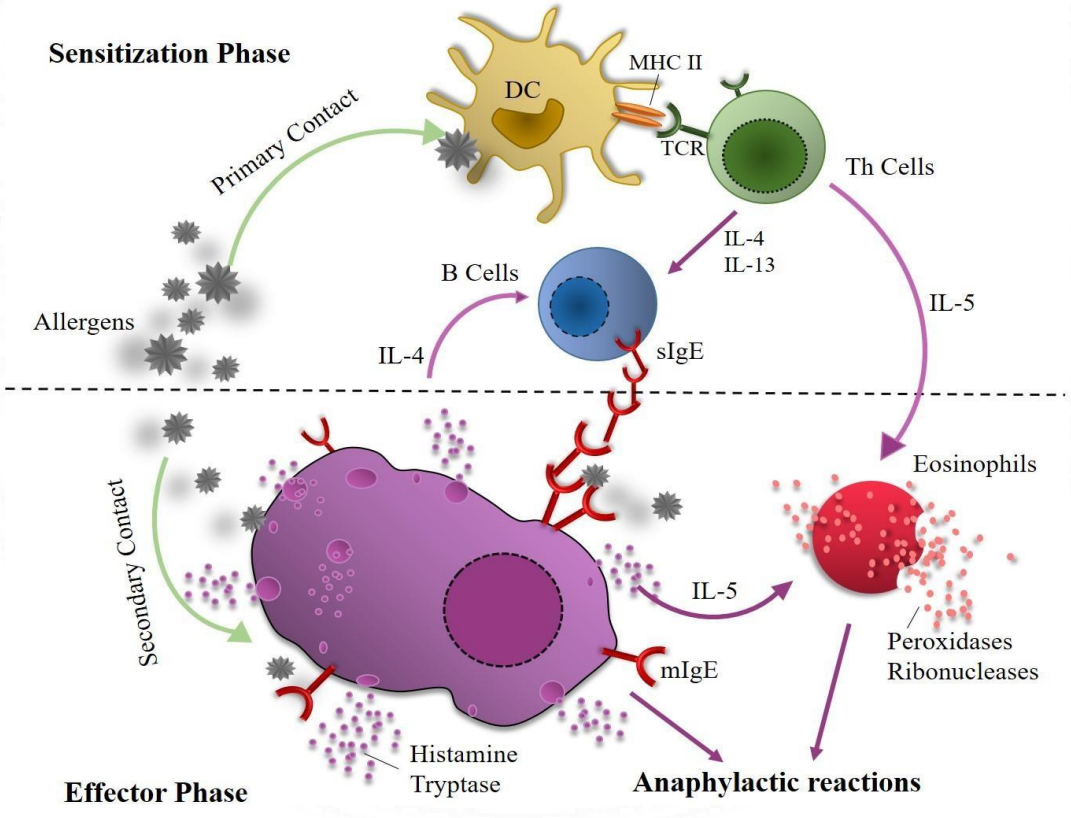

Figure 1.3: Cellular response to foreign invaders

Activated B-cells on interaction with foreign antigens differentiate through various cytokine-mediated signalling into antibody-secreting plasma cells. T and B-cells together constitute the adaptive immune response through which there is a generation of immunological memory. Secondary infection with the same or antigenically similar infection can be defended immediatelyby the memory cells (Crotty and Ahmed, 2004). Another important innate lymphoid cell includes; cytotoxic NK cells, which acts non-specifically to various classes of antigens, but their defence does not lead to the production of memory cells for future protection. Other than immunologically active cells like lymphocytes, neutrophils, macrophages, mast cells and eosinophils, non-immune cells like erythrocytes, vascular endothelial cells and platelets also contribute to inflammatory responses.

1.4. IMMUNOTOXICOLOGY

Immunotoxicology is a branch of toxicology originated in early 1970s which deals with the study of effects of toxic substances on the immune system. External toxic substance or xenobiotics include industrial chemicals, heavy metals, agrochemicals, pharmaceuticals, drugs, ultraviolet radiation, air pollutants and some biological materials. They can alter the homeostatic stage of the immune system. This may lead to hypersensitivity, immune suppression or autoimmune reactions. Over the past forty years, significant research efforts in the field of immunotoxicology resulted in development of specific assays and identification of sensitive endpoints. These assays measure the effects of xenobiotics on human immune response. Regulatory agencies have developed specific immunotoxicity testing guidelines based on various interdisciplinary research data on immunotoxicity.

According to FDA and World Health Organization (WHO) guidelines, immunotoxicity studies are performed according to the type of material being evaluated. These are assessed for a basic and higher level of toxicity. The Type I assay includes basic and expanded level of evaluation. Level I immunotoxicity evaluation have haematology, biochemistry, histopathology and body weight measurements. The use of animals for toxicity analysis after exposure of antigens, vaccines, infectious agents, or tumour cells is coming under Type 2 or Level II immunotoxicity assays. If there are alterations in routine haematological, biochemical and histopathological

parameters in a short term or sub chronic range, the same substance is subjected for more specific and advanced toxicity studies in selected animals (Luster *et al*., 1988).

White blood cell count, differential count, eosinophil counts are the routine haematological assays, whereas serum protein and liver enzyme assays are coming under biochemical analysis. Histopathology of the spleen, lymph node, thymus, GALT, liver and kidney are also included in type 2 analysis. If the Xenobiotic seems to be immunotoxic, expanded assays will be performed. It involves the cytometric analysis of B and T lymphocytes, electrophoretic analysis of serum protein fractions and immunoglobulins (IgG, IgM, IgA and IgE). Functional activity of NK cells, mitogenic stimulation assay for B and T-cells, macrophage phagocytic potential analysis, Bone marrow stem cell progenitor population assay are the extended *in vitro* assays performed if the substance caused changes in the function of cells.

Delayed hypersensitivity test with dinitrochlorobenzene (DNCB) and mixed lymphocyte reaction assay also included in Type 2 assay. Assays using syngeneic tumour cells were involved in type 2, where PYB6 sarcoma assay and the B16F10 melanoma assay in the mouse are the validated tests. Inoculation of the lung tumour model and the MADB106 tumour cell line has also been validated for immunotoxicity studies (Bick, 1982). Evaluation of immunotoxicity mainly focused on inflammation, immune suppression, autoimmunity reactions and changes in both cellular and humoral responses.

1.5. EVENTS OF INFLAMMATION

Inflammation is the first line of defence offered by the immune system to noxious conditions like pathogenic infection, tissue injury, invasion of toxic compounds and irradiation with harmful chemicals. Usually, inflammation is a well-regulated process that initializes cellular and molecular interactions. This mitigation process ultimately results in the elimination of infectious and non-infectious agents and helps restore tissue homeostasis. According to the Roman doctor Cornelius Celsus's definition in the 1st century AD, inflammation is characterized by *rubor et tumour cum calore et dolore*, which means redness and swelling with heat and pain (Medzhitov, 2010).

Inflammatory events propagate in four stages and are mentioned below;

1. Entry of inducers
2. Activation of innate immune cells
3. Release of inflammatory mediators and
4. Initiation of response signals in target cells.

Initial detection of immune invaders by specific PRRs on immune cells results in high production of pro-inflammatory cytokines. This is the clinical indication of normal versus inflammatory biological response (Xu and Larbi, 2018).

The physiological events of inflammation begin with the detection of foreign bodies, bacteria, virus, fungus or their specific PAMPs by PRR. Injured or damaged cells within the body trigger signals via PRRs and induce inflammatory reactions. When an active signalling cascade begins within the immune cells, it propagates the intensity of inflammation by releasing various pro-inflammatory molecules. During inflammation, there is an activation of intracellular signalling through mitogen-activated protein kinase (MAPK), nuclear factor Kappa B (NFκB), Janus kinase (JAK) signal transducer and activator of transcription (STAT) pathways. Inflammation also triggers the release of inflammatory mediators such as cytokines, chemokines, C-reactive protein (CRP), haptoglobin, serum amyloid A, fibrinogen and alpha 1-acid glycoprotein. Enzymes such as high-mobility group box 1 (HMGB1), superoxide dismutase (SOD), glutathione peroxidase (GPx), NADPH oxidase (NOX), inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) acts at different stages of inflammatory response to execute various intracellular reactions (Chen *et al*., 2018).

1.6. IMMUNE SUPPRESSION

Immune suppression is the process of inactivation or suppression of the immune system that weakens the body's ability to fight against foreign invaders and diseases. Inactivation of immune cells, organs or both increases the susceptibility for multiple infection and concurrent diseases. Immune suppression is either a parallel effect of persistent illness or the side effect of some medication. Even though immune suppression is dangerous, some medical conditions such as

auto-immune reaction and tissue or organ transplantation strictly recommend immune suppression with immunosuppressive drugs (Mukherjee and Mukherjee, 2009). To reduce the chance of rejection in transplantation, it is necessary to suppress individuals' immune system using appropriate drugs. Immune suppression is common in individuals undergoing chemotherapy, antibody therapy, corticosteroid therapy *etc.* It is also associated with patients who consume TNF-α inhibitor drugs as part of treatment for auto-inflammatory diseases. Medical conditions such as asplenia, primary immunodeficiency syndromes and human immune deficiency virus infection can induce immune suppression. Various immune suppressants are available to inactivate the immune system by targeting specific molecules or metabolic pathways. Some of the immune suppressants are anti-TCR agents, calcineurin inhibitors, complement inhibitors, cytokine inhibitors, chemokine inhibitors, JNK inhibitors, mTOR inhibitors and inhibitors of DNA/RNA synthesis (Wiseman, 2016).

1.7. AUTO-IMMUNE REACTIONS

An auto-immune reaction is an abnormal condition in which the immune system attacks self-cells or tissues by considering it as a foreign substance. Destruction of the body's own cells or tissues may cause an imbalance in homeostasis and ultimately results in severe auto-immune diseases. In some cases, auto-immune diseases are genetically transferable from generation to generations. Systemic lupus erythematosus is the best example of auto-immune disease transferable from parents to their offspring. Treatment for auto-immune reactions is designed according to the severity and type of immune reactions. Basically, the human immune system and its actions are operated in a regulated manner. The production of immune cells (T and B-cells) from bone marrow is a controlled process. These cells that interact with self-antigens are removed or inactivated at the time of production or release. Failure of this mechanism will produce self-reacting T-cells and self-reacting antibodies (Rosenblum *et al.*, 2015).

In addition to defective immune regulation, environmental factors such as recurrent infection and Ultra Violet (U.V) radiation also trigger auto-immune reactions. Auto-immune reactions propagate severe inflammation at specific tissue, organs or other parts of the body. The most common auto-immune reactions affecting the human population worldwide include rheumatoid

arthritis, celiac disease, autoimmune hemolytic anaemia, type 1 diabetes mellitus, Graves' disease, Sjögren's syndrome, Hashimoto's thyroiditis, myasthenia gravis, inflammatory bowel disease, multiple sclerosis, psoriasis *etc.*,. Generally, a combination of anti-inflammatory drugs is used for the treatment of auto-immune diseases. At present non-steroidal anti-inflammatory drugs is the most accepted immune suppressant for auto-inflammatory conditions. New modalities of treatment are in place for auto-immune diseases. These new modalities include targeted therapy to regulate T-cell production and activation, B-cell differentiation and antibody secretion, cytokine release and signalling (Rosenblum *et al.*, 2012). Analysis of specific and non-specific markers such as ESR, complete blood cell count, CRP, anti-nuclear antibodies and pro-inflammatory cytokines helps in the early detection, diagnosis and treatment of auto-immune diseases.

1.8. IMPORTANCE OF PRO-INFLAMMATORY MARKERS

Pro-inflammatory markers are signalling molecules released from immune cells at the time of initiation of inflammation or disease. Pro-inflammatory markers help in the detection of initial activation of the immune system and diagnosis of diseases. The increase or decrease in the concentration of pro-inflammatory markers depends on the type of stimuli and the system that gets affected. Inflammation results in the elevation of different kind of pro-inflammatory markers. Pro-inflammatory cytokines act as biomarkers to identify and resolve the disease at the initial stage. The pro-inflammatory cytokines also have a cardinal role in therapeutic interventions.

Following are the clinically important and commonly analyzed pro-inflammatory markers:

- C reactive protein (CRP)
- Erythrocyte sedimentation rate (ESR)
- Soluble vascular cellular adhesion molecule (sVCAM-1)
- Cytokines (IL-1β, TNF-α, IL-6, and IL-10)
- Apolipoprotein A1 (Apo-A1)

- Apolipoprotein B (Apo-B)
- Complement component 3 (C3)
- Adiponectin
- Fibrinogen and
- MetS/MetS components.

These pro-inflammatory markers are seemed to be present in samples (blood or other organs) at acute phase of inflammation or disease. Some of the pro-inflammatory markers are non- specific and are very sensitive in the prediction/commencement of immune response. CRP is a non-specific acute phase protein synthesized and released from the liver and seems to be elevated in almost all inflammatory processes. The releases of pro-inflammatory markers are interconnected with the signalling of cytokines. Expression of genes specific for pro- inflammatory markers will get activated on particular cells, organ or tissue at the time of inflammation (Kir *et al.*, 2019).

1.8. EMPHASIS ON INTERLEUKIN 1-BETA (IL-1β)

Interleukins are inflammatory mediators released by leukocytes. They act as communication molecules between immune cells during inflammation or injury. The interleukins family is an eleven membered cytokine family played crucial role in acute and chronic inflammatory responses. They include IL-1α, IL-1β, IL-1Ra, IL-18, IL-33, IL-36Ra, IL-36α, IL-37, IL-36β, IL-36γ and IL-38 (Dinarello, 2011). Among these, IL-1β is the most prominent and characterized cytokine involved in inflammatory signalling and is the potent pleiotropic cytokine that participates in fever response and pain induction.

Monocytes, macrophage and dendritic cells predominantly produce IL-1β. At the time of synthesis, IL-1β exists as a biologically inactive precursor known as pro-IL-1β (31kDa). When PAMPs or DAMPs interact with Toll Like Receptors (TLR), which trigger the processing or secretion of active IL-1β (17kDa) from immune cells. The protein NLRP3 (NLR Family Pyrin Domain Containing 3) along with the adaptor molecule, Apoptosis-associated Speck-like protein containing a CARD (Caspase activation and recruitment domain) and inactive pro-caspase-1

forms a complex within a specialized multi-protein called the inflammasome. Activated caspase-1 released from inflammasome cleaves inactive pro-IL-1β and release mature IL-1β. Mature IL-1β is rapidly secreted out from the immune cells to propagate inflammatory response (**Figure 1.4**). This activation process is initiated by NFκB mediated transcription of cytokine genes within the cell nuclei (Martinon *et al*., 2009). Alternate activation of pro-IL-1β was present in neutrophils, where cleavage was attributed by elastase and proteinase-3 enzymes. Activated IL-1β interacts with IL-1 receptors present on target cells. IL-1β and their receptors stimulate the gathering of various intracellular enzymes and adaptor molecules. This will ultimately result in the transcription of NFκB dependent genes and further biological signaling events (Netea *et al*., 2015).

The primary role of IL-1β is the initiation and propagation of inflammatory reactions. IL-1β pleotropically interact with other cytokines, chemokines and growth factors. The sickness syndromes like fever, chills, fatigue, loss of concentration *etc*., are associated with elevated IL-1β in blood and brain. It is also noted that the up-regulated IL-1β directly or indirectly affects the homeostasis of CNS functions. Cytokines reach CNS via diffusion through the fenestrated epithelial lining of the blood-brain barrier (BBB). IL-1β crosses the BBB and principally affects the circumventricular organ of the hypothalamus and alters the thermoregulatory mechanism. In some instances, cytokines' signals pass through vagal neuronal synapses originating from a peripheral organ like the liver. Direct or indirect signalling of IL-1β to CNS alters the thermoregulatory mechanism of the hypothalamus and results in fever and febrile reactions (Roerink *et al*., 2017).

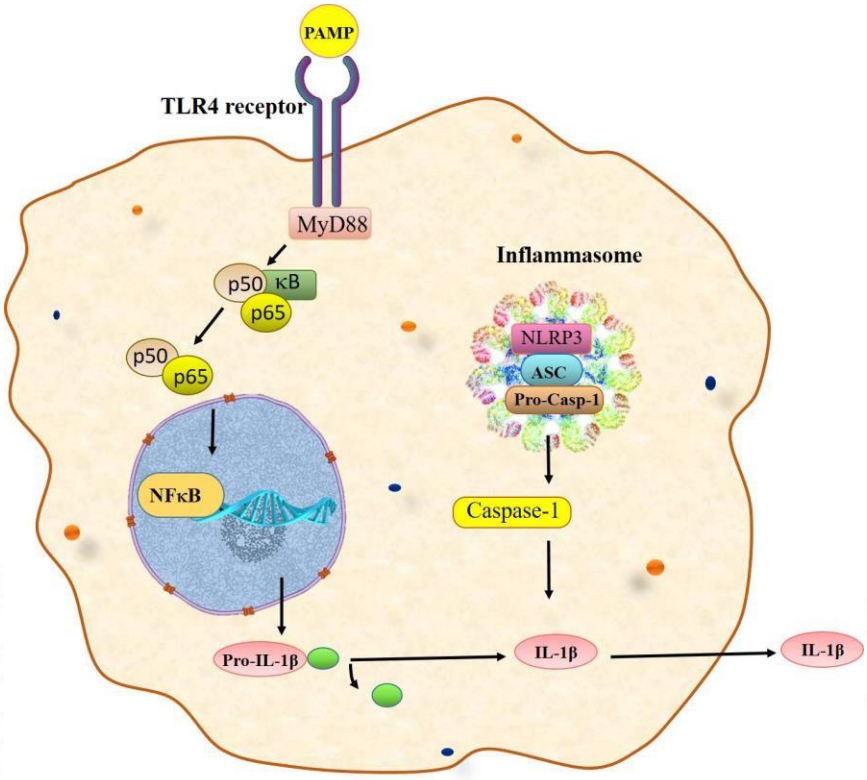

Figure 1.4: Synthesis and release of interleukin-1β

IL-1β displays momentous interaction between humoral and cellular immunity, which has a cryptic role in developing auto-inflammatory disorders. Following are the major auto inflammatory disorders;

- Periodic Fever
- Aphthous Stomatitis, Pharyngitis and Adenitis (PFAPA) syndrome
- Rheumatoid arthritis
- Type 2 diabetes mellitus
- Uveitis
- Pericarditis
- Behçet's disease

- Gout
- Sjögren's syndrome
- Interstitial lung diseases and
- Still's disease.

The severity of auto-inflammatory diseases seemed to be reduced in the absence of IL-1β secretion (Lopalco *et al*., 2015). Cytokines are used as therapeutic agents for cancer treatment since they involve the growth and transformation of cells. Dysregulation of IL-1β shown to be associated with the following human malignancies (Litmanovich *et al*., 2018);

- malignant gliomas
- pancreatic cancer
- hepatocellular carcinoma
- oesophagal squamous cell carcinoma
- breast cancer
- multiple myeloma
- genitourinary cancers *etc*.,

Reports indicated rapid up-regulation of IL-1β associated with multiple inflammatory changes in rodents at the time of experimental brain injury. IL-1β up regulation induced neutrophil infiltration, BBB damage, astrogliosis and neovascularisation in experimental animals. Administration of IL-1β receptor antagonist (IL-1Ra) reduces focal cerebral ischaemia, traumatic and excitotoxic injury in rodents. Antagonist blocks the interaction of IL-1β with their specific receptors on brain cells. This observation directs the fundamental role of IL-1β in the initiation of inflammatory brain damage (Relton and Rothwell, 1992). IL-1β is reported to induce inflammatory changes in endothelial as well as vascular smooth muscle cells. It alters the function of cardiac myocytes and impairs contractile nature of heart muscles. It was evident that IL-1β plays a major role in atherogenesis and associated cardiac complications. Targeting of IL-

1β ushers a new era of anti-inflammatory therapies for various inflammatory diseases, including atherosclerosis (Abbate *et al*., 2013).

1.9. MOTIVATION OF THE STUDY

In the present scenario, medical research focused on the safe and effective implementation of diagnostic and therapeutic strategies to ensure the quality of the health care system. Regular monitoring and validation of safety aspects of medical products are of great importance to minimize the risk associated with its use. Assessment of health care products such as pharmaceuticals, injectables, implants, surgical devices, and recombinant proteins for possible pyrogen contamination is the primary criterion to be fixed to accomplish the safety of individuals under diagnosis or treatment.

The microbial or non-microbial pyrogens that can induce fever or febrile reaction in the living system lead to immune activation, septic shock or even death in extreme condition. Over the past fifty years, large numbers of rabbits were consumed to assess pyrogenic contamination and the RPT is still going on as the primary test system. Because of the ethical consideration and minimizing animal consumption, research focused on an alternative test system for pyrogenicity evaluation. An *in vitro* system LAL, that detects Gram-negative endotoxin was developed in 1980 and was widely accepted as an effective system for pyrogen detection. Even though the LAL test is an *in vitro* assay, it also uses hemolymph collected from horseshoe crab to detect the bacterial endotoxin.

It was well reported that there are several advantages and disadvantages for RPT and LAL test. Hartung and Wendel developed a new *in vitro* system that utilizes human blood as the test system to detect pyrogen contamination. The human whole blood assay emerged as a specific and sensitive test system for pyrogen detection either in solid or liquid form. Human blood has the advantage over RPT and LAL assay, which include species similarity, easy availability, low cost and detection of various classes of pyrogens. The induction of the release of pyrogenic cytokines from human blood cells on challenge with exogenous pyrogen mimics the actual immune response of the human defence system against that pyrogen. Hence, the whole blood assay is considered the most suitable alternate for conventional pyrogen assay. The monocyte activation test was included in the European pharmacopoeia since 2010. The thesis work is

focused on the detection of IL-1β release from pooled human blood on challenge with bacterial, biological and chemical pyrogens. Apart from these, the molecular toxicity of these toxicants was studied using THP-1 monocyte cells. Pooling of multiple blood samples allows minimizing inter-individual variation in cytokine response to pyrogens and reduces multiple sampling for different class of pyrogens.

1.10. OBJECTIVES

The objective of the present study is the investigation of inflammatory cytokine (Interleukin-1β) released from pooled human blood stimulated with bacterial and non-bacterial pyrogens using indigenously developed ELISA. The molecular interaction of pyrogens was studied using human monocytic cell line THP-1 cells. Pyrogenic and immunotoxic response of endotoxin was studied in rabbits.

There are three objectives and are mentioned below;

Objective 1: Evaluation of Inflammatory cytokine release from pooled human blood on challenge with toxicants (LPS, LTA, PHA and TNP) using an indigenously developed ELISA kit.

Phase I: Induction of cytokine release from pooled human blood

Phase II: Detection of IL-1β using ELISA

Objective 2: Molecular toxicity of toxicants (LPS, LTA, PHA and TNP) using THP-1 cells

Objective 3: Administration of endotoxin to rabbits to determine pyrogenicity

The overall summary of PhD work is depicted in **Figure 1.5.**

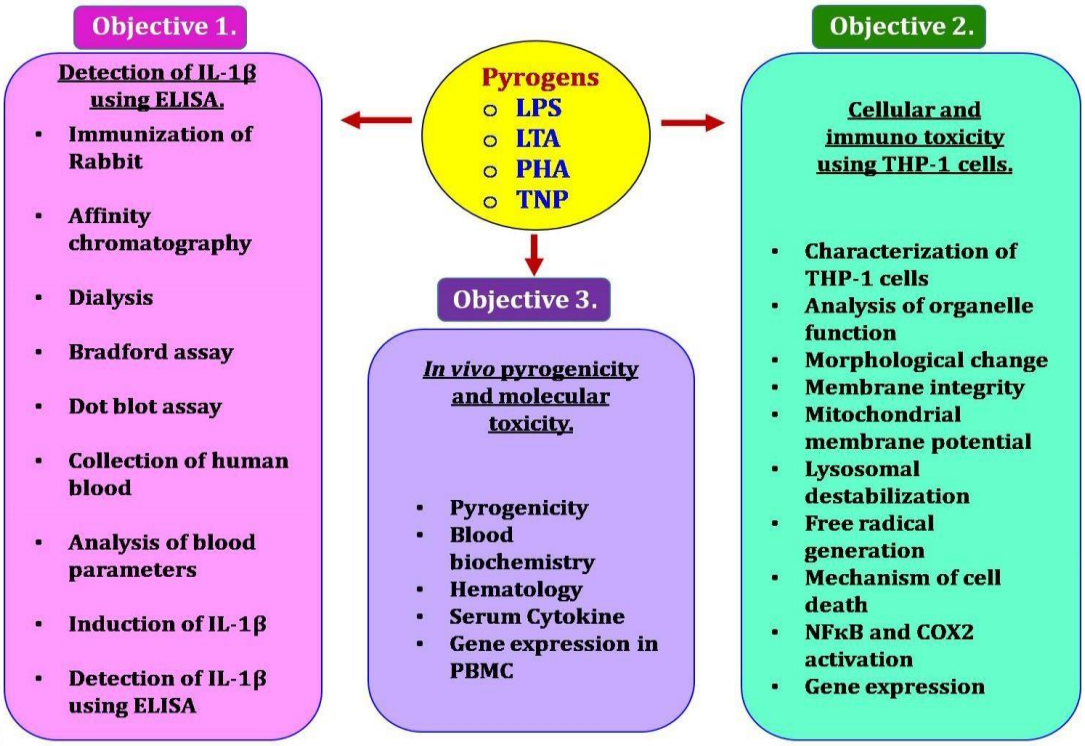

Figure 1.5: Experimental design of the thesis

1.11. THESIS OUTLINE

The thesis is divided into six chapters. The **first chapter** is the **introduction** about the background and significance of the study. This chapter briefs about the immune system, immunotoxicology, inflammation and role of inflammatory cytokines. A concise description of Interleukin-1β is emphasized here.

The **second chapter** is the **review of literature** on pyrogens and their classification. Mechanism of fever induction and detection of pyrogenic substance were also reviewed in this section. The chapter briefly appraised the techniques of cytokine analysis and detection.

Materials and method and is divided into three and is explained in the **third chapter**. The first part deals with the detection of IL-1β using indigenously developed ELISA. Immunization and purification of IgG from rabbit blood were done using affinity chromatography. ELISA plate was coated with purified antibodies and was used for the analysis of IL-1β. Induction of cytokine

release from pooled blood was done using four classes of pyrogens. In the second part, a preliminary molecular and cytotoxicity evaluation of LPS, LTA, PHA and TNP was carried out using THP-1 and MDM cells. This section evaluates the differentiation and characterization of THP-1 cells to MDM cells. This section also detailed about the methodology adapted for analysis of molecular and cytotoxicity of selected toxicants using THP-1/MDM cells. The third section of this chapter represents the pyrogenicity of endotoxin using rabbits. This section dedicates to the biochemical, haematological and immunotoxic response after endotoxin administration. Endotoxin-induced cytokine gene expression of PBMC isolated from rabbit blood is also explained in this section.

Fourth chapter represents the **results** of the study. The first part details results of the detection of IL-1β from pooled human blood. The chapter also explains the outcomes of the cellular interaction studies using THP-1 and MDM cells. The third section of this chapter describes the pyrogenic and immune response of endotoxin in rabbits. The biochemical, immunological and haematological changes in response to endotoxin and gene expression using PBMC is depicted in this section.

Discussion of the results obtained was explained in the **fifth chapter** contains. This discusses the results obtained from ELISA plate development and detection of IL-1β from pooled human blood. The second section elaborates on the results obtained after the interaction of pyrogens with THP-1 and MDM cells. This part suggests the changes in organelle function in THP-1/MDM cells and details the cellular and molecular mechanism underlying LPS, LTA, PHA and TNP interaction with THP-1/MDM cells. Further, this section detailed the immunotoxic mechanism of pyrogens. The third section elaborates the findings on the biochemical, haematological, immunological and pyrogenic response of endotoxin.

Sixth chapter detailed about the **Summary and conclusion** of the thesis work.

CHAPTER 2: REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. EFFECT OF TOXICANT/NON-TOXICANTS ON IMMUNE SYSTEM

2.1.1. PYROGENS

Fever inducing substances, either living or non-living state, are collectively termed as pyrogens. External agents that invade the immune barrier and activate immediate defence stage of immunity are called exogenous pyrogens. Cytokines released from host cells in response to external agents and initiate fever/febrile reactions are called endogenous pyrogens or pyrogenic cytokines(Dinarello, 2004). Broad classification of pyrogens is depicted in **Figure 2.1.**

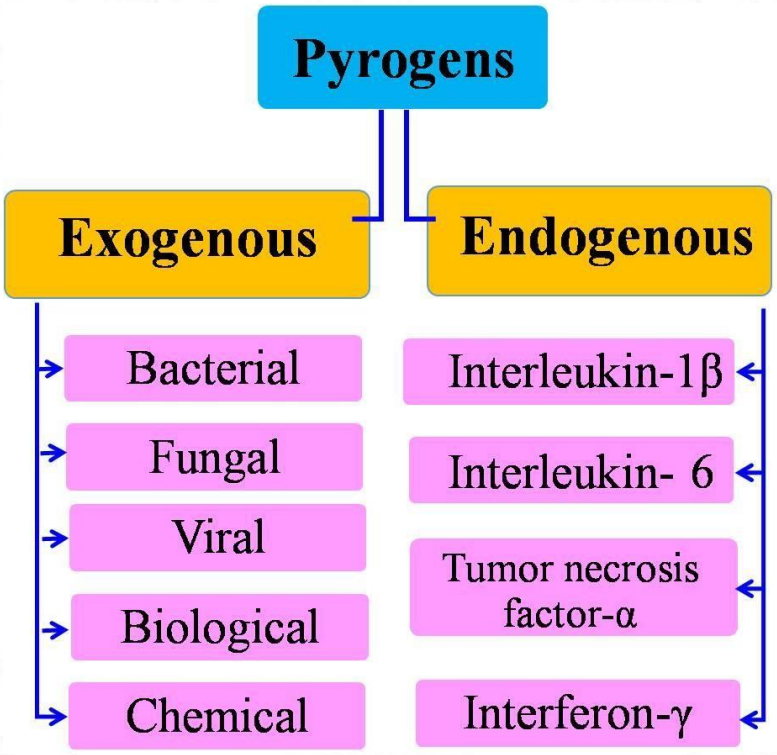

Figure 2.1: Classification of pyrogens

2.1.1.1. EXOGENOUS PYROGENS

The external antigens such as bacteria, virus, fungi, chemicals and leachable from medical device acts as exogenous pyrogens. Lipopolysaccharide (LPS) is the most potent and harmful exogenous pyrogen. LPS is an endotoxin, originated from the cell wall of Gram-negative

bacteria. Similarly, Lipoteichoic acid (LTA) is a non-endotoxin pyrogen that originated from Gram-positive bacteria and plays a key role in initiating fever reactions. Following are the other class of non-endotoxin pyrogens (Borton and Coleman, 2018).

- peptidoglycans
- Metals (nickel salts)
- uncoupling agents of oxidative phosphorylation (Trinitrophenol, Dinitrophenol, 4, 6-dinitro-o-cresol)
- N-phenyl-β-naphthylamine
- Disruptors of the thermoregulatory Center (LSD, cocaine, morphine) and
- inducers (Polyadenylic, polyuridylic, polybionosinic and polyribocytidylicacids)

2.1.1.1.1. LIPOPOLYSACCHARIDES (LPS)

Lipopolysaccharides are the phosphorylated glycolipid present in the outer membrane of Gram-negative bacteria and are universally termed as endotoxin (**Figure 2.2**). LPS is a vital part of the structural and functional integrity of Gram-negative bacteria.

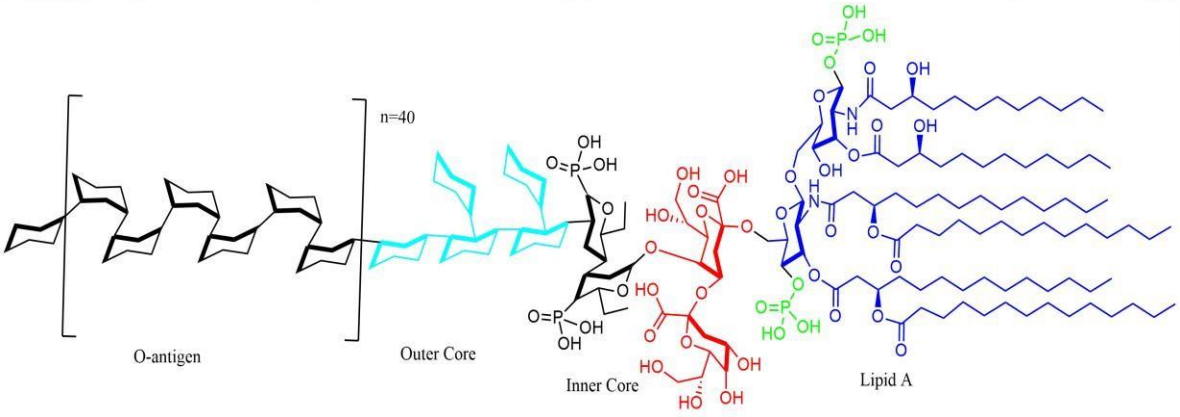

Figure 2.2: Lipopolysaccharides

It contains a hydrophilic polysaccharide chain and hydrophobic lipid moiety termed Lipid A. Lipid A is the most conserved and potential toxic component of LPS, responsible for the

induction of immune response in the living system. Lipid A moiety of LPS interact with innate immune receptors called TLR. Lipid A is covalently linked with non-repeating units of sugar moieties called core polysaccharide. Common sugars such as heptoses and 2-deoxy-d-mannooctulosonic acid functionalized with phosphate or ethanolamine groups are the principal units of core polysaccharides. The distal end of LPS containing repeating units of three to five sugar residues that designate the virulence nature of Gram-negative bacteria is called O-polysaccharide antigen (Stromberg *et al*., 2017).

2.1.1.1.2. LIPOPOLYSACCHARIDE PATHWAY

The innate immune system of human provides defence against pathogenic invasion by various mechanisms and is the first line of protection against any injury or infection. When an external antigenic substance enters into the body, they are recognized by PRRs present over the innate immune cells and trigger inflammatory reaction cascades. Among various PRRs, TLRs play a significant role in discrimination of self and non-self antigens. Membrane anchored domain of transmembrane leucine-rich repeat (LRR) and intracellular TLRs of immune cells recognize PAMPs and signals through cytoplasmic Toll/IL-1 receptor (TIR) domain (Barton and Medzhitov, 2003).

The shedding of LPS from the bacterial cell wall acts as a warning signal for bacterial entry into the host system. LPS is transferred by LPS binding protein (LBP) present in the serum to the TLR receptor complex. Among the 13 different TLR families, TLR4 is more specific for LPS recognition along with an accessory protein myeloid differentiation 2 (MD2). LBP and membrane-anchored glycoprotein CD14 facilitates the conversion of polymeric LPS micelle to monomeric units. This interaction initiates TLR4-MD2 complex activation. Downstream signalling via TIR domain recruits adaptor proteins such as (Molteni *et al*., 2016);

- Myeloid differentiation primary response protein 88 (MyD88),
- TIR-associated protein (TIRAP),
- TIR domain-containing adaptor protein-inducing IFN-β (TRIF) and
- TRIF-related adaptor molecule (TRAM)

MyD88 dependent signalling is activated through phosphorylation of inhibitor of NFκB (IκB), utilizing IL-1 receptor-associated kinase 4 (IRAK-4), TNF receptor associated factor 6 (TRAF6), NFκB and mitogen-activated protein kinase (MAPK). Series of signalling process results in the release of inflammatory cytokines from immune cells. MyD88 independent signalling from activated TLR4 present in the endosomes transmits signals via TRIF and TRAM. The endosome transmitted signals activate interferon regulatory factor 3 (IRF3) transcription factor and ultimately result in enhanced production of Type I IFN in response to LPS stimulation (Lu *et al*., 2008). The pathway of LPS mediated immune signalling is depicted in **Figure 2.3**

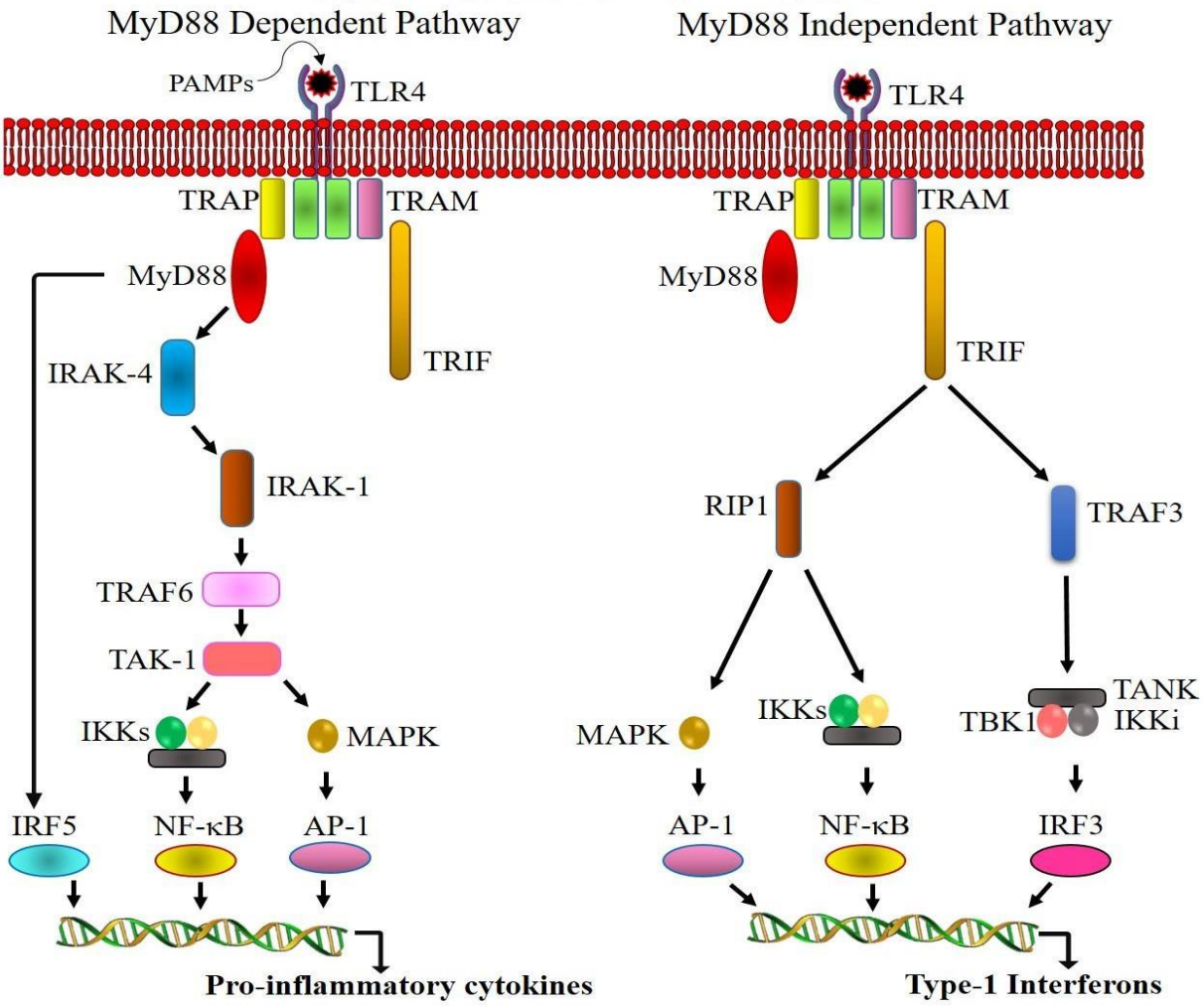

Figure 2.3: Pathway of LPS mediated immune signalling

2.1.1.1.3. LIPOTEICHOIC ACID (LTA)

Lipoteichoic acid is the surface anchored adhesion molecule made of repeating units of phosphodiester-linked polyols (**Figure 2.4**). LTA is the part of the outer membrane of Gram-positive bacteria, including *Staphylococcus aureus, Streptococcus pneumonia, Bacillus subtilis* and *Bacillus anthracis* (Schneewind and Missiakas, 2019).

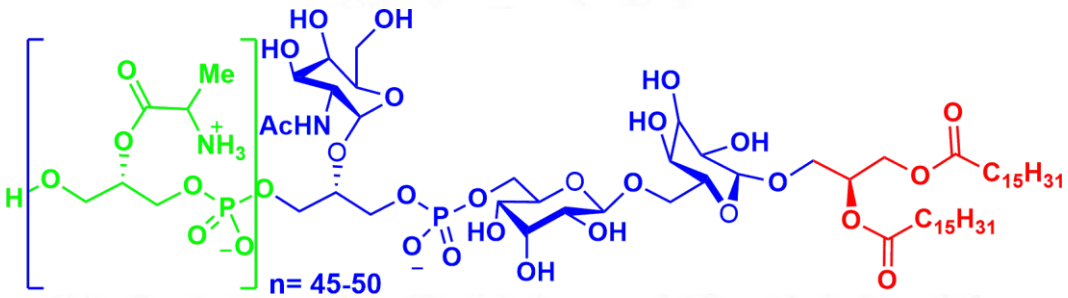

Figure 2.4: Lipoteichoic acid

LTA is an envelope protein that supports bacterial growth, cell division, separation, ion hemostasis, envelope assembly and integrity. Teichoic acid anchored in the cell wall and associated with membrane glycolipids. LTA is classified into five types depending upon the type of sugar and phospholipid ester linkage with the polymer, as LTA I-V (Schneewind and Missiakas, 2014).

2.1.1.1.4. PYROGEN-VIRUS ORIGIN

An entire virus or its components can act as exogenous pyrogen (viral pyrogen). It was reported that the PR8 strain of influenza A and Newcastle disease virus (NDV) when administrated intravenously into rabbits, induced fever and the associated release of endogenous pyrogens (Atkins and Huang, 1958). IL-1 dependent fever reactions found in rats after intraperitoneal injection (i.p) of viral poly I: C (Polyinosinic: polycytidylic acid). A synthetic double-stranded RNA exhibited a significant rise in IL-1β and COX2 mRNA expression in the hypothalamic region of the rat brain (Fortier *et al*., 2004). IL-1, IL-6 and TNF-α are the central endogenous cytokines that facilitate fever induction (pyrogenic response). On the contrary, reports are suggesting *a* lack of correlation of cytokine release in ferret or human phagocytes after influenza

viral infections [virulent (H3N2) or attenuated (H1N1)]. The profile of conventional cytokines IL-1, IL-6 and TNF-α was found to be different from EP obtained from ferret or human phagocytes (Jakeman *et al*., 1991). In some instance, the haemagglutinin and/or neuraminidase content of influenza virosomes initiate pyrogenic stimuli in ferrets (Pickering *et al*., 1992, Alluwaimi *et al*., 1994).

2.1.1.1.5. PYROGENS-FUNGUS ORIGIN

Fungal cell wall components (β-glucan and mannan) are known stimulator of innate immune responses. It was reported that *i.v* injections of viable *Candida albicans, Blastomyces dermatitidis, Histoplasma capsulatum* and *Sporotrichum schenckii* induced fever. Similar to LPS induced fever response, fungal induced fever response started within 1 to 3h after injection and lasted up to 8h. However, pyrogenic tolerance was not detected with repeated exposure to fungi (Braude *et al*., 1960). Nagase *et al.,* (1984) established the role of physicochemical properties of mannan components on pyrogenic fever. Influence of molecular weight, the density of branching moieties and alkali labile nature of mannan components were studied in rabbits. Intracerebroventricular administration of mannan activates its binding receptors present on the third ventricle of the hypothalamus. Thermoregulatory centres of the hypothalamus activated via receptor-mediated signalling. This signalling induces fever response in rabbits against mannans from *S. Cerevisiae.* Similarly, it was reported that mannan proteins from *C. Albicans* and *S. Cerevisiae* caused fever in rat with significant production of PGE2, Nitric Oxide and pyrogenic cytokines (Ataŏglu *et al*., 2000).

2.1.1.1.6. PYROGENS-BIOLOGICAL ORIGIN

Plant lectins are proteins that can interact with the carbohydrate moiety of glycoproteins and glycolipids present on the cell membrane. Glycoprotein-lectin interaction causes agglutination of cells and activates inflammatory signalling. Concanavalin and Phytohemagglutinin (PHA) are the most popular plant lectins that accelerate thymocyte proliferation. PHA is a well-known lectin found in red kidney beans *Phaseolus vulgaris* and can agglutinate leukocytes and red blood cells (**Figure 2.5**). PHA is extensively used as a plant mitogen and is emerging as an ingredient of pharmaceutical products intended for metaphase and cell-stimulating preparations (Movafagh *et al.,* 2011).

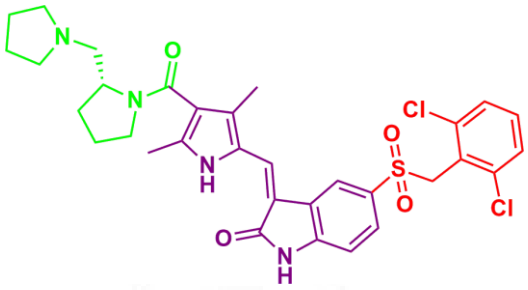

Figure 2.5: Phytohemagglutinin

PHA mediated induction of cytokines was reported by De Groote *et al.,* in 1992, where the whole blood collected from human volunteers respond well with marked production of IL-1β, IL-6 and TNF-α compared to the isolated peripheral blood mononuclear cells (PBMC). Pyrogenic response of plant mitogen concanavalin A is also reported in rabbits. It was evident from the report of Atkins *et al.,* 1973 that concanavalin A is a potent pyrogen, producing biphasic fevers similar to those of endotoxin with an increase in temperature of 1.5-2^{o}C after 15-30 min of intravenous administration. It was noted that concanavalin A significantly induces the release of endogenous pyrogens from leukocytes in a dose-dependent manner.

2.1.1.1.7. PYROGENS-CHEMICAL ORIGIN

The role of liver in the propagation of pyrogenic fever after bacterial, viral and chemical pyrogen was studied since 1966. It was found that the administration of lysergic acid diethylamide and dinitrophenol caused persistent fever in rabbits (Venulet and Desperak-Naciαzek, 1966). The uncoupling agent of oxidative phosphorylation, dinitrophenol, was also known to cause hyperthermia by alternating amino acid metabolism and enhancing the release of EP in the rat brain (Liu *et al*., 2013).

Similarly, the pyrogenic potential of 2, 4, 6- trinitrophenol (picric acid) was studied by Mohanan *et al*., in 2011 using human blood (**Figure 2.6**). It was found that there is a time-dependent release of IL-1β from whole blood and revealed that chemicals such as trinitrophenol could also act as exogenous pyrogen when it interacts with the immune cells.

Figure 2.6: 2, 4, 6, trinitrophenol

2.1.2. DETECTION OF PYROGENS

It is mandatory to detect the pyrogenic contaminations in pharmaceuticals, drugs, recombinant products, and medical devices before their administration into the living system. Detection of pyrogens should minimize the risks associated with immune activation against these external antigenic contaminations. For more than 30 years, rabbit pyrogen tests are in practice for evaluating the pyrogenic contaminations in pharmaceuticals, drugs and medical devices. Presently there are several *in vitro* methods available for the detection of pyrogen. Following methods are widely used for the detection of pyrogenic contamination.

- Rabbit pyrogen test (RPT)
- Limulus Amebocyte Lysate test (LAL)
- Monocyte activation test (MAT)
- Whole blood *in vitro* pyrogen test (IVPT)

2.1.2.1. RABBIT PYROGEN TEST

Rabbit pyrogen test (RPT) is the recommended test for detecting pyrogenic contaminations in parenteral products since 1940. New Zealand or Belgian white rabbits are commonly used for RPT. Rabbits are the preferred animal model for pyrogen detection because of their sensitivity and similarity to endotoxin response. RPT is a qualitative method, which measures the rise in temperature following the intravenous administration of parenteral products or leachable (**Figure.2.7**). According to ISO 10993 / USP, a group of three rabbits was required for the assay. The body temperature of these animals should be in the range of 38 to 39.8^{0}C. 10ml/kg of body

weight is the ideal dose/volume of administration. Change in rectal temperature has to be read in every 30min interval for 3h. RPT test is deemed positive if the body temperature exceeds 0.5°C or more in individual animals (Roberts, 2007).

RPT is a gold standard for detecting pyrogenic contamination and is the first method that ensures safety of parenteral drugs and other biologicals, including recombinant products for human use. However, Vipond *et al*., 2016 reported that an inconsistent result of RPT was observed when meningococcal outer membrane vesicle based vaccines exposed to rabbits. Even after years, the RPT method is still widely used to detect endotoxin and non-endotoxin pyrogens.

2.1.2.2. LIMULUS AMEBOCYTE LYSATE TEST

Limulus Amebocyte Lysate (LAL) test is based on the activation of coagulation enzymes present in the amebocyte lysate prepared from Limulus Polyphemus (horseshoe crab). When this lysate come in contact with endotoxins, the inactive coagulase enzyme gets activated. The activated enzyme converts coagulogen to active coagulin responsible for the formation of a gel clot (**Figure 2.7**). The speed of formation of gel clot is proportional to the concentration of endotoxin present in the sample and is a quantitative assay (Young and Prendergast, 1972). Following are the methods for the detection of endotoxin using LAL reagent

- gel-clot method
- turbidimetric kinetic method
- thrombogenic kinetic method
- chromogenic and
- turbidimetric end-point method.

Formation of lysate clot after interaction with endotoxin is the principle behind the gel clot method. Similarly, the turbidity of the test mixture or colour change of a chromogenic substrate in the test system is the principle behind turbidimetric and chromogenic assay (Blechova and Pivodova, 2001).

Fever, rigours, hypotension and vomiting is the significant complications reported by hemodialzing patients during or after dialysis and was found to be associated with accidental encountering of endotoxins into their blood circulation. In the early 1990s, Pegues *et al.*, experimented with detecting pyrogenic reactions in patients undergoing dialysis. The presence of endotoxin in the filtered and unfiltered dialysate was analyzed using turbidimetric LAL assay. It was reported that dialysate filtered through polysulfone high-flux hemodialyzer was free of 90% of pyrogenic contaminations compared to the unfiltered. Mostly, endotoxins from Gram-negative bacteria are problematic with septic syndromes. LAL test is sufficient to detect their presence even in negligible concentrations (Pegues *et al*., 1992). When comparing with RPT, the LAL test is cost effective and easy to perform. Drugs like hypnotics and radiolabelled pharmaceuticals unsuitable for pyrogen testing with RPT were suitable for LAL reagent and gave high sensitivity results. More importantly, temperature fluctuation of individual rabbits irrespective of pyrogenic reactions makes inconsistent results in RPT. Such influence was absent in the LAL test.

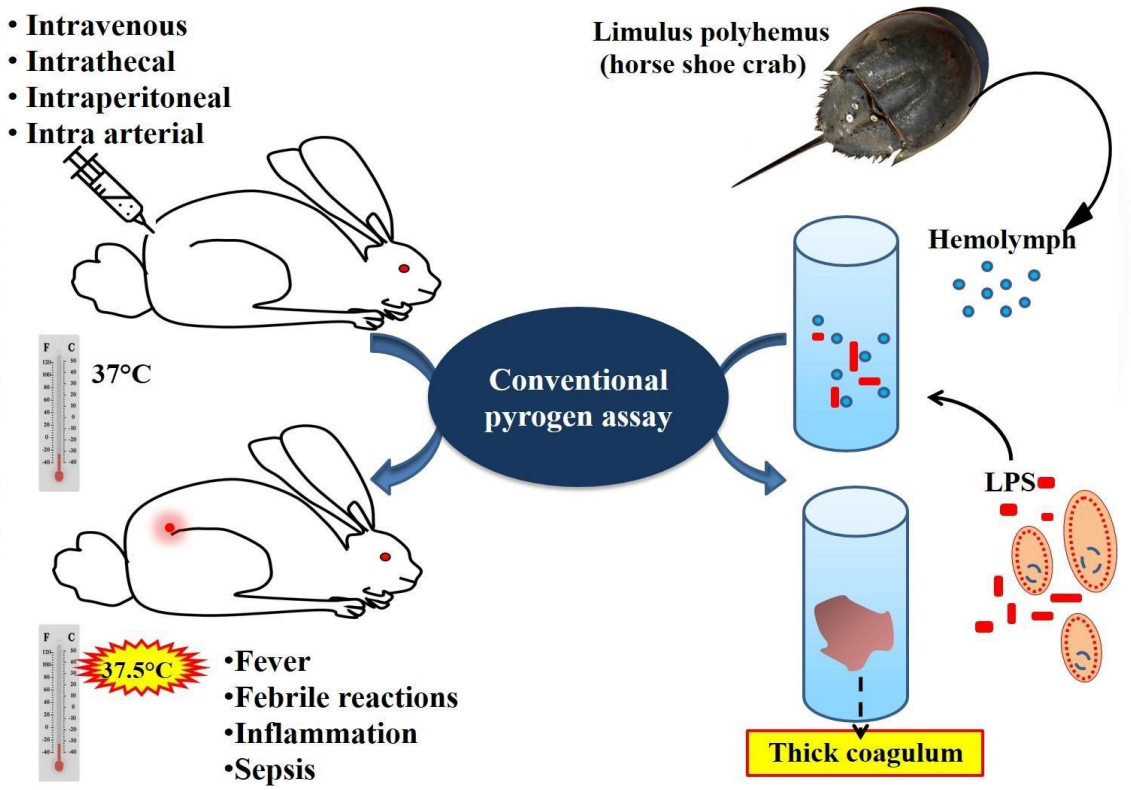

Figure 2.7: Rabbit pyrogen test and Limulus amoebocyte lysate assay (Courtesy Prajitha *et al.*, 2019)

Even though there are considerable advantages in the LAL test, there remain some drawbacks. The intensity of chromogenic substance after reaction with endotoxins is measured by Kinetic chromogenic LAL assay. The colour produced was interfered with LPS coated with different formulation. It also depends on the buffer system operating at varying pH and temperature. The binding of LPS with Factor C of the LAL test is affected by surfactant molecules and components of chelating buffers. It was also noted that there was time-dependent reduction in detection of LPS aggregates formed with LAL reagents. This undetected endotoxin with potential toxic recovery after a period of time has a chance of pyrogenic contamination even in tested samples (Reich *et al*., 2016).

Similarly, pharmaceuticals like ampicillin, methylprednisolone, and erythropoietin C showed inconsistent results with gel clot LAL reagent compared to the chromogenic LAL assay (Silveira *et al*., 2004). LAL test detects only bacterial endotoxins leaving all other types of pyrogenic contamination and is a major drawback. The test is not suitable for non-endotoxin pyrogens. It gives false-positive results towards endotoxin-binding components and several herbal products (Hartung, 2015).

2.1.2.3. MONOCYTE ACTIVATION TEST AND WHOLE BLOOD *IN VITRO* PYROGEN TEST

The cells that act primarily to invading foreign antigens are phagocytes. Immune cells of our body react against these antigens by releasing potent chemical mediators. Researchers focused on developing a new *in vitro* test system to detect both endotoxin and non-endotoxin pyrogens in the last few decades. Recently, in 2010 monocyte activation test (MAT) was included in the European Pharmacopoeia as an alternative method to RPT.

MAT is based on detecting inflammatory cytokines released by monocytes on challenge with endotoxins or non-endotoxin using an ELISA kit. Monocytes and other phagocytic cells present in human whole blood or monocyte isolated and maintained in an *in vitro* culture acts as the test medium. These test system is suitable for pharmaceuticals, biological and other chemical pyrogens and reduces the consumption of animals for pyrogen evaluation (Hasiwa *et al*., 2013). **Figure 2.8** represent the principle behind MAT and IVPT assays.

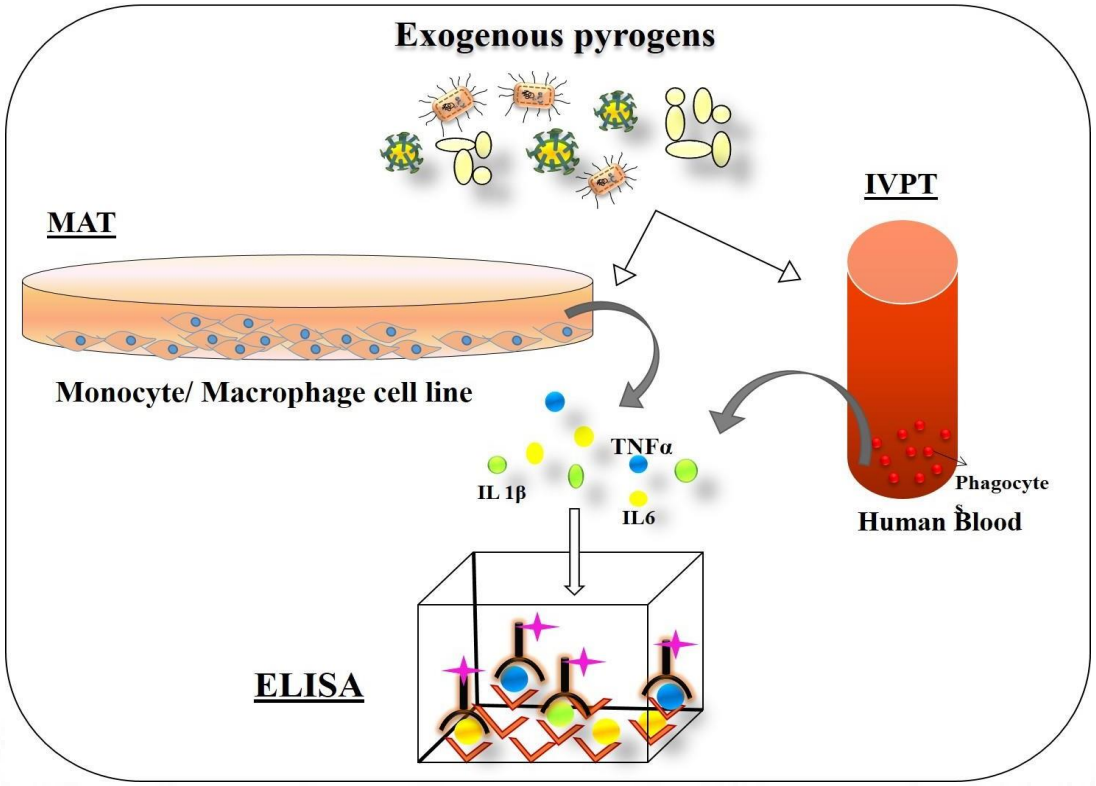

Figure 2.8: Monocyte activation test and *in vitro* pyrogen assay

Chao *et al.,* in 1977, studied pyrogenic response in Guinea pigs previously sensitized with bovine gamma globulin. Sensitized lymphocytes release lymphokines, which are capable of inducing a biological response *in vitro.* Chao *et al.,* isolated phagocytic cells from peritoneal exudates of sensitized animal and these cells were cultured *in vitro* and treated with heat-killed Staphylococcus. Bacterial endotoxin challenge to cultured phagocytes induces production and release of EP. Hence, the cell culture supernatant was analyzed for the presence of EP using RPT. It was observed that there is fever and febrile reaction in rabbits following administration of cell culture supernatant (Chao *et al*., 1977). Among the phagocytes, monocytes and macrophages are the principal producers of pro inflammatory cytokines. Experiments with macrophages were merely helpful to evaluate the LPS mediated inhibition of phagocytosis. Human monocyte-macrophage cell line (Mono-Mac-6 or MM6) is used as the test system for endotoxin detection in monocyte activation test (MAT). It was reported that MM6 cell lines responded well with increased released release of IL-1, IL-6, and TNF-α on stimulation with endotoxin from *E. coli*, peptidoglycan, *S. aureus* Cowan 1 (SAC) and poly I·C even at

lower concentration. ELISA method is efficient for the detection of cytokines released from these cells. Hence, the technique is called MAT (Nakagawa *et al*., 2002).

Lekshmi *et al*., 2012 reported that lymphocytes isolated from fresh as well as pooled human blood release IL-1β on challenge with LPS and LTA. Blood and blood components are taken from a healthy individual to interact with exogenous pyrogens and release IL-1β; similar to what happens in the natural *in vivo* system. Excellent sensitivity and reproducibility were obtained with ELISA technique for detection of IL-1β release. It was observed that both freshly withdrawn blood (Lekshmi *et al*., 2012) and cryopreserved blood (Megha *et al*., 2011) is suitable for performing IVPT. Pyrogens present on solid materials as well as in liquid materials can be detected by IVPT. The immune response against pyrogens requires synergetic action of various cytokines released by immune cells. The interaction of multiple immune cells is necessary for the maximum response to a pyrogen. In IVPT technique, the cellular composition of blood was maintained as that of an *in vivo* system. So the *in vitro* response should be more or less comparable to that of an *in vivo* cytokine induction cascade. MAT and IVPT tests are reported to be more convenient, cost effective, accurate and sensitive for detecting different classes of pyrogens (Daneshian *et al*., 2009).

Limitations of conventional pyrogen assay compared to IVPT is depicted in **Table.1**

	RPT	LAL	IVPT
Endotoxin	Yes	Yes	Yes
Chemical Pyrogen	Yes	No	Yes
Chemotherapeutic agents	No	Yes	Yes
Radioactive Drugs	No	Yes	Yes
Biological Products	Yes	No	Yes
Animal Consumption	Yes	No	No
False Positive/Negative Results	Yes	Yes	No
Cost-Effective	No	Yes	Yes
Easiness Of Assay	No	Yes	Yes
Quantitative assay	No	Yes	Yes

Table 1.1: Limitations of conventional pyrogen assay (Courtesy Prajitha *et al.,* 2019)

2.1.3. ENDOGENOUS PYROGENS

Endogenous pyrogens are pro-inflammatory cytokines, acts as mediators of inflammation. Endogenous pyrogens/cytokines commonly participate in pathways related to fever induction. Major pyrogenic cytokines involved in fever induction are IL-1α, IL-1β, TNF-α, IL6, and IFN-γ. Other than these major cytokines, some minor cytokines like IL-8, IFN- α and macrophage inflammatory Protein 1 (MIP1) induce pyrogenic response. The molecular weight of these cytokines is between 17 to 30kDa. These cytokines are produced by different cells in response to appropriate stimuli. Since the mechanism and action of cytokines are regulated by various hormones and other factors released from the cells, the role of individual cytokines during the initiation of fever is challenging to establish fully. Cytokines acts individually or together to convey signals from site of stimuli to the centre of action.

2.1.3.1. INTERLEUKIN-1β (IL-1β)

Interleukin-1β (IL-1β) or leukocytic pyrogen is the well-established member of IL-1 family. IL-1β is the major pro-inflammatory cytokine, participating in various cellular functions such as prostaglandin synthesis, neutrophil influx/activation, T-cell activation/cytokine production, B-cell activation/antibody production and fibroblast proliferation/collagen production. The synthesis and release of IL-1β from immune cells is a two-step process. This process requires initial priming and secondary activation of the inflammasome complex. When monocytes or macrophages initially get exposed to a foreign pathogen, it activates the synthesis of inactive pro-IL-1β from these cells. Priming events begins with the interaction of PAMPs and PRR's present on macrophages that induce expression of the pro-IL-1β gene. Pro-IL-1β is synthesized and released by the immune cells on activation. Secondary infection of the same primed cells with similar PAMP or DAMP (danger-associated molecular pattern, or endogenous molecules released from dead cells) triggers inflammasome assembly and associated signalling cascades. Several Nod-like receptor proteins act as the sensor molecule for inflammasome assembly.

Inflammasome complex contains adaptor proteins, adaptor apoptosis-associated speck-like protein containing a caspase-recruitment domain (CARD) (ASC) and the cysteine protease caspase-1. Among the inflammasome complex, NLRP3 inflammasome assembles and mediate the activation of enzyme pro-caspase 1 to caspase1. These enzymes cleave pro-IL-1β, ultimately results in the release of mature IL-1β (Lopez-Castejon and Brough, 2011). There are reports suggesting IL-1β induced expression of Cycloxygenase 2 (COX2) mRNA in cultured cells isolated from rat brain. Rat trigeminal ganglia (group of neuronal cells, satellite cells and Schwann cells) culture supernatant showed a time-dependent rise in expression of COX2 on exposure to IL-1β. COX2 expression leads to the release of functionally active molecule prostaglandin E2 (PGE2) from the culture supernatant. PGE2 is the eicosanoid mediator of fever induction (Neeb *et al.*, 2011).

2.1.3.2. TUMOR NECROSIS FACTOR- α (TNF-α)

Tumour Necrosis Factor-α or cachectin is an acute phase signalling molecule released from immune cells at the time of inflammation. There are nineteen more members identified in the TNF family. TNF-α and TNF-β are the principal members of TNF cytokines. Activated

monocytes and macrophages synthesize and release TNF-α. TNF-α exists in membrane-bound or soluble form. Initially, the primary form of TNF-α (26kDa glycoprotein) is synthesized from macrophages and was proteolytically cleaved by matrix metalloproteinase enzymes [TNF-α converting enzyme (TACE)] to release active TNF-α (17kDa protein). Similar to other cytokines, TNF-α also interacts with two specific transmembrane receptors on the cell surface. These receptors are designated as TNF receptor 1 (TNFR1, also known as p55 or p60) and TNF receptor 2 (TNFR2, also known as p75 or p80) (Parameswaran and Patial, 2010).

TNF-α signalling enables resistance to infection, survival, differentiation, proliferation, migration and programmed cell death. TNF-α receptors contain highly conserved cysteine-rich domains in their extracellular region. Tyrosine or serine/ threonine kinase domains are present at the intrinsic region of TNFRs. TNF-α interacts with receptors on cells and executes different cellular events. Activation of NFκB, AP-1, RelB, c-Rel, IRF-1 and GAS eventually results in regulatory expression of corresponding genes. This signalling leads to pathophysiological effects of TNF-α (Karsan and Leong, 2000).

TNF-α triggers inflammatory response in rabbits following *i.v.* administration with increased release of PGE2. It was also noted that the fever induction potential of TNF-α is similar to IL-1β. TNF-α can induce COX2 and PGI2 expression in vascular endothelial cells (Nakamura *et al*., 1988). Even though TNF-α exhibit protective action against intracellular bacteria, virus, fungus and parasites, uncontrolled release of TNF-α will mediate the development of metabolic syndromes, auto-inflammatory, allergic, cardiovascular and neuronal diseases (Popa *et al*., 2007).

2.1.3.3. INTERLEUKIN-6 (IL-6)

Interleukin-6 (IL-6) is a pleiotropic cytokine transiently produced during inflammation, hematopoiesis, infections and other immune reactions. It is a 21-26 kDa glycoprotein produced by immune (B cells, macrophages, dendritic cells, and mast cells) as well as non-immune cells (endothelial cells, mesenchymal cells, fibroblasts, astrocytes, epithelial cells and several malignant cells). IL-6 has a significant role in producing acute-phase proteins from hepatocytes and activation or differentiation of T and B cells, respectively. IL-6 mediates signalling through interaction with a specific transmembrane receptor (IL-6R) and downstream activation of JAK-STAT3 and JAK-SHP-2 mitogen-activated protein (MAP) kinase pathway (Tanaka *et al*., 2014).

There are reports suggesting the endogenous production of IL-6 along with IL-1β in rats after subcutaneous exposure of LPS. Rise in body temperature and febrile reactions were reported with the release of endogenous pyrogens and their entry into the CNS (Cartmell *et al*., 2000). There are other contrary reports indicating the absence of direct influence of IL-6 on COX2 and PGE2 synthesis. IL-6 acts as an external mediator of fever elucidated by PGE2 on the thermoregulatory centre of the hypothalamus on exposure to LPS (Nilsberth *et al*., 2009)

2.1.3.4. INTERFERON-γ (IFN-γ)

Interferon-γ acts as a central coordinator of immune response with stringent anti-viral, anti-tumour and pro-apoptotic activity. IFN are classifieds into three types, namely interferon α, β and γ, depending upon their sequence identity, genetic loci, origin, nature and distribution of receptors. There are two major classes of IFNs, such as Type I IFN (α, β) and Type II IFN (γ). IFN-γ is principally secreted from T cells and NK cells. Along with these, IFN-γ is also produced from B cells, dendritic cells and macrophages.

IFN-γ receptors 1 and 2 (IFNGR 1 and 2) are specific for interacting with IFN-γ, which activates the JAK-STAT signalling pathway. These cytokine signals propagate through a cascade of molecular activation, ultimately results in the expression of IRF-1transcription factors. The effectual output of these signals may lead to various immune system modulations, including leukocyte activation, immune cell adhesion, anti-microbial responses, cell cycle regulation and apoptosis (Teixeira *et al.,* 2005).

It was reported that a dose-dependent rise in body temperature and acute phase responses was observed when human recombinant IFN-γ administrated (*i.v.* or *i.c.v*) in rabbits. Further, it was noted that an instantaneous temperature rises observed within 20 min of *i.c.v* administration, whereas *i.v.* administration induces fever only after 1h of exposure. IFN-γ also caused febrile reactions in rabbits on endotoxin challenges (Morimoto *et al*., 1987). Similarly, it was reported that there is a release of IFN-γ from NK and T-cells isolated from mouse spleen following challenge with *E. coli* endotoxin. The concentration of IFN-γ reached maximum within 8h following endotoxin administration. The release and action of IFN-γ was found to be dependent on the presence of IL-15, IL-18, IL-12 and co-stimulatory signals (B7) (Varma *et al*., 2002).

2.2. DETECTION OF PRO-INFLAMMATORY CYTOKINES

The production and release of cytokines are distinct in acute and chronic diseases. Elevation of some cytokines begins at the initial stage, where others begin only at the end-stage. Detection of these cytokines at various stages of disease helps to identify the treatment strategies. This also helps to analyze the prognosis of diseases up to some extent.

There are different methods available to check the level of cytokines in blood, tissues, cells and other body fluids (Whiteside, 2002). *In vitro* assays are the most acceptable method than *in vivo* because of their low risk and easiness of performance. *In vitro* assays include immunoassays and bioassays. Immuno assays detect the level of cytokines, whereas bioassays detect their activity. Bioassays principally detect the role of different cytokines on proliferation, cytotoxicity, chemotaxis and other vital function of cells.

The concentration, as well as activity of cytokines, will be measured by the following assays;

- ELISA
- Radioimmunoassay (RIA)
- Chemiluminescence
- Multi-parametric flow cytometry
- Magnetic beads based quantitation or isolation of cytokine-producing cells
- Quantitative reverse transcriptase linked – polymerase chain reaction (RT-PCR)
- Northern blotting
- *In situ* hybridization (ISH)
- RNase protection assays (RPA)
- Intra-cytoplasmic cytokine staining (ICC)
- Enzyme-linked immunospot (ELISPOT)

- Immunofluorescence (IF)
- Immunocytochemistry
- DNA microarrays and
- Protein microarrays

The most commonly used assays for the detection of cytokines are mentioned below;

2.2.1. ENZYME-LINKED IMMUNOSORBENT ASSAY

Enzyme-Linked Immuno Sorbent Assay (ELISA) is the most popular and simple immune assay based on antigen or antibody detection. ELISA is widely accepted for detecting cytokines present on body fluids and *in vitro* cell culture supernatants (**Figure 2.9**). Antigen or antibody-coated microplates are available for the detection of cytokines. The detection matrix in ELISA is based on a specific reaction between an enzyme-labelled antibody and its chromogenic substrate. Because of the use of enzyme-labelled antibodies, ELISA is termed to be more specific and sensitive with relatively low cost. ELISA can be classified as indirect, direct, sandwich or competitive based on the target antigens captured or detected. The performance of ELISA depends on the quality of capture as well as detection antibodies used.

2.2.2. FLOW CYTOMETRY

Flow cytometry is a simple and efficient method for analyzing the presence of more than one cytokines. It works on the principle of detection of emitted light from specific fluorescent-labelled antibodies (**Figure 2.9**). Multiplexed fluorescent bead-based assays are exploring the field of cytokine detection using flow cytometry. The flow cytometer can discriminate between individual microspheres based on the size, fluorescent intensity and fluorescent wavelength. Therefore, flow cytometry opens up the possibility for multi analytical assays. Flow cytometer in cytokine assay depends on proper sampling methods, quality of reagents, simplicity of analytical software along with the instrument and the performance of the tests (Sachdeva and Asthana, 2007).

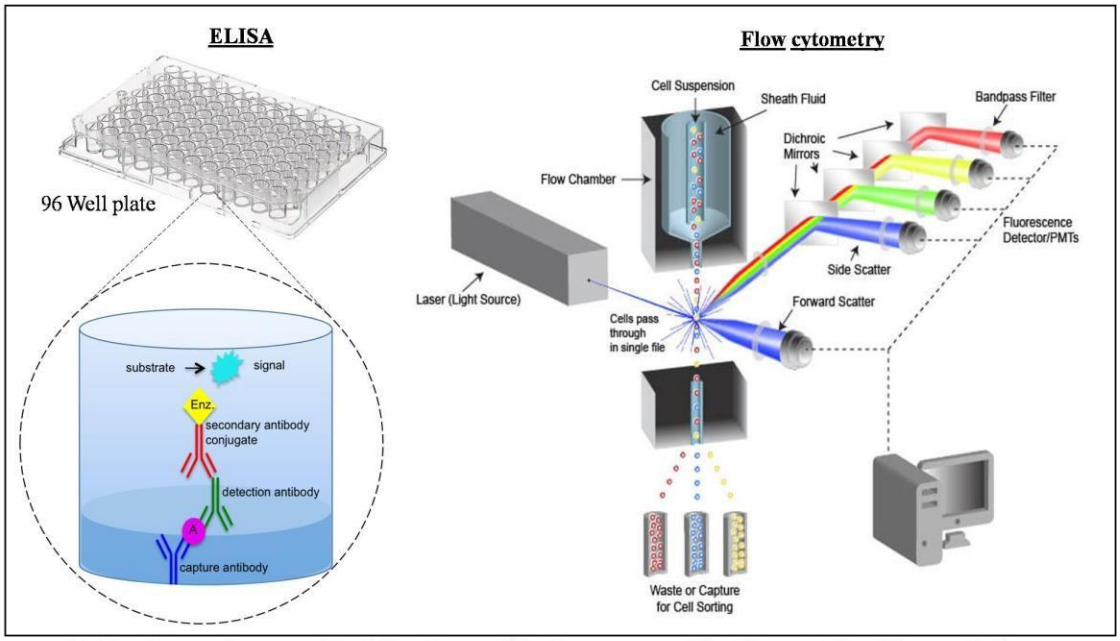

Figure 2.9: ELISA and Flow cytometry

2.2.3. IMMUNOSTAINING

Immunostaining includes immunocytochemistry and immunohistochemistry. Individual cells are analyzed for cytokine production or release by immunocytochemistry. At the same time, the tissue section is stained for the detection of specific cytokines by immunohistochemistry analysis. Either monoclonal or polyclonal antibodies labelled with an enzyme or fluorescent probe is used for the detection. The cytokines, which cannot undergo denaturation following fixation, is suitable for analysis with these techniques. Fluorescently tagged antibody produced against target cytokine will help detect multiple cytokines from a single sample with different fluorescence signals. This technique also helps to localize the cells that secrete cytokines in a tissue sample compared with an isotype control.

2.2.4. BLOTTING TECHNIQUE

The blotting technique is a golden standard for detecting proteins, RNA, DNA *etc.*, in an unknown sample. It helps both qualitative and quantitative analysis of biological samples. Western blotting is the technique widely accepted for the detection of proteins. Sodium dodecyl sulphate-poly acrylamide gel electrophoresis (SDS-PAGE) is the basic procedure for

separation of proteins. These proteins are transferred into a nitrocellulose/PVDF membrane for further detection. Monoclonal or polyclonal antibodies are available to bind with the target cytokine blotted in a nitrocellulose membrane. Secondary antibodies labelled with enzymes, fluorescent probe or chemiluminescence probe help visualize separated proteins as specific bands. Cytokines either in their precursor (high molecular weight) or in their active form (low molecular weight) can be identified using this technique. For example, IL-1β is synthesized as a 31kDa molecule where their active form is a 17kDa protein. Indirect assessment is possible by comparing specific blots of cytokines using a suitable molecular weight marker. Similarly, dot blot is a simple technique for qualitative analysis of cytokines present in the biological samples. On the other hand, this technique is less specific for cytokine analysis compared to other immunoassays.

2.2.5. POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction especially reverses transcriptase PCR (RT-PCR), is employed mainly to detect cytokine expression in cells. By comparing the expression level of housekeeping genes in a sample, one can quantitatively assess the targeted cytokine gene expression by this method. RT-PCR is suitable for studying both *in vivo* and *in vitro* production of cytokines. Initially, the cytokine mRNA released from the cells after expression of a specific gene is isolated and are converted into its complementary DNA (cDNA). PCR require a reaction mix containing Taq polymerase enzyme, nucleotides and co-factors necessary for DNA amplification. DNA present in the sample is amplified by programmed cycles of denaturation, annealing and extension. Fold change in expression of the cytokine gene is quantitatively analyzed by relative normalized expression compared to control. The use of double-stranded DNA (dsDNA) specific probes or dsDNA intercalating fluorescence dyes in RT-PCR makes it more sensitive and specific for detecting gene expression in biological samples. **Figure 2.10** depicts the components and steps necessary for conducting a PCR reaction.

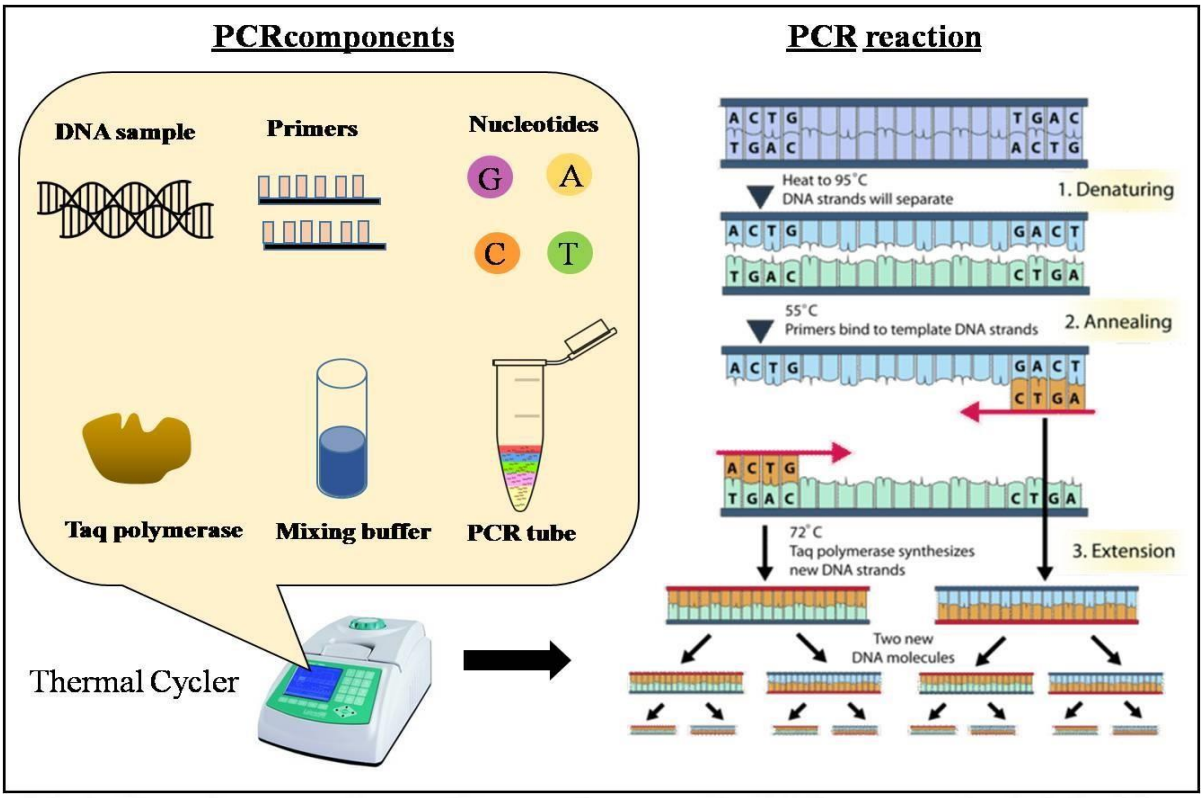

Figure 2.10: Polymerase chain reaction

2.2.6. MICROARRAYS

Microarray is a new technology that relay on the principle of interaction of biological molecules with its complementary sequences impregnated in a chip or a bead (**Figure.2.11**). Microarray helps in the detection of thousands of samples at a time. Both DNA and protein microarray technique is suitable for cytokine analysis. When a cytokine gene is expressed after an immune stimulus, it releases the corresponding mRNA into the cells. In DNA microarray, the cytokine mRNA released in cells can be detected using a cDNA impregnated chip. Each cytokine mRNA or cDNA specifically binds to the corresponding nucleotide sequence on the microarray chip and can be detected as specific fluorescence signals. Since not all the mRNA produced will translate into its active form, protein microarray is more suitable and sensitive for detecting functional cytokines. Similar to DNA microarray, proteins or peptides impregnated on a glass surface or bead or microplate are used in protein microarray. Usually, antibodies raised against specific proteins labelled with chemiluminescent, fluorescent, or chromogenic signals are used for

detection. Microarray is widely accepted as a simple technique for the detection of large numbers of DNA or proteins from a single specimen with high sensitivity (Stenken and Poschenrieder, 2015)

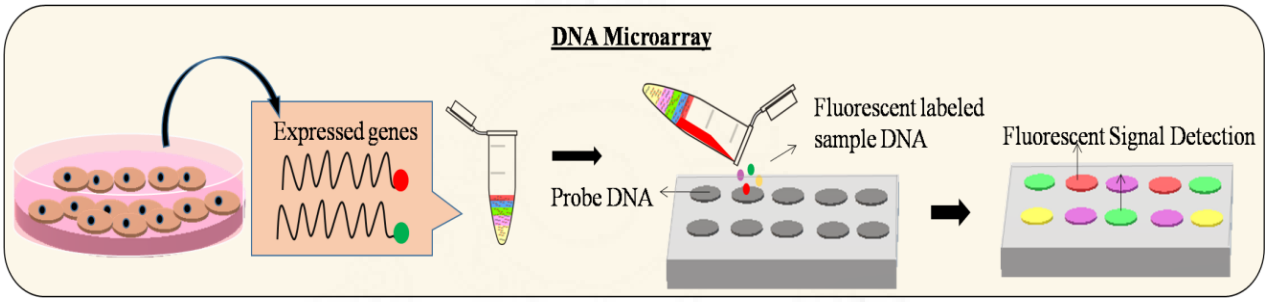

Figure 2.11: DNA Microarray

2.3. IMMUNOTOXICANTS

Several environmental, chemical, pharmaceutical and biological toxicants are known to modulate the immune response in humans. These toxicants have consequences on the health of individuals, including their capability to resist pathogenic infections. Observations of impaired immunocompetence and increased disease incidence highlight the potential relevance of the effects of immunotoxicants. Hence, it is imperative to consider the impact of immunotoxicants in the toxicological risk assessment program.

2.3.1. ROUTE OF ADMINISTRATION

The route of administering a substance into the host system is critical for initiating and executing inflammatory reactions. Fever and febrile reactions are reported following the *i. p, i.v* or *intra-arterial* administration of recombinant cytokines IL-1β, TNF-α, IL-6, IL-8 and MIP-1 in experimental animals. It was also reported that the administration of IL-1 through the intra thecal route promotes secretion of PGE2 faster than their *i.v* administration. In comparison, endotoxins are active irrespective of the route of administration (Coceani *et al*., 1986). Pyrogen induced fever has several effects on human metabolism. Fever and the febrile reaction were reported, following *i.v.* injection of lipexal (endotoxin derived from *Salmonella abortus equi*) in healthy human volunteers. When the drug salicylamide was given along with lipexal to human volunteers, it was found that there is a reduction in the rate of metabolism and excretion of

salicylamide. This observation points out the effect of pyrogenic stimuli on the normal biochemical function of the human hepatic system (Song *et al.*, 1972).

2.3.2. IMMUNOTOXICITY AT CNS LEVEL

Immuno-toxic response against most of the pyrogens begins with the release of inflammatory cytokines and elevation of temperature above the normal physiological range. Pyrogens induced variation of body temperature is achieved by signalling through cytokines and release of eicosanoid mediators at CNS level. Eicosanoids are signalling molecules produced by the oxidation of polyunsaturated fatty acid like arachidonic acid. The eicosanoid PGE2 plays a central role in the elevation of temperature at the time of the pyrogen attack. Prostaglandin H/G synthase or COX is a myeloperoxidase enzyme that catalyzes the rate-limiting step of prostaglandin biosynthesis from arachidonic acid. COX1 and COX2 are the two isoforms of cyclooxygenases. COX2 is regulated by growth factors, IL1-β, IL-6 or TNF-α, consequently over expressed during inflammation. COX2 mediates the production of PGD2, PGE2, PGI2 and thromboxane A2, where PGE2 exerts significant effects on CNS (Sobolewski *et al*., 2010). Reports are suggesting the elevated expression of COX2 mRNA in various area of the rat brain, including leptomeninges, neocortex, cingulate cortex, piriform cortex, hippocampus, lateral amygdala, thalamus, hypothalamus, striatum, preoptic area, blood vessels, perivascular monocytes and endothelial cells following administration of LPS (Quan *et al*., 1998, Cao *et al*., 1995).

PGE2 is the most abundant lipid autacoid released during inflammation. The action of two enzymes generally releases PGE2. Phospholipase A2 acts on membrane phospholipid and COX2 acts on arachidonic acid. A small amount of PGE2 was found to be liberated from 2-acyl glycerol upon LPS challenge by the action of monoacylglycerol lipase (Kita *et al*., 2015). Since PGs are lipophilic, they can easily cross the BBB to interact with specific receptors on neuronal cells. Four types of G-protein coupled receptors EP1, EP2, EP3 and EP4 are present in the circumventricular organ of the brain. The interaction of PGE2 with EP receptors transmits pyrogenic signals into the median preoptic area (MnPO), organumvasculosum of the lamina terminalis (OVLT) and ventromedial pre-optic nucleus (VMPO) of the brain. Even though all the EP receptors are in action, the EP3 receptor plays the principal role in PGE2 mediated signalling

at the time of pyrogenic stimuli (Lazarus, 2006). The pathway of fever induction by endogenous pyrogens is depicted in **Figure 2.12.**

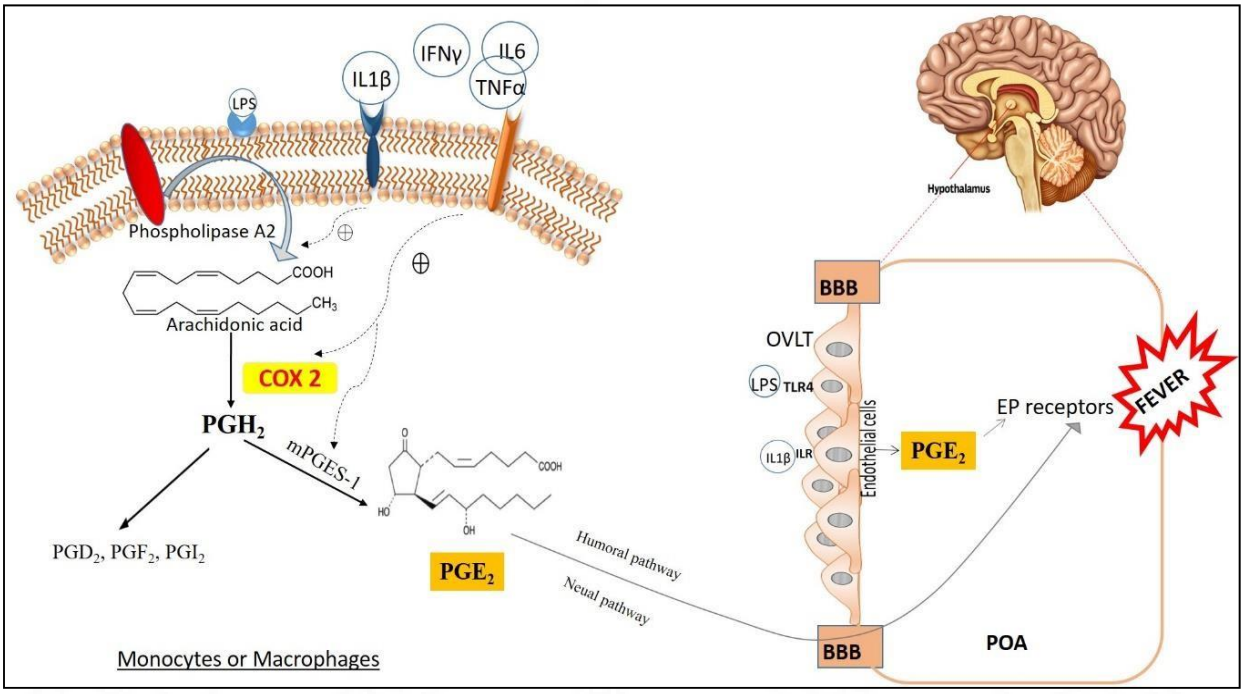

Figure 2.12: Fever induction pathway (Courtesy Prajitha *et al.*, 2019*)*

Fever induction by PGE2 requires the release of neurotransmitter cAMP from the hypothalamic cells. Interaction of IL-1β, IL-6 and TNF-α with PGE2 activates signalling at the thermoregulatory centre and alter the thermostatic set point of the hypothalamus (Bernheim, 1986). The induction of pyrogen associated fever is mediated through two interconnected pathways include humoral and neuronal pathways. Activation of the humoral pathway induces the production of endogenous cytokines concerning the entry of exogenous pyrogens. These inflammatory cytokines both directly or indirectly enter into the circumventricular organ of the brain and interact with receptors present on micro endothelial cells. This cytokine-receptor interaction induces the release of PGs. Since PGs are lipophilic, they easily cross the BBB and mediate further signals of fever generation. The neural pathway involves the communication of CNS with peripheral signals. Sensory nerves of cutaneous surfaces and vagus nerve from the hepatic system act as a route of peripheral signals (Dantzer *et al.*, 2000). There were reports of fever and febrile reactions in guinea pigs followed by LPS injection into subcutaneously

implanted Teflon chambers. There found augmented production of PGE2, IL-1β, IL-6 and TNF-α. Neural signals of pyrogenic fever reached the thermoregulatory centre of the brain via localized afferent nerve fibers. In turn, these peripheral signals activate the release of pyrogenic cytokines and PGE2 (Rummel *et al*., 2005). Sub-diaphragmatic vagal nerve endings possess receptors where IL-1β can bind and initiate the neural signals to the POA. It was evident from the study by Simons *et al.,* 1981 that non-vagotomised rats showed a dose-dependent rise in body temperature following *i.p.* injection of recombinant human IL-1β. However, rats that underwent vagotomy showed non-responsiveness to pyrogen stimuli due to the defective transduction of pyrogenic signals into the brain through the peripheral vagus nerve system (Simons *et al*., 1998).

2.4. IMPORTANCE OF OTHER INFLAMMATORY CYTOKINES

In general, cytokines initiates and propagate inflammatory signals within the immune network of the body. However, some other cytokines work against the action of pro-inflammatory cytokines and are called anti-inflammatory /antipyretic cytokines. Other mechanisms present in the body to counterbalance the effect of inflammatory cytokines include internalising endotoxin-receptor complexes, downregulation of cytokine-receptor complexes, releasing soluble receptors of IL-1/TNF, the release of antagonists of IL-1 and its receptor (IL-1ra) (McCarthy, 1994). IL-4, IL-10, IL-11, IL-13, IL-18 and TGF-β are the major anti-inflammatory cytokines produced to resolve the devastating effect of persisting inflammation.

IL-10 has a high level of anti-inflammatory action compared to other anti-inflammatory cytokines, where they inhibit the expression and release of IL-1, TNF-α and IL-6. IL-10 up regulates the expression of IL-1ra so that the propagation of inflammatory signals after the interaction of IL-1 with their receptors gets inhibited. IL-10 not only prevents the interaction of IL-1 but also reduce the production of IL-1β. IL-10 can act as an inhibitor of caspase-1 dependent inflammasome activation in macrophages to reduce inflammatory cytokines production. It can also inhibit the mammalian target of rapamycin (mTOR) signalling pathway necessary for the metabolic rearrangement of cells at the time of infection or inflammation (Prajitha *et al*., 2019).

TGF-β acts as an anti-inflammatory cytokine through inhibition of T helper cells and cytokine production from macrophages. There are three isoforms of TGF- β such as TGF- β1, TGF- β2 and TGF- β3 in mammals. Like IL-10, TGF- β counteract with the production of IL-1, IL-2, IL-6 and TNF to resolve inflammation. TGF- β1 is found in various regions of the CNS and involved in a negative feedback mechanism to limit the activation of glial cells. TGF- β showed antagonistic action on nitric oxide production and is a mediator of neuropathic pain during inflammation (Sanjabi *et al*., 2009).

2.5. INTRODUCTION OF CELL LINE USED FOR STUDY

THP-1 is a cancerous monocyte like cells derived from the peripheral blood of a 1-year-old boy who had acute monocytic leukaemia (purchased from National Centre for Cell Science, Pune, India). The cells are phagocytic for sensitized erythrocytes. THP-1 express Fc receptors and C3 complement receptors. THP-1 monocytes lack immunoglobulin either on their surface or on their cytoplasm.

2.5.1. CULTURE AND PASSAGING: THP-1 cells grow well in RPMI-1640 medium modified to contain 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate, 4500mg/L glucose, 1500mg/L sodium bicarbonate and 10% fetal bovine serum. It is maintained at a density between $1x10^5$ to $1x10^6$cells/ml at 37^0C with 5% CO_2. The same were subcultured once a week when cell concentration reached $8x10^5$ cells/ml and not exceeds $1x10^6$ cells/ml. Revived a new vial of frozen cells after every five passages to maintain healthy cells.

2.5.2. CHARACTERISTICS OF CELL LINE: THP-1 cells are non-adherent suspension cells. They possess large, round, single-cell morphology with an approximate size of 18 to 21µm. These cells are phagocytic and lack cytoplasmic and surface immunoglobulins. The average doubling time of THP-1 cells is between 19 to 50h.

2.5.3. DIFFERENTIATION OF THP-1 MONOCYTES TO MACROPHAGES: Differentiation of THP1 monocytes to macrophage phenotype is widely accepted as the best *in vitro* system to elucidate human immune response to inflammatory stimuli. Phorbol 12-myristate 13-acetate (PMA) is a mitogen that acts as an inducer of monocytes to macrophage differentiation. Treatment with PMA (100nM) induces terminal differentiation of THP-1

monocyte cells to macrophage called monocyte-derived macrophages (MDM). Differentiated macrophages get to adhere to the culture plate and resemble the morphology of real macrophage.

2.6. JUSTIFICATION OF SELECTION OF THP-1 CELL LINES

The human immune system is a vast network of organs and cells that defend against a range of foreign invaders, injury or infection. The cells that play a major role in defence are the monocytes, macrophage, dendrite cells *etc.* In mammals, circulating monocytes protect most of the bacterial toxins. LPS and LTA are the toxins released during the attack of Gram-negative and Gram-positive bacteria. When circulating monocytes encounter these toxins, they migrate into the lymph node and present antigenic determinants to B and T-cells. This activates humoral as well as cell-mediated immune response. Monocyte also differentiated into macrophage and moved into the tissue to phagocytose the infectious agents entering the tissue surface. The initial stage of an immune response begins with inflammation. Monocyte and other granulocytes enter at the site of inflammation and release potentially active molecules like chemokines and cytokines that help propagate inflammation.

Immune activation takes place after the interaction of antigen with their specific receptors present on the immune cells. TLR are the most common receptor found on almost all cells of the innate immune response. Monocytes possess TLR4 and are the receptor for LPS. LPS induce signalling through CD14-TLR4-MD2 complex. The most active pro-inflammatory cytokine released during this signalling is the IL-1β, which mediates various responses against the invading microorganisms. Primary responses like fever and febrile reaction are associated with profound secretion of inflammatory cytokines and eicosanoids at the thermoregulatory centre of the CNS.

THP-1 cells are a suitable model for immuno-toxicity studies because they possess the characteristics of human monocytes. THP-1 cells are ideal for studying the morphological and functional aspects of monocytes in healthy and diseased conditions. Recently, Długosz *et al.,* 2019 reported the cytokine signalling via THP-1 monocytes following infection with the parasite *Toxocara canis* and its glycans. To prove the possible route of the immune response against parasitic infection, Długosz *et al.,* chose THP-1 cell as an *in vitro* study model. PMA induced

differentiation of THP-1 monocytes is a well-established technique that helps to analyze *in vitro* response of macrophages to various toxins, including pandemic viral agents (Sokolova *et al.,* 2018).

2.7. JUSTIFICATION OF SELECTION OF POOLED HUMAN WHOLE BLOOD FOR INTERLEUKIN-1β MEASUREMENT

Pyrogens, either endogenous or exogenous, may cause fever and febrile reaction in humans and other organisms. For the past several years, RPT is the only option to evaluate pyrogenic contamination in a parenteral drug, injectables and medical devices. As an alternative to these, researchers develop LAL test (from crab species Limulus Polyphemus) and can only detect endotoxin from Gram-negative bacteria. To reduce the utilization of animals for pyrogen test, researchers focus on the release of EPs from human monocytes after challenge with exogenous pyrogens *in vitro*. *In vivo* assays are costly and less sensitive to various kinds of pyrogens. To overcome these lacunae, MAT, as well as IVPT, is underway (Nakagawa *et al.*, 2002).

The reliability of IVPT is based on the release of pro-inflammatory cytokine from human monocytic cells after challenge with exogenous pyrogens. Monocyte culture is the test system in MAT whereas, freshly drawn human blood is used as a test system in IVPT. The whole blood collected from healthy individuals mimics the in vivo environment and represents an accurate test system to reproduce pyrogen-induced cytokine release. Reports highlight the use of human whole blood from a single donor, fresh and cryopreserved lymphocytes isolated from single/pooled blood for conducting IVPT. The release of inflammatory cytokine with respect to stimuli varies with the physiological and health status of an individual. Pooled human blood is considered to be an excellent choice to reduce the fluctuation in results with individual variation in cytokine release. The use of pooled blood also increases the chance of analyzing multiple Pyrogens. Pooled blood system also reduces multiple sampling.

CHAPTER 3: MATERIALS AND METHODS

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3.1. CHEMICALS

Purified human recombinant interleukin 1β protein (Sigma Aldrich), Freunds' complete and incomplete adjuvants (Sigma Aldrich), Pyrogen-free 0.9% saline (Bendtt, India), Sterile filtered pyrogen-free RPMI1640 media (Himedia, India), Endotoxin standards from *E. coli* strain 055:B5 lipopolysaccharide (Sigma Aldrich), Lipoteichoic acid from Bacillus subtilis (Sigma Aldrich), Phorbol Myristic acid (Sigma Aldrich), Reagent-grade 2,4,6-trinitrophenol (Sigma Aldrich), Phytohaemagglutinin lectin from Phaseolus vulgaris (Sigma Aldrich), Sterile pyrogen-free 96-well flat-bottom microplates (Nunc Maxisorp) (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)/MTT, Griess reagent, Cytopainter phalloidin were obtained from Abcam (UK). Fetal bovine serum (FBS), antibiotic/antimycotic, glutamine, phosphate-buffered saline, 0.25% trypsin were obtained from gibco®, Life technologies (USA), Calcein-AM and DilC1 (5) Dye (Mitochondrial Membrane Potential Probe) were purchased from ThermoFisher Scientific (USA). 30% Hydrochloric acid (Merck, India). Neutral red, Propidium iodide, Acridine orange and DAPI (4', 6-diamidino-2-phenylindole) were purchased from Himedia Laboratories, India. Alexa Flour 488 Annexin V/Dead cell apoptosis kit (Invitrogen, India). 2, 7-dichlorodihydrofluorescein diacetate (H2DCFH-DA), Sodium carbonate and Trisodium citrate were purchased from Sisco Research Laboratories (SRL), India. Rabbit anti-Human NFκB antibody, Rabbit anti-Human COX2 antibody and Goat anti-rabbit secondary antibody-FITC were purchased from Abcam, Cambridge, UK. RNeasy Mini Kit (Qiagen, Germany), PrimeScript 1st strand cDNA synthesis kit and PCR master mix were purchased from Takara Bio (Japan). Human IL-1β ELISA kit (Thermofischer Scientific, India). Biochemistry reagents for blood analysis were purchased from Erba mannhiem (Germany). Reagents for haematological analysis were procured from Horiba (Japan). Sterile cell culture plastic wares were purchased from Nunc.

3.2. EQUIPMENTS

Affinity Chromatography AKTA prime plus FPLC system (GE Healthcare), Fluorescent microscope (Axio Scope A1 Carl Zeiss, Germany), Phase contrast microscope (Leica Microsystem, Germany), Haematology analyser: Horiba Vet abc (Japan). Biochemistry analyser: Erba Mannheim XL300 (Germany). Laminar airflow (Mark Air particulars, India). CO2 incubator (Sanyo, Japan), CytoFlex Flow cytometry (Beckman Coulter, India) and Amnis flow cytometer, Germany, controlled by 'FloMax' software (version 2.4; Partec, Münster, Germany), Incubator shaker (New Brunswick Scientific, USA), Biophotometer (Eppendorf, Germany), Steam sterilizer (Nat Steel, India). Monochromator based multimode microplate reader (BioTeck Instruments, USA), Thermomixer (Eppendorf, Germany). Transilluminator, Micro-plate reader (Infinite F Nano+, Tecan, Switzerland), Refrigerated centrifuge (Eppendorf, USA). Real-time PCR system CFX 384 (Biorad, USA), Handheld thermometer, Laminar flow hood

3.3 ANIMAL HUSBANDRY AND WELFARE

New Zealand White Rabbits were procured from the Division of Laboratory Animal Sciences, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (Govt. of India), Trivandrum. The animals were maintained in a 12h light and dark cycle with a constant temperature of 22 ± 2°C and relative humidity of 30-70%. Animals were provided with a standard pellet diet and water *ad libitum*. Individual animals were identified by animal number. Additionally, each animal cage was labelled for name, experiment number, number of animals, date of commencement, and experimental period. All the animals were acclimatized for 7 days before beginning the experiments. The animals were routinely monitored for health by cage side observation. All the animals were handled humanely, without causing any pain or distress and with due care for their welfare. The care and management of the animals were carried out in compliance with the Committee for Control and Supervision of Experiments on Animals (CPCSEA).

3.4 ANIAML ETHICS

All the experiments were carried out after getting approval from the Institute Animal Ethics Committee (IAEC). Animal experiments conformed to the guidelines of IAEC regulations approved by the CPCSEA, Govt. of India. Clearance was obtained from IAEC for carrying out the study. IAEC approval No: SCT/IAEC-384/NOVEMBER/2020/107.

3.5. METHODS

Investigation of IL-1β released from pooled human blood stimulated with LPS, LTA, PHA and TNP were done using an indigenously developed ELISA kit. The development of the ELISA detection system is depicted in **Figure 3.1.**

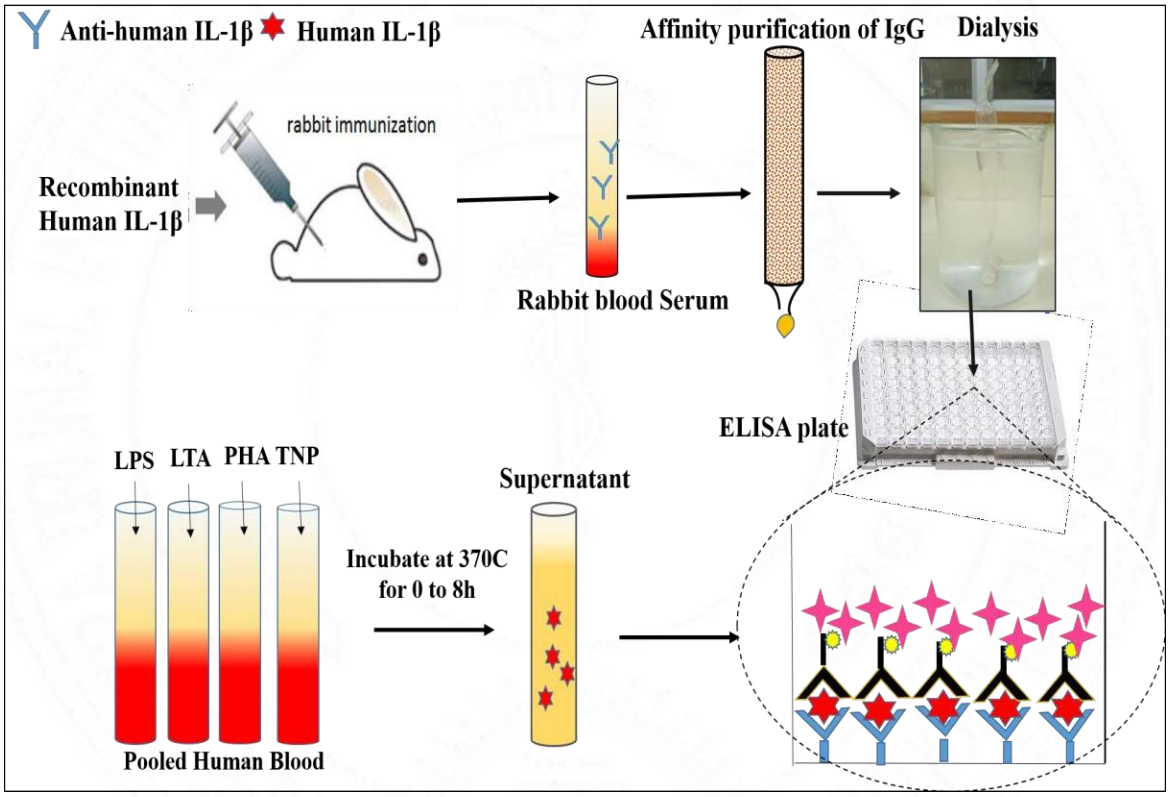

Figure 3.1: Schematic diagram depicting the stages of ELISA plate development

3.5.1. IMMUNIZATION OF RABBIT

A healthy male New Zealand white rabbit was immunized with purified human recombinant interleukin 1β (Sigma). The active production antibodies to human IL-1β (anti-human IL1β) were persuaded by scheduled booster doses at an interval of 28 days. The micellar solution of recombinant human IL-1β was prepared by resuspending the lyophilized powder in phosphate-buffered saline (PBS) with an equal volume of Freunds' complete adjuvant. The solution was administrated intramuscularly at the hind limb area. Booster doses were given every 28 days after primary immunization, using 1:1 emulsion of Freunds' incomplete adjuvant.

3.5.2. PURIFICATION OF IgG FROM RABBIT BLOOD SERUM

Blood was collected from the marginal ear vein of the immunized rabbit every 12^{th} day of booster doses. The blood was allowed to clot at 37 °C for 1h and retract at 4 °C overnight. Clotted blood was centrifuged at 2000 rpm for 10 min at 4 °C and serum separated was stored at -20 °C. The IgG antibody present in rabbit blood serum was purified with AKTA prime plus FPLC system (GE Healthcare) using HiTrap protein A affinity chromatography column as per the manufacturer's instructions. Briefly, the column was equilibrated with binding buffer, pH 7.0 (20mM sodium phosphate buffer). Serum diluted with binding buffer was injected into the column using a syringe pump. The IgG antibody bound explicitly with the protein A column was eluted using an elution buffer (100mM citric acid, pH 3.0). The eluted fractions were collected in centrifuge tubes containing 100µl of 1M Tris-Cl (pH 9.0) to neutralize the pH of the eluting buffer. The fractions showed maximum peak value for IgG antibody were pooled together and dialyzed against PBS overnight at 4 °C. The concentration of the dialyzed fraction was estimated by the micro-Bradford colourimetric method. The experimental details are described in **Table 3.1.**

Standard BSA (µl)	Distilled Water(µl)	Bradford reagent(ml)
0	**100**	**1**
2	**98**	**1**
4	**96**	**1**
6	**94**	**1**
8	**92**	**1**
10	**90**	**1**
20	**80**	**1**
Test (2 µl)	**98**	**1**
Mix the solutions well and incubate at dark for 5 min. Read the absorption at 590 nm		

Table 3.1: Estimation of protein concentration by micro Bradford assay

3.5.3. DOT BLOT ASSAY

Dot blot assay was done to confirm the specificity of the anti-human IL-1β antibody raised in rabbits against recombinant human IL-1β. 5µl of recombinant human IL-1β (Sigma) was spotted as an antigen on a nitrocellulose membrane and allowed to dry. The unbound area of the nitrocellulose membrane was blocked with 1% BSA for 1hr at room temperature. After blocking with BSA, the membrane was washed three times with PBS and incubated with affinity-purified IgG antibodies (1: 100 dilutions) from pre-immunization and post-immunization (booster dose) serum. The membrane is kept at room temperature for 30 min. Washed with PBS and incubated with secondary antibody HRP conjugate for 30 min at room temperature. After washing with PBS, the chromogenic substrate 4-chloro-1 naphthol was added and incubated for 30 min to develop purple colour.

3.5.4. ANTIBODY-ENZYME CONJUGATION

The affinity-purified anti-human IL-1β antibody was conjugated with amine-reactive horseradish peroxidase (HRP) using the EZLink ®Plus Activated Peroxidase kit (Thermo Scientific, USA) as per the manufacturer's instructions. Briefly, 100µl ultrapure water was added to the

lyophilized activated peroxidase powder and mixed to dissolve. Purified IgG was added to this solution and mixed well. 45mM sodium cyanoborohydride was added to the mixture and incubated for 1h at room temperature. The conjugation reaction was quenched with 54mM ethanolamine and the antibody-HRP conjugates were purified using the FreeZyme™ conjugate purification kit (Thermo Scientific, USA). Initially, the immobilized iminodiacetic acid column was equilibrated with five volumes of tris buffered saline (TBS) (25mM Tris, 0.15M NaCl, pH 7.2) and activated using Ni^{2+} aqueous column activator buffer. The column was then washed with five volumes of TBS to remove non-bound nickel. The antibody-HRP conjugate was poured on the top frit of the column and drained with three volumes of TBS. The bound antibody-HRP conjugate was eluted from the column by adding elution buffer and were collected. The enzymatic activity of collected fractions was analyzed using the substrate trimethyl blue. The fraction showing the highest activity was aliquotted and preserved at -20 °C.

3.5.5. *IN VITRO* PYROGEN ASSAY BY ELISA

3.5.5.1. HUMAN WHOLE BLOOD

Healthy human volunteers who did not suffer from any disease, nor taken any medication, nor afflicted with physical injury for at least two weeks were selected for the study. Informed consent was obtained from the volunteers for collecting blood for the study (IAEC approval No: SCT/IEC-1366/APRIL/2019, IEC Reg No: ecr/189/Inst/KL/2013/RR-16)). Fresh venous blood was drawn from individuals and transferred into heparinized as well as plain tube. Individual blood samples were pooled within 15 min of collection. Blood samples were used to induce cytokine production within 1h of blood collection.

3.5.5.2. HEMATOLOGICAL AND BIOCHEMICAL ANALYSIS

The collected individual and pooled blood were checked for parameters such as Platelet count, WBC count, RBC count, and Hemoglobin within an hour of blood collection. The complete blood cell count was performed using an automated haematology analyzer (SYSMEX K4500). The serum separated from an individual as well as pooled blood was analyzed for biochemical parameters such as Glucose, Cholesterol, Triglycerides, Alanine transaminase (ALT), Aspartate

transaminase (AST), Alkaline phosphatase (ALP), Total Bilirubin, Total Protein, Albumin and Creatinine using Biochemistry analyser: Erba Mannheim XL300

3.5.5.3. INDUCTION OF CYTOKINES

Pooled human whole blood was used to induce a pyrogenic response. The reaction mixture was taken in a 1.5ml pyrogen-free microcentrifuge tube. LPS from *Escherichia coli* (1and 5EU/ml), LTA from *Bacillus subtilis* (0.1 and 1µg/ml), PHA from *Phaseolus vulgaris* (15 and 30µg/ml) and 2, 4, 6 trinitrophenol (TNP) (15 and 30µg/ml) were used as stimulators of pyrogenic response. The experimental details are described in **Table 3.2**.

	Control	**Test**
Heparinized Blood	250µl	250µl
RPMI 1640 media	250µl	250µl
Pyrogen	-	10µl
Physiological saline	500µl	490µl
Incubate the reaction mixture at 37°C /5% CO_2 for 0 to 8h. ⇩ Collect supernatant in every 2h intervals/separate by centrifugation at 500 g for 2 min at 4 °C. Store supernatants at -20 °C until analysis.		

Table 3.2: Induction of IL-1β release from pooled human blood

3.5.5.4. MEASUREMENT OF INTERLEUKIN-1β (IL-1β)

An indigenously developed ELISA kit was used for the estimation of IL-1β released from pooled whole blood. Briefly, the ELISA plate was prepared by coating affinity purified anti-human IL-1β antibodies in a sterile 96 well microplate. 50µl of anti-human IL-1β in 50mM carbonate bicarbonate buffer (pH 9.6) was added to each well of a 96 well plate and incubated overnight at 4°C. The unbound surface was blocked with blocking solution (137mM NaCl, 2.7mM KCl, 10 mM Na_2HPO_4, 2mM KH_2PO_4, 1% BSA, pH 7.2) for 1h at room temperature. After washing with

washing buffer (137mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.2), 50µl of the diluted serum samples (1:20 in blocking solution) were incubated for 2h at room temperature. The wells were washed again and incubated with 100µl of diluted anti-IL1β-HRP conjugate for 2h at room temperature. After washing, the wells were incubated with TMB substrate for 30min in the dark. 100µl of 1M H_2SO_4 was added to stop the colour reaction and absorbance reading was taken using ELISA plate reader (ASYS Expert plus) at 450 nm and 620 nm corrective filter. The mean OD values and the % CV were calculated for each IL-1β concentration points on the standard graph.

3.5.6. MOLECULAR AND CYTOTOXICITY ANALYSIS

Cellular interaction of LPS, LTA, PHA and TNP was studied in PMA differentiated THP-1 cells or MDM cells. The cell viability, mitochondrial activity, lysosomal integrity, free radical production, lysosomal activity, apoptosis and immunotoxicity in the presence of toxicants were studied using different assays, as shown in **Figure 3.2.**

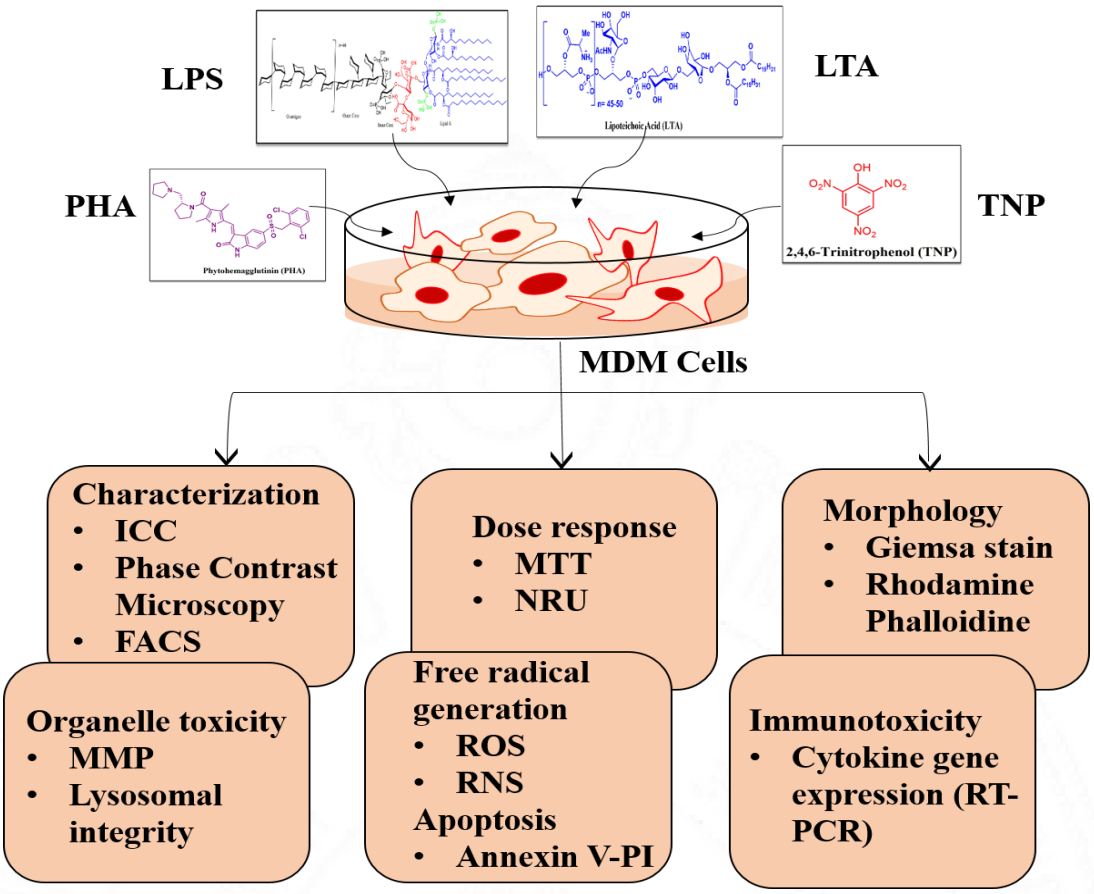

Figure 3.2: Flow chart of cellular interaction of LPS, LTA, PHA and TNP using THP-1/MDM cells

3.5.6.1. CHARACTERIZATION OF PMA DIFFERENTIATED MACROPHAGE

3.5.6.1.1. IMMUNOCYTOCHEMISTRY

Macrophage marker CD68 was used to characterize PMA differentiated macrophages. THP-1 monocyte cells were seeded over a coverslip placed in a single well dish at an initial density of $1x10^5$ cells. 100nM PMA was added to the well and incubated at 37^0C/5% CO_2 for 48h. After incubation, the medium was removed and the cells were fixed with 4% formaldehyde for 5 min. Cells were washed three times with sterile PBS. Cells were treated with 1% BSA for 1h to block non-specific binding and permeabilised with 0.1% Triton X100 for 5 min at room temperature. After removing the permeabilization solution, rabbit anti-human CD68 primary antibody (1:500 dilutions in PBS) was added to the wells and incubated overnight at 4°C. The cells were washed

thrice with PBS (in a 3 min gap) and incubated with Goat anti-rabbit IgG-FITC secondary antibody (1:500 dilutions in PBS) for 2h at dark. The cells' nucleus was demarcated using 1µl of DAPI (1mg/ml) for 5min. The cells were washed thrice with 1X PBS and observed under a fluorescent microscope using a blue and green filter (Axio Scope.A1, Carl Zeiss, Germany).

3.5.6.1.2. FLUORESCENT ACTIVATED CELL SORTING (FACS)

To confirm the differentiation of THP-1 monocytes to MDM, the cells were analyzed using flow cytometry. In flow cytometry, forward scattered (FSC) light indicates the size of the cell and side-scattered (SSC) light indicates the internal complexity of cellular granularity (McKinnon, 2018). Differentiation of monocytes to macrophage will leads to an increase in granularity and the corresponding increase in SSC. In this experiment, $1x10^6$ cells were seeded in a single well plate. The cells were treated with 100nM PMA and kept at 37^0C/5% CO_2 for 48h. After incubation, the cells were harvested using a cell scraper and centrifuged at 1200 rpm for 2 min. The cell pellet was resuspended in 500µl PBS and analyzed using CytoFlex Flow cytometry (Beckman Coulter, India)

3.5.6.1.3. PHASE CONTRAST MICROSCOPY

Cell imaging using a phase-contrast microscope is a simple and convenient method for visualizing the morphology of THP-1 cells before and after differentiation. Briefly, $1x10^5$ cells with or without PMA was seeded over a coverslip to analyze the morphology of differentiated and undifferentiated cells. Cells were incubated at 37^0C/ 5% CO_2 for 48h. After incubation, cells were washed with 1X PBS and observed under a phase-contrast microscope (Leica Microsystem, Germany). The image was captured with 40X magnification.

3.5.6.2. MITOCHONDRIAL ACTIVITY BY MTT ASSAY

MTT assay was used for the assessment of cell viability in terms of mitochondrial activity. The mitochondrial oxidoreductase enzyme reduces MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) reagent to an insoluble purple coloured product formazan. The amount of formazan crystal formed is directly proportional to the number of active cells. For the assay, $1x10^4$cells were seeded into a 96 well plate and incubated overnight at 37°C and 5% CO_2. PMA at a concentration of 100nM is added to the cell suspension to activate differentiation of

THP-1 monocytes to MDM cells. After incubation, the cells were treated with different concentration of LPS (0.25, 0.5,1,2 and 5EU/ml) and other toxicants LTA, PHA and TNP (0.1, 1, 10,100 and 1000µg/ml). Cells were again incubated for 24h, 48h and 72h at 37°C/5% CO2. After incubation, MTT (0.05mg/ml) dye was added and incubated for 3h in the dark. The insoluble formazan crystals formed was solubilised by adding100µl DMSO and kept for 30 min at room temperature. Absorbance was measured at 540 nm using ELx 808, BioTek Instruments, USA.

3.5.6.3. LYSOSOMAL ACTIVITY BY NEUTRAL RED UPTAKE (NRU) ASSAY

The uptake of Neutral Red dye by lysosomes within a healthy cell was analyzed to detect the viability in terms of lysosomal activity. The method introduced by Borenfreund and Puerner (Borenfreund and Puerner 1985) measures the amount of dye taken up by the cells after treatment with toxicants. Briefly, cells were cultured at an initial density of $1x10^4$ cells in a 96 well plate and incubated at 37°C/ 5% CO_2 for 24h. After incubation, the supernatant was removed and added with different concentration of LPS, LTA, PHA and TNP. Treated cells were incubated for 24h, 48h and 72h. 10µl of 1% neutral red was added to each well and incubated in the dark for 3h. After incubation, the supernatant containing excess dye was removed and the dye taken up by the cells was solubilized using 100µl acid alcohol (1%, v/v, acetic acid and 50% ethanol). Cells were kept on an incubator shaker at 60 rpm at 37°C for 30 min. Absorbance was measured at 540nm using an ELX 808 multiwell plate reader (Bio-Tek, Winooski, USA).

3.5.6.4. CYTOSKELETAL DESTABILIZATION BY RHODAMINE PHALLOIDIN STAINING

Actin is the major protein necessary to maintain the integrity of the cell cytoskeleton. Phalloidin is an F- actin-binding protein isolated from toxic mushroom *Amanita phalloides*. The non-fluorescent phalloidin is conjugated with tetramethylrhodamine to get fluorescence detection property, which gives red fluorescence at an excitation/emission wavelength 540/565 nm. $1x10^5$cells/well were seeded over a coverslip placed in a 6 well plate and incubated overnight to get attached. The cells were exposed to LPS, LTA, PHA and TNP for 24h. After treatment, the cells were washed with PBS and fixed using 4% formaldehyde for 10min. Excess aldehyde was quenched for 5min using 0.1M glycine in PBS. Cell membrane permeabilised using 0.1% Triton-X 100 (in PBS) for 1 min. Permeabilized cells were incubated with Rhodamine-phalloidin

(1:250 dilutions in PBS) dye for 15 min at room temperature. The nucleus was counterstained by using the blue-emitting dye DAPI. Excess dye was removed by washing with PBS and the stained cells were observed under blue (461 nm) and red (620 nm) filter of the fluorescence microscope (AxioScope.A1, Carl Zeiss, Germany).

3.5.6.5. MORPHOLOGY ANALYSIS BY GIEMSA STAINING

Giemsa stain is used to analyze the change in morphology of cells after treatment with toxicants. Giemsa stains the nucleus as dark purple and cytoplasm in light. For the assay, cells were counted at a density of $1x10^5$ cells and seeded over sterile coverslips placed in 6 well plates. Plates were kept overnight in a CO_2 incubator for attachment. Cells were exposed to varying concentration of LPS, LTA, PHA and TNP for 24h. The next day, cells were washed with PBS and fixed using 4% formaldehyde for 15 min. Fixative was removed and washed again with PBS. Cells were treated with 10% Giemsa stain for 5min and washed three times using PBS. Morphology of the control and treated cells was observed under a compound microscope (Leica, Japan).

3.5.6.6. DETECTION OF REACTIVE OXYGEN SPECIES (ROS) BY DCFH-DA

The fluorescent probe 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) was used to analyze reactive oxygen species generation within the cells. The esterified form of DCFH-DA dye is freely permeable through the cell membrane. The dye undergoes deacetylation by intracellular esterase and forms 2′, 7′-dichlorofluorescein (DCFH) within the cells. When ROS is generated inside the cells, it will oxidize DCFH to a fluorescent molecule dichlorofluorescein (DCF). The fluorescence generated is directly proportional to the amount of ROS produced inside the cells. In brief, the cells at an initial density of $1x10^4$ cells/wells were seeded in a 96 well plate were allowed to attach overnight. Cells were exposed to LPS, LTA, PHA and TNP along with 5µM DCFH-DA for 1h, 3h, 24h, 48h and 72h. After incubation, the supernatant medium was replaced with PBS. The fluorescence was read using fluorescence plate reader Infinite F Nano+, Tecan at an excitation and emission wavelength of 485 nm and 530 nm, respectively.

3.5.6.7. NITRILE RADICALS BY GRIESS REAGENT ASSAY

The nitric oxide production in MDM cells was analyzed by a modified Griess assay that relies on a diazotization reaction originally described by Griess in 1879. Griess reagent contains naphthyl ethylenediamine dihydrochloride suspended in water and sulphanilamide in phosphoric acid. This reagent reacts with nitrite released from cells to form a purple azo product, the absorbance of which is measured at 546 nm. In brief, cells were seeded at a density of $1x10^4$cells/well in a 96 well plate and incubated overnight. Cells were then exposed to LPS, LTA, PHA and TNP for 24h, 48h and 72h. 50µl of supernatant was taken and mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. The absorbance was read at 540 nm using a multiwell plate reader (Bio-Tek, Winooski, USA). The concentration of nitrite released within the sample was calculated from the standard graph plotted using sodium nitrite.

3.5.6.8. LYSOSOMAL INTEGRITY BY ACRIDINE ORANGE (AO) STAINING

Lysosomes are membrane-bound spherical organelle found in the cell cytoplasm. Destabilizations of lysosomes will result in leakage of hydrolytic enzymes into the cytosol. Since AO is a lipophilic, cationic fluorochrome, it diffuses through the cell membrane and enters an acidic lysosome compartment. The dye differentially stains lysosomes and cytosol depending on the pH. The dye gets protonated inside the acidic vesicles and emits orange-red fluorescence, whereas, in the cytoplasm, it remains green in color. The cells were seeded at an initial density of $5x10^4$cells/well in a 6 well plate and exposed to LPS, LTA, PHA and TNP for 24h. After incubation, the cells were washed with PBS and incubated in 1ml AO solution (2µg/ml in PBS) for 20 min at 37°C. The fluorescence image was taken under a Green and Red filter using Axio scope A1, Carl Zeiss fluorescent microscope.

3.5.6.9. MITOCHONDRIAL MEMBRANE POTENTIAL BY DILC1 (5) FACS ANALYSIS

The MitoProbe™ DiIC1 (5) Assay Kit provides solutions of the cyanine dye DiIC1 (5) (1, 1′, 3, 3, 3′, 3′-hexamethylindodicarbo - cyanine iodide) and CCCP (carbonyl cyanide 3-chlorophenylhydrazone) for the study of mitochondrial membrane potential (MMP). DiIC1 (5) is a cationic cyanine dye that penetrates the cytosol of eukaryotic cells. Dye accumulates primarily

in mitochondria with active membrane potentials and emits red fluorescence. DiIC1 (5) stain intensity decreases when cells are treated with reagents that disrupt mitochondrial membrane potential like CCCP. Here, the loss of MMP after treatment with LPS, LTA, PHA and TNP was evaluated in MDM cells. Briefly, $1x10^6$ cells were seeded in a 6 well plate and incubated overnight at $37^0C/5\%CO_2$. The cells were treated with LPS (0.25EU/ml, 1EU/ml and 5EU/ml), LTA, PHA and TNP (0.1μg/ml, 10μg/ml and 100μg/ml) for 24h. On the next day, the cells were harvested and centrifuged at 1200 rpm for 3 min. Pellets were washed with PBS and stained with 0.5μM concentration of DilC1(5) for 30 min at 37^0C. 1μl CCCP was added to the tube marked as the positive control. After incubation, cells were washed with PBS and FACS analysis was done using an excitation/emission filter of 638 nm/ 658 nm, respectively.

3.5.6.10. FLUORESCENCE IMAGING OF MITOCHONDRIAL MEMBRANE POTENTIAL BY JC1 DYE

JC1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimi- dazolylcarbocyanine iodide) is a cationic carbocyanine dye used to detect mitochondrial membrane potential. The dye is suitable for qualitative and quantitative assessment of membrane potential irrespective of size, shape and density of mitochondria. JC1 is a lipophilic dye, which enters into the mitochondria and polymerises to form J-aggregates in a concentration-dependent manner. J-aggregates emit red fluorescence, wherein the cytoplasm JC1exists as a monomer giving green fluorescence. In brief, the cells were seeded at an initial seeding density of $5x10^4$cells/well in a 6 well plate. Culture at confluency was exposed to LPS, LTA, PHA and TNP for 24h. At the end of exposure, 1μM JC-1dye was added to each well and incubated for 20 min at room temperature. The cells were washed three times with PBS and observed under a fluorescence microscope (Axio Scope.A1, Carl Zeiss, Germany). Photographs were taken using a green and red filter.

3.5.6.11. MEMBRANE PERMEABILISATION BY LDH ASSAY

The CyQUANT LDH cytotoxicity Assay kit was used to measure extracellular LDH released from the cells to media. LDH is a cytosolic enzyme present in cells and acts as an indicator of cellular toxicity. Destruction of the cell membrane due to the action of toxicants results in the release of LDH into the surrounding cell culture medium. In this assay, extracellular LDH can be

quantified by a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via NAD+ reduction to NADH. Diaphorase then uses NADH to reduce a tetrazolium salt (INT) to a red formazan product measured at 490 nm. The amount of formazan formation is directly proportional to the amount of LDH released into the medium. For the assay, $1x10^4$ cells were seeded into a 96 well plate and incubated overnight. Cells were then treated with LPS, LTA, PHA and TNP for 24h, 48h and 72h. After incubation, the cell culture supernatant was aspirated from each well and analyzed for the released LDH as per the manufacturer's instruction.

3.5.6.12. LIVE-DEAD ASSAY BY CALCEIN-AM/PROPIDIUM IODIDE

Calcein AM and propidium iodide (PI) dual staining was used to assess live- dead stage of a cell with respect to its membrane integrity. Calcein AM is a hydrophobic, permeable dye with good cell retention capabilities and insensitivity to fluctuations in the physiological pH range. Acetoxymethyl ester of calcein upon hydrolysis by intracellular esterase enzyme converted into a polyanionic fluorescein derivative emits green fluorescence in viable cells. PI, on the other hand, stains dead cells with compromised membrane integrity and emits red fluorescence. In this assay, cells were seeded at an initial density of $1x10^5$ cells/well in a 6 well plate and incubated at 37°C for 24h to get attached. The next day the medium was removed and the cells were treated with LPS, LTA, PHA and TNP for 24h, 48h and 72h. The medium was discarded and the cells were washed with PBS. Initially, Calcein AM (1µg/ml) was added to the wells and incubated at 37°C for 40 min. The cells were then treated with PI (2.5µM) for 5 min. The cells were washed with PBS and observed under a fluorescent microscope, Axio Scope.A1 (Carl Zeiss, Germany), using a green and red filter. Quantitative measurement was done using a fluorescent microplate reader using 485/ 595 nm filter (Infinite F Nano+, Tecan).

3.5.6.13. APOPTOSIS BY ANNEXIN V/ PI STAINING

Annexin/PI apoptosis kit is widely accepted to assess programmed cell death or apoptosis after exposure to various chemicals or toxicants. Annexin V is an impermeable Ca2+ dependent phospholipid-binding protein having a high affinity to phosphatidylserine (PS). PS is a phospholipid generally located at the inner surface of the plasma membrane. During apoptosis, there is flipping up of PS to the outer surface of the plasma membrane. Annexin V tagged with

green fluorescent dye Alexa flour 488 interacts only with PS present on apoptotic cells where PI stains dead or necrotic cells. Necrotic cells emit red fluorescence given by PI. The current experiment was carried out as per the manufacturer's instruction with slight modification. $1x10^6$cells were seeded in a 6 well plate and kept at 37°C/ 5% CO2 for 24h. After incubation, cells were exposed to LPS, LTA, PHA and TNP for 24h. The cells were harvested by trypsinization and centrifuged at 1200 rpm for 5 min. The pellets were washed three times with cold PBS. After washing, the cells were stained with 5µl of Annexin V constituted in 1X Annexin binding buffer (ABB) for 10 min. 1µl of 100µg/ml PI was added to each tube and incubated for 5min prior to the beginning of the assay. The samples were kept on ice and immediately analyzed using FACS (amnis) at fluorescence emission of 530 nm and 575 nm. Gating was done using four controls: cells without Annexin or PI, cells with Annexin V only, cells with PI only and finally cells with Annexin V and PI.

3.5.6.14. IMMUNOCYTOCHEMISTRY ANALYSIS OF NUCLEAR FACTOR KAPPA B (NFκB) p65

Nuclear translocation of activated NFκB p65 in MDM cells after LPS stimulation was analyzed using a fluorescent microscope. For the imaging, cells were seeded over a coverslip placed in a single well dish and differentiated with PMA for 48h. LPS at 0.25, 0.5, 1, 2 and 5EU/ml concentration was added to corresponding wells and incubated for 24h. After LPS exposure, the cells were washed with PBS and fixed with 4% formaldehyde for 5 min. Washed with PBS and permeabilized with Triton X100. Blocking of unwanted sites was done with 1%BSA. Washed three times with PBS and incubated overnight with anti-NFκB p65 primary antibody (1:500 dilution) at 4^0C. After incubation, the cells were washed three times with PBS. FITC tagged secondary antibody (1:500 dilution) was added and kept for 2h at room temperature. Washed with PBS and image was taken under a fluorescent microscope (Axio Scope). Actin filaments and the nucleus of the cells were counterstained with Rhodamine phalloidine and DAPI, respectively.

3.5.6.15. ANALYSIS OF NFκB p65 ACTIVATION BY FLOW CYTOMETRY

NFκB is an ancient transcription factor that regulates the expression of various immune mediators like cytokines and immunoglobulins. Flow cytometry was conducted to analyze NFκB p65 activation in MDM cells after exposure with LPS, LTA, PHA and TNP. Briefly, $1x10^6$ cells were seeded in 6 well plates and incubated at $37^0C/5\%$ CO_2. The cells were treated with LPS (0.25EU/ml, 1EU/ml and 5EU/ml), LTA (0.1µg/ml, 10µg/ml and 1000µg/ml), PHA (0.1µg/ml, 10µg/ml and 1000µg/ml) and TNP ((0.1µg/ml, 10µg/ml and 1000µg/ml) for 24h. Untreated cells were served as negative control. Cells were harvested from the wells and centrifuged at 1200 rpm for 3 min. Cell pellets were washed with sterile PBS. After washing, the cells were fixed with 80% ethanol for 5 min. Permeabilzation was done with 0.1% Triton X100. After permeabilization, the cells were washed three times with PBS and incubated with anti-NFκB p65 primary antibody (1:1000 dilution) overnight at 4^0C. Cells were washed with PBS and stained with FITC tagged secondary antibody. They were kept at room temperature for 2h. FACS analysis was done using a green laser.

3.5.6.16. CYCLOXYGENASE 2 (COX2) ACTIVATION BY FLOW CYTOMETRY

COX2 is the enzyme involved in the pathogenesis of fever through the induction of PGs secretion. Flow cytometry analysis was done to analyze COX2 activation in MDM cells after exposure with LPS, LTA, PHA and TNP. Briefly, $1x10^6$ cells were seeded in 6 well plates and incubated at $37^0C/5\%$ CO_2. The cells were treated with LPS (0.25EU/ml, 1EU/ml and 5EU/ml), LTA (0.1µg/ml, 10µg/ml and 1000µg/ml), PHA (0.1µg/ml, 10µg/ml and 1000µg/ml) and TNP ((0.1µg/ml, 10µg/ml and 1000µg/ml) for 24h. Untreated cells were served as the negative control. Cells were harvested from the wells and centrifuged at 1200 rpm for 3 min. Cell pellets were washed with sterile PBS. After washing, cells were fixed with 80% ethanol for 5 min. Cells were permeabilised with 0.1% Triton X100 for 1 min. After permeabilization, the cells were washed three times with PBS and incubated overnight with anti-COX2 primary antibody (1:1000 dilution) at 4^0C. Cells were washed with PBS and stained with FITC tagged secondary antibody. Kept at room temperature for 2h. FACS analysis was done using the green laser.

3.5.6.17. CYTOKINE GENE EXPRESSION- QUANTITATIVE REAL-TIME PCR (qRT-PCR) ANALYSIS

Cytokine expression from MDM was quantified by real-time qRT-PCR. THP-1 cells were cultured and differentiated as described previously in section 2.5.1

3.5.6.17.1. RNA ISOLATION

Total RNA was isolated from MDM cells exposed to LPS, LTA, PHA and TNP for 24h using RNeasy Mini Kit-QIAGEN, according to the manufacturer's instruction. RNA concentration was estimated by measuring the absorbance at 260 nm using a Biophotometer (Eppendorf). RNA isolated from control and test samples were kept frozen at -20°C until use.

3.5.6.17.2. cDNA SYNTHESIS

First-strand complementary DNA (cDNA) was reverse transcribed from 200ng of total RNA from the samples using the reverse transcriptase core kit, according to the manufacturer's instructions. cDNA prepared from control and test samples were stored at -20 °C until use.

3.5.6.17.3. qRT-PCR

The qRT-PCR reaction was performed in a BioRad instrument. Expression of IL-1β, TNF- α, NFκB and COX2 was analyzed. Primer sequences of all genes are given in **Table 3.3.** Polymerase chain reactions were performed using the following amplification program: initial denaturation at 95°C for 1 min, 35 cycles of 3s denaturation at 95◦C, 30s annealing at 51-62°C and finally 15s extensions at 72°C. The program was terminated with 10 min at 72°C. Reference gene β-actin (ACTB) was used for normalizing the gene expression data. Amplification of genes and data analysis was done by using the system software. Melting curve analysis was done to monitor the specificity of the amplified product.

S.No	Genes	Primer Sequence
1.	IL-1β	FP: 5'-ATAAGCCCACTCTACAGCT-3' RP: 5'-ATTGGCCCTGAAAGGAGAGA-3.'
2.	TNF-α	FP: 5'-CAGAGGGAAGAGTTCCCCAG-3' RP:-5'-CCTTGGTCTGGTAGGAGACG-3'
3.	NFκB	FP: 5'-CGCCGCTTAGGAGGGAGA-3.' RP: 5'-AGGTATGGGCCATCTGCTGT-3.'
4.	COX2	FP: 5'-TCATCAACACTGCCTCAATTC-3' RP: 5'-CTCTGGATCTGGAACACTGA-3'
5.	β-actin	FP: 5'-AACTACCTTCAACTCCATCA-3' RP: 5'-GAGCAATGATCTTGATCTTCA-3'

Table 3.3: Primers for Human Cytokine genes

3.5.7.DETECTION OF PYROGENICITY USING RABBITS

Study of *in vivo* immune response after LPS administration was carried out using Newzeland White rabbits. The detailed flow chart is given in **Figure 3.3.**

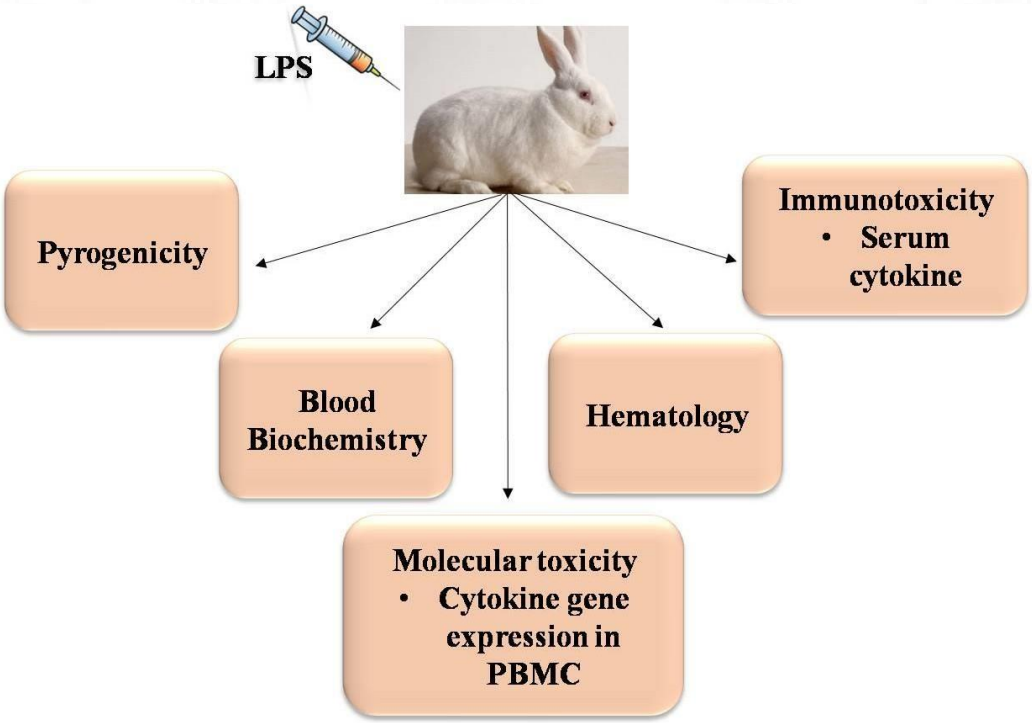

Figure 3.3: Flow chart of pyrogenicity and molecular toxicity studies

3.5.7.1. EXPOSURE OF ENDOTOXINS TO RABBITS

Healthy Newzeland White rabbits of either sex weighing 2-3 Kg were used for the study.

Rabbits before administration of LPS were considered as the control sample. Animals were conditioned for 7days and rectal temperature was recorded every 30 min for 3h. Before conditioning, venous blood was withdrawn from the marginal ear vein for biochemical, haematological and immunological analysis. The rectal temperature of all three animals was recorded 30 min prior to the administration of endotoxin. Animals received *i.v.* administration of LPS in saline (10ml/kg body weight) was the test group. Test animals were observed for temperature fluctuation at every 30 min interval for a period of 180 min after LPS administration. 3h later, blood was taken from each animal for biochemical, haematological and immunological analysis.

3.5.7.2. MEASUREMENT OF PYROGENICITY

The pyrogenic potential of LPS through intravenous administration was assessed as per USPA1/NF36, 2018. Pyrogen free syringes, needles and glassware were used for the study. LPS prepared in normal physiological saline was warmed at 25^{o}C for 30 min before *i.v.* administration. All rabbits were housed in an area of uniform temperature and their rectal temperature was taken using a thermometer. Rabbit, whose temperature does not vary by more than 1^{o}C from each other, was selected for the study. Rectal temperature is taken 30 min before the injection serves as control temperature. The ear vein of rabbits was disinfected with 70% alcohol and LPS (1EU/kg) containing solution was administrated into the ear vein of each of three rabbits at a dose of 10ml/kg body weight within a period of 10 min. The rectal temperature of each rabbit was recorded at every 30 min interval for a period of 3h subsequent to the injection. Rabbit shows an individual rise of 0.5^{o}C or more above its respective control temperature was considered as positive. The experimental design is given in **Table 3.4.**

Experimental Details	
Basal Temperature recording	The rectal temperature was recorded 30 min prior to the injection. This served as the control temperature.
Test Substance	Physiological saline containing lipopolysaccharides
No. of animals	3
Name of species	Rabbit
Name of strain	Newzealand white
Sex of animals	Male
Body weight	2400 – 2700 g
Route of injection	Intravenous (marginal ear vein)
Dosage of injection	10ml/kg body weight

Table 3.4: Experimental details of induction of pyrogenic response in rabbits

3.5.8. EVALUATION OF BLOOD PARAMETERS

3.5.8.1. HAEMATOLOGY ANALYSIS

Fresh venous blood was collected from each animal in heparinized blood collection tubes and was subjected to haematology analysis within an hour of collection. Haematology blood parameters, including haemoglobin (HGB) level, haematocrit (HCT), red blood cell (RBC), white blood cell (WBC) and platelet (PLT) count, were analyzed using an automated haematology analyser, Horiba Vet ABC (Japan).

3.5.8.2. BIOCHEMICAL ANALYSIS

Venous blood collected from each animal was collected into plain blood collection tubes and were allowed to clot at room temperature. The serum was separated by centrifugation at 3500

rpm for 15 min. Routine biochemistry analysis including blood sugar, total protein, albumin, creatinine, bilirubin, cholesterol, alkaline phosphatase (ALP), aspartate aminotransferase (SGOT), alanine transaminase (SGPT) *etc.*, were analysed by an automated biochemistry analyser, Erba Mannheim XL300 (Germany).

3.5.9. IMMUNOTOXICITY ANALYSIS

3.5.9.1. MEASUREMENT OF SERUM CYTOKINE LEVEL

The major inflammatory cytokine released from immune cells of rabbits before and after *i.v.* administration of LPS was assessed using commercially available ELISA kits. Serum collected from clotted blood samples were analyzed for cytokines IL-1β, IL-8 and TNF-α as per the manufacturer's instruction.

3.5.9.2. ISOLATION OF PERIPHERAL BLOOD LYMPHOCYTES

Venous blood collected from the marginal ear vein of the animal was transferred into a heparinised tube to prevent clotting. Blood was diluted with an equal volume of PBS (pH 7.4) and mixed by tilting. Diluted blood was layered over Histopaque 1077 solution and centrifuged at 900xg for 30 min at room temperature to achieve density gradient separation of blood components. The supernatant containing plasma and platelets were removed by aspiration. The buffy coat layer containing mononuclear cells in the interface between the plasma and packed RBCs were aspirated carefully and transferred into a fresh centrifuge tube. Collected PBMCs were washed twice with an excess volume of sterile PBS and resuspended in RPMI-1640 medium. Cell viability was assessed by staining with trypan blue dye.

3.5.9.3. CYTOKINE GENE EXPRESSION FROM RABBIT PBMC-qRTPCR

Cytokine gene expression from rabbit blood PBMC was done as described in section 3.5.6.17

3.5.9.3.1. RNA ISOLATION

RNA from PBMC was isolated as described in section 3.5.6.17.1

3.5.9.3.2. cDNA SYNTHESIS

cDNA synthesis was done as described previously in section 3.5.6.17.2

3.5.9.3.3. qRT-PCR

The qRT-PCR reaction was performed in a CFX 384 BioRad PCR instrument. Expression of NFκB, IL-1β and IL-8 was analyzed. Polymerase chain reactions were performed using the following amplification program: initial denaturation at 95°C for 1 min, 35 cycles of 3s denaturation at 95◦C, 30s annealing at 51–62°C, and finally 15s extensions at 72◦C. The program was terminated with 10 min at 72°C. Reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalizing the gene expression data. Amplification of genes and data analysis was done by using the system software. Melting curve analysis was done to monitor the specificity of the amplified product. The primer sequence for all cytokine genes is given in **Table 3.5.**

S.No	Genes	Primer Sequence
1	IL-1β	FP: 5'-TTGAAGAAGAACCCGTCCTCTG-3' RP: 5'-CTCATACGTGCCAGACAACACC-3'
2	NFκB	FP: 5'-CAAGAAGTCCACAAACAC-3' RP: 5'-ACCGATATGTCCTCTTTC-3'
3	IL-8	FP: 5'-GCTAAGAATACTGGAATTGT-3' RP: 5'-TAGGATGTTGGCTGATAC-3'
4	GAPDH	FP: 5'-TGACGACATCAAGAAGGTGGTG-3' RP: 5'-GAAGGTGGAGGAGTGGGTGTC-3'

Table 3.5: Primers for rabbit cytokine genes

CHAPTER 4: RESULTS

4. RESULTS

4.1. BLOOD COLLECTION FROM IMMUNIZED RABBITS

Venous blood was collected from the marginal ear vein of immunized rabbits after administering the second booster dose of recombinant human IL-1β. 10ml blood was collected and kept at 4^0C overnight. The serum was separated by centrifugation at 2000 rpm for 10 min. Approximately 6 ml of serum was separated and stored at -20^0C until use. 1ml of serum was subjected to IgG purification each time using HiTrap protein A affinity chromatography column on AKTA START FPLC system (GE Healthcare).

4.2. PURIFICATION OF IgG FROM RABBIT BLOOD SERUM

The IgG antibody released from immunized rabbits against recombinant human IL-1β was purified using the Hitrap Protein A column. Hitrap Protein A column is specific for binding with monoclonal or polyclonal IgG antibodies. Protein A is prepared from a selected strain of *Staphylococcus aureus* and it consists of six different regions. Out of six, five regions are specific for binding with Fc-part of IgG. **Figure 4.1** indicates the single peak of purified IgG from rabbit serum and the purified IgG was collected in fraction collection tubes. These fractions were pooled, dialyzed and stored at -20^oC for further coating on ELISA plates.

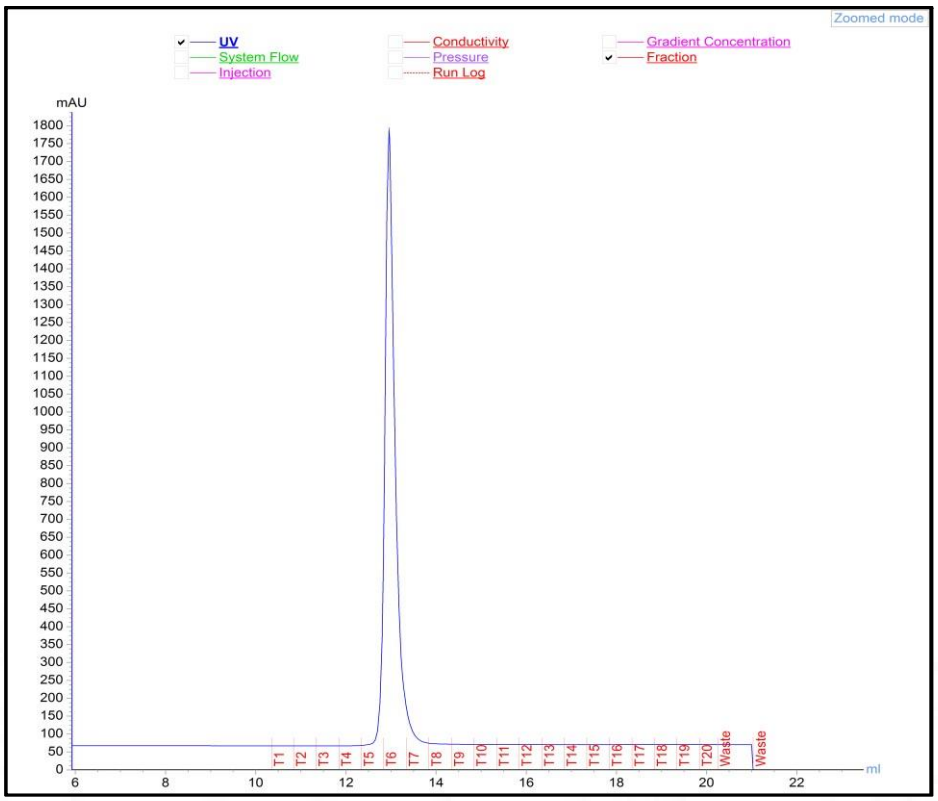

Figure 4.1: Purified anti-human IL-1β IgG antibodies by affinity chromatography

4.3. DOT BLOT ASSAY

The specificity of antibody produced against human IL-1β was confirmed by Immuno dot-blot assay (**Figure 4.2**). Anti-human IL-1β raised in rabbits interacts explicitly with human IL-1β and produce purple colour dots. Booster dose with human IL-1β enhanced the purple colour of the dot. It is due to the increased titer of anti-human IL-1β in rabbit blood following subsequent booster doses of recombinant human IL-1β.

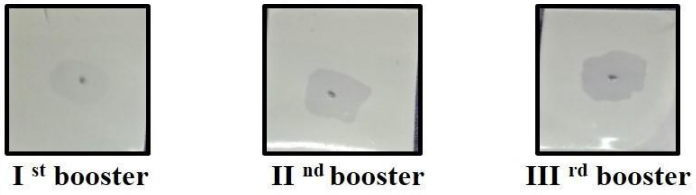

Figure 4.2: Immuno dot-blot assay for detection of anti-human IL-1β from rabbit serum

4.4. ESTIMATION OF PROTEIN CONCENTRATION FROM DIALYZED FRACTION

Micro-Bradford assay was done to analyze the protein concentration of dialyzed fraction of purified IgG. Bovine serum albumin Fraction V is a known protein, which is used as the standard. From the standard graph (**Figure 4.3**), the protein concentration present in the dialyzed fraction was calculated and was found to be 2.16µg/µl. The same protein fraction was used for HRP conjugation and was used as a secondary antibody for detection.

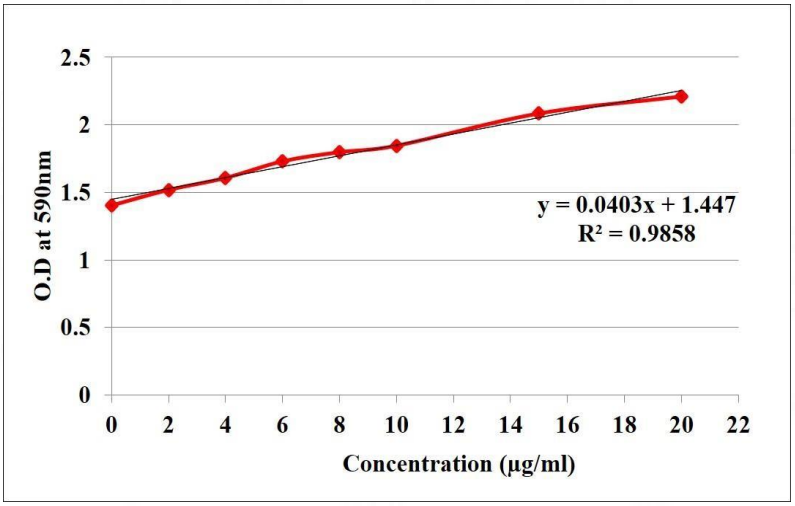

Figure 4.3: Standard graph of micro-Bradford assay

4.5. COATING OF ANTI-HUMAN IL-1β ON ELISA PLATE

Purified IgG or anti-human IL-1β from rabbit serum was used for surface coating on ELISA plate at a concentration of 10µg/ml. The required volume of protein was diluted in carbonate-bicarbonate buffer at pH 9.8. 50µl of protein solution was added to each well of 96 plates and allowed to attach overnight at 4^0C. These coated plates will be subjected to the measurement of IL-1β from the sample.

4.6. COLLECTION AND POOLING OF BLOOD FOR DETECTION OF IL-1β USING ELISA

Venous blood from four volunteers as per methodology 3.5.5.1 was collected in heparinised and clot activator tubes. Blood was pooled immediately after collection. Individual and pooled blood samples were subjected to biochemical analysis using a Biochemistry analyser, Erba Mannheim XL300 and haematological analysis using an automated haematology analyzer.

4.6.1. BLOOD BIOCHEMISTRY

Biochemical parameters such as glucose, cholesterol, triglycerides, ALT, AST, ALP, total bilirubin, total protein, albumin and creatinine were monitored in individual and pooled blood. All the biochemical values analyzed were under the standard/reference range (**Table 4.1**).

S. No	Parameter	Donor 1	Donor 2	Donor 3	Donor 4	Pooled	Mean ±SD	Reference range
1	Glucose	103	109	91	105	119	102±7.0	70-110 mg/dl
2	Cholesterol	176	182	145	146	168	162±19	<200 mg/dl
3	Triglycerides	77	107	30	55	73	67±32	<150mg/dl
4	Alanine transaminase	17.9	32.7	18.1	15.7	19.4	21.1±7.8	7-55 IU/L
5	Aspartate transaminase	24.9	35.6	24.1	20.3	22.4	26.2±6.5	8-48 IU/L
6	Alkaline phosphatase	29	73	36	53	50	47±19	40-129 IU/L
7	Total Bilirubin	0.59	0.4	0.3	0.1	0.24	0.34±0.2	0.1-1.2 mg/dl
8	Total Protein	9.8	10.6	11.1	9.5	10.7	10.2±0.7	6-8.3 g/dl
9	Albumin	6.4	7.2	7.4	6.3	7.2	6.8±0.5	3.5-5 g/dl
10	Creatinine	1.15	1.46	1.01	1.1	1.23	1.18±0.1	0.84-1.21 mg/dl

Table 4.1: Biochemical parameters of individual and pooled blood. n=4, Data represent Mean±SD

4.6.2. HEMATOLOGY ANALYSIS

Venous blood collected from individual volunteers and pooled blood was analyzed for complete blood cell count using an automated haematology analyzer within an hour of blood collection. Haematology parameters of all donors and pooled blood were found to be within the standard/reference range (**Table 4.2**).

Parameter	Donor 1	Donor 2	Donor 3	Donor 4	Pooled	Mean ±SD	Reference range
WBC ($x10^3$/µl)	7.54	8.3	6.72	7.12	6.72	7.42±0.67	4–10
Lym%	40.7	36.1	52.3	33.3	38.8	40.6±8.3	18-45
Gran%	54.1	60.3	41.3	62.9	57.2	54.65±9.6	50-70
Mid%	5.2	3.6	6.4	3.8	4.0	4.75±1.3	3-10
RBC ($x10^6$/mm3)	4.33	6.02	4.03	4.33	4.06	4.67±0.9	4.50–5.50
Hb (g/dl)	13.7	16.7	12.5	10.8	14.1	13.4±2.4	13.0–16.0
HCT (%)	38.8	47.6	36.1	28.3	32.7	37.7±7.9	40.0–54.0
MCV (µm3)	89.7	79.0	89.7	65.5	80.7	80.9±11.4	75–95
MCH (pg)	31.7	27.7	31.2	22.7	34.7	28.3±4.1	30–35
MCHC (g/dl)	35.4	35.0	34.7	34.7	43.1	34.9±0.3	30–35
PLT ($x10^3$/µl)	331	244	216	344	259	283±63	150–500

Table 4.2: Hematology parameters of individual and pooled blood.WBC; white blood cells, RBC: red blood cells, HGB; haemoglobin, HCT; haematocrit, MCV; Mean corpuscular volume, MCH; Mean corpuscular haemoglobin, MCHC; mean corpuscular haemoglobin concentration. n=4, Data represent Mean±SD.

The haematological and biochemical parameters of individual and pooled blood were found to be under normal range and this pooled blood was used as a test system for detection of IL-1β

4.7. DETECTION OF IL-1β BY ELISA

4.7.1. STIMULATION OF POOLED HUMAN BLOOD WITH LIPOPOLYSACHARIDES (LPS)

The release of IL-1β is estimated using indigenously developed ELISA, following the stimulation of pooled human blood with 1 and 5EU/ml concentration of LPS for a period of 0 to 8h. The optical density (O.D) values were measured at 0, 2, 4, 6 and 8h using an ELISA plate reader. The O.D. values were plotted against time for estimating IL-1β released from pooled blood and is mentioned in **Figure 4.4**. Compared to the non-stimulated control, LPS stimulated blood showed a significant increase in the release of IL-1β. It was noted that the release of IL-1β begins at 2h following stimulation with 1EU/ml of LPS and elevated until 8h. Exposure of 5 EU/ml of LPS induced rapid release of IL-1β within 1h and reaches the maximum at 2h.

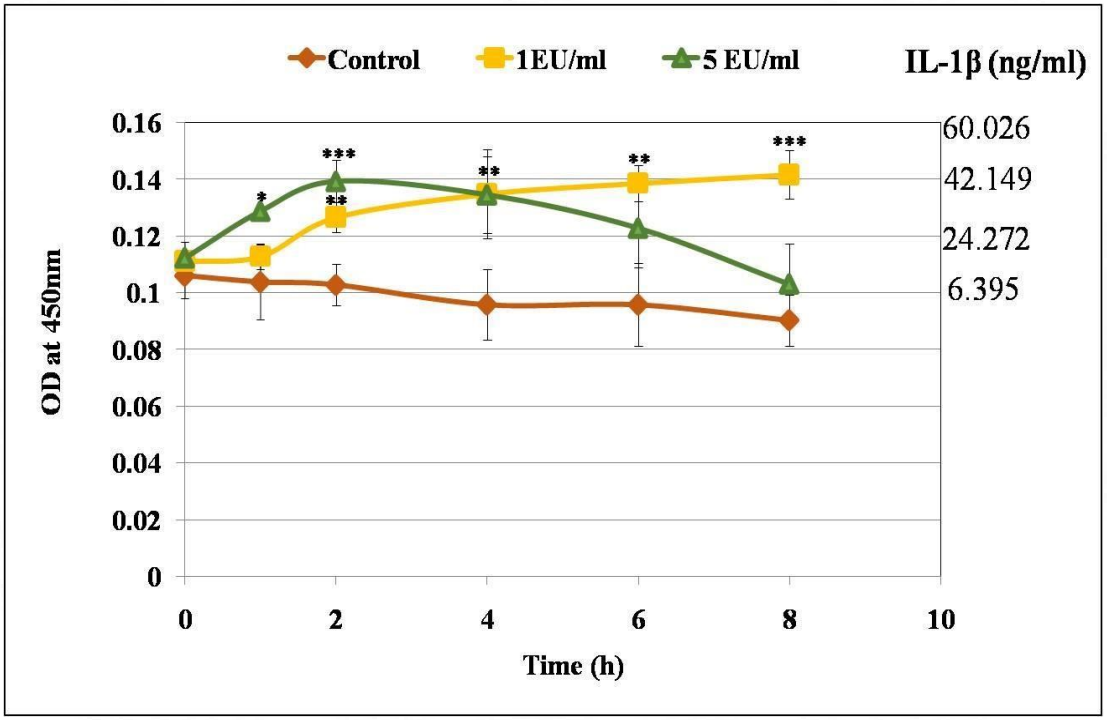

Figure 4.4: The IL-1β release from pooled blood by stimulating with LPS (1 and 5EU/ml) using ELISA. Reactions were monitored for 8h at 2h intervals. n=3, Data represents Mean±SD, *p<0.05, **p<0.01, ***p<0.001

4.7.2. STIMULATION OF POOLED HUMAN BLOOD WITH LIPOTEICHOIC ACID (LTA)

The pyrogenic potential of LTA from Gram-positive bacteria *Bacillus subtilis* is estimated using indigenously developed ELISA for IL-1β detection. The present study investigated the release of IL-1β from pooled human blood following stimulation with 0.1 and 1µg/ml concentration of LTA. Reaction supernatant containing IL-1β released from blood cells were evaluated for a period of 8h. It was observed that LTA at 0.1µg/ml induced maximum cytokine release between 2h to 6h, whereas exposure of 1µg/ml LTA caused a sudden rise in IL-1β and reached a maximum within 2h. A decline of IL-1β release was observed from 4 to 8h following stimulation with 1µg/ml of LTA (**Figure 4.5**)

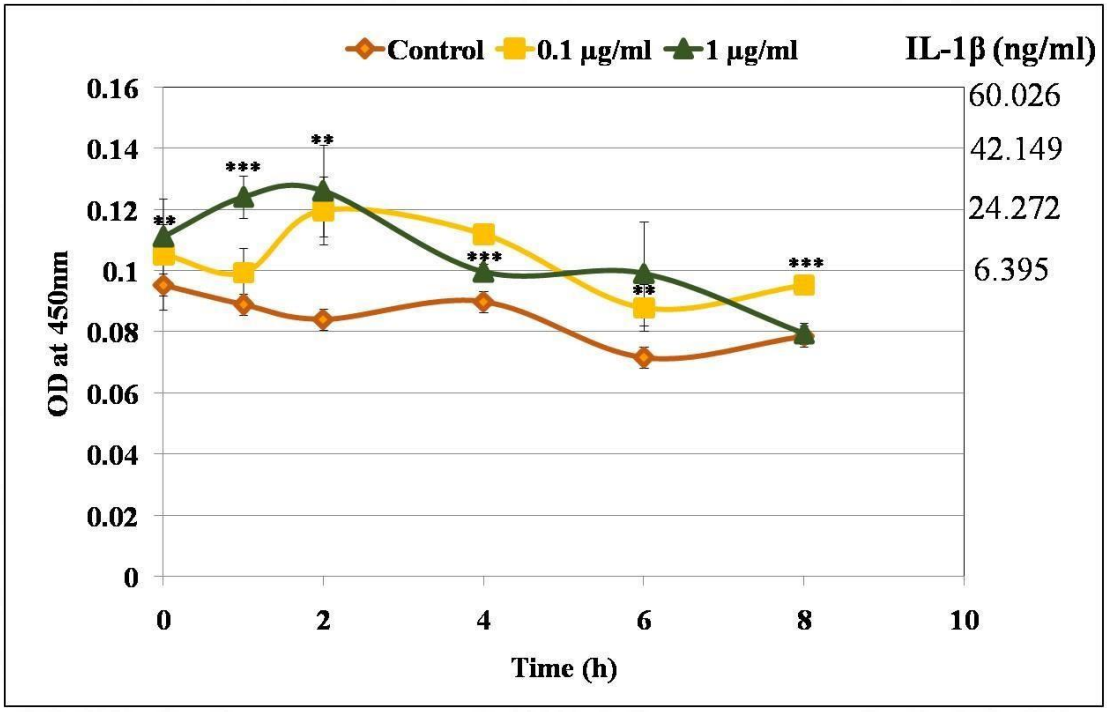

Figure 4.5: The IL-1β release from pooled blood by stimulating with LTA (0.1 and 1µg/ml) using ELISA. Reactions were monitored for 8h at 2h intervals. n=3, Data represents Mean±SD, *p<0.05, **p<0.01, ***p<0.001

4.7.3. STIMULATION OF POOLED HUMAN BLOOD WITH PHYTOHEMAGGLUTININ (PHA)

The present study investigated the potential of PHA to induce IL-1β release within a specified time interval. Here, a statistically significant rise of IL-1β was observed following the exposure of 15µg/ml of PHA during the period of 2 to 8h. Maximum release of IL-1β was observed at 4h. There was a rise in IL-1β observed at 1h when exposed to 30µg/ml PHA. Afterwards, the level of IL-1β begins to drop and reached baseline at 8h. **Figure 4.6** represent the release of IL-1β following PHA stimulation.

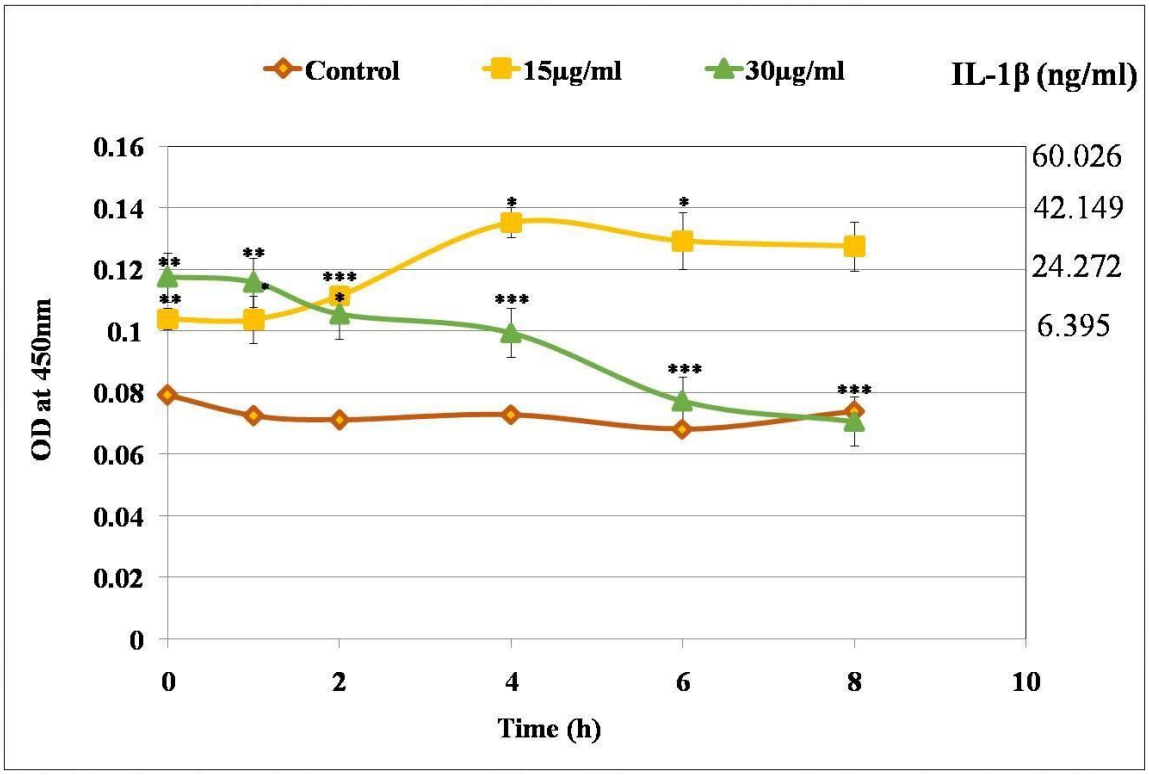

Figure 4.6: The IL-1β release from pooled blood by stimulating with PHA (15 and 30µg/ml) using ELISA. Reactions were monitored for 8h at 2h intervals. n=3, Data represents Mean±SD, *p<0.05, **p<0.01, ***p<0.001

4.7.4. STIMULATION OF POOLED HUMAN BLOOD WITH 2, 4, 6, TRINITROPHENOL (TNP)

2, 4, 6, trinitrophenol (TNP) is selected as a chemical pyrogen to induce IL-1β release from pooled blood. It was noted that TNP is a potent stimulator of pyrogenic response *in vitro*. Maximum IL-1β release was found at 2h following the exposure of 30µg/ml PHA and declined after 4h, as shown in **Figure 4.7**. Compared to 30µg/ml TNP, a concentration of 15µg/ml TNP caused a steady rise in IL-1β release from blood cells. Following 15µg/ml treatment, IL-1β began to rise at 2h and remained elevated up to 8h.

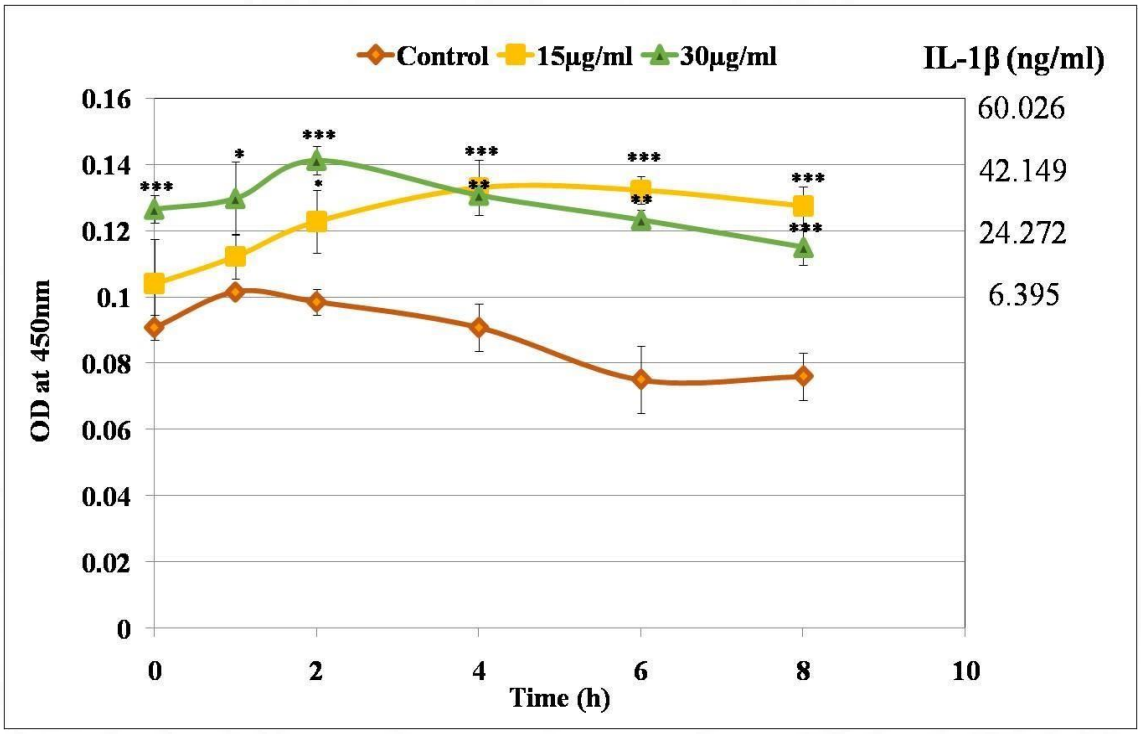

Figure 4.7: The IL-1β release from pooled blood by stimulating with TNP (15 and 30µg/ml) using ELISA. Reactions were monitored for 8h at 2h intervals. n=3, Data represents Mean±SD, *p<0.05, **p<0.01, ***p<0.001

4.8. MOLECULAR AND CYTOTOXICITY ANALYSIS

4.8.1. CHARACTERIZATION OF THP-1 CELLS

4.8.1.1. IMMUNOCYTOCHEMISTRY

Differentiation of THP-1 cells to MDM cells was done by incubating the cells with 100nM PMA for 48h. It was characterized by immunocytochemistry using a CD68 marker. CD68 is a protein highly expressed in phagocytic cells. CD68 expression is minimal in undifferentiated THP-1 cells and there was no green fluorescence in THP-1 cells. THP-1 cells are negative for the CD68 marker. Green fluorescence observed in MDM cells indicates its functional differentiation and is positive for macrophage marker CD68 **Figure 4.8**. The cell nucleus was counterstained with blue fluorescent dye DAPI.

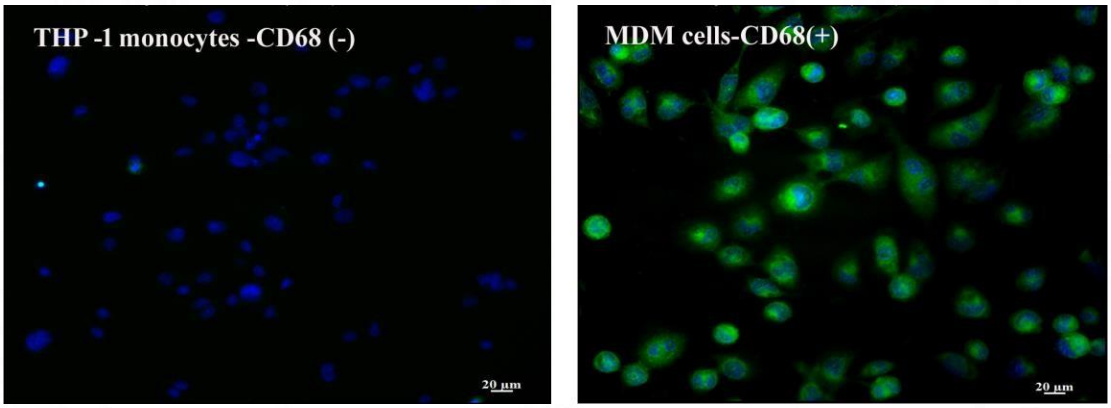

Figure 4.8: Characterization of MDM cells using CD68 macrophage marker. Green fluorescence represents CD68 expression in MDM cells. The nucleus was counterstained with DAPI and give blue florescence. Scale bar 20µm, Magnification 20x

4.8.1.2. FLOW CYTOMETRY

PMA mediated differentiation of THP-1 cells to MDM cells was assessed by flow cytometry. An increase in SSC parameters suggests high cellular granularity of MDM cells compared to THP-1 monocytes (**Figure 4.9**).

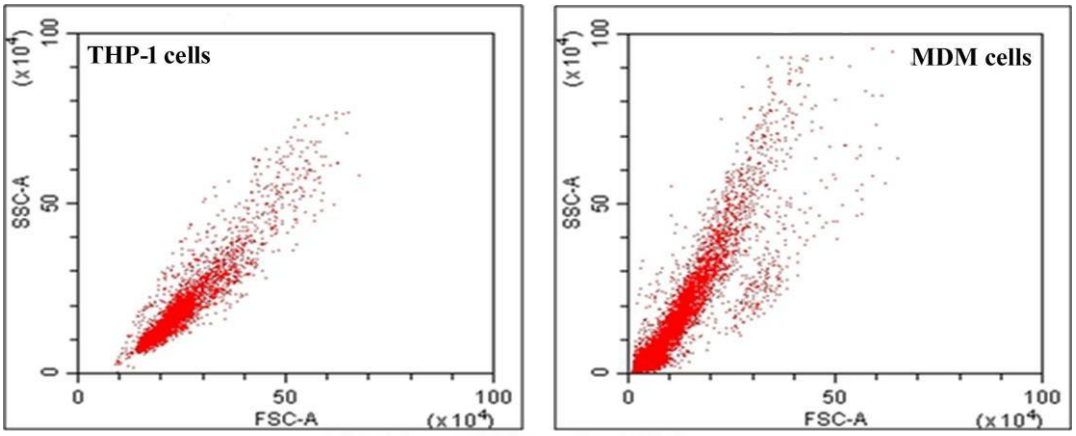

Figure 4.9: Macrophage differentiation by flow cytometry analysis. Compared to THP-1 cells, change in the SSC pattern of MDM cells indicate increased granularity following PMA induced differentiation. SSC: side scatter, FSC: forward scatter.

4.8.1.3. PHASE CONTRAST MICROSCOPY

The PMA activated differentiation of THP-1 monocyte was observed under a phase-contrast microscope. Undifferentiated THP-1 cells are round in shape and are not get attach to the surface of the culture flask. PMA differentiated THP-1 cells, or MDM, is attached to the bottom of the culture flask and showed macrophage-like phenotype. The cells become less refractive to light microscopy and some of the cells exhibited elongated morphology. MDM cells exhibit increased cytoplasmic ratio, pseudopodia and extended vacuolar system. As shown in **Figure 4.10,** the morphology of differentiated macrophages is non-uniform in nature.

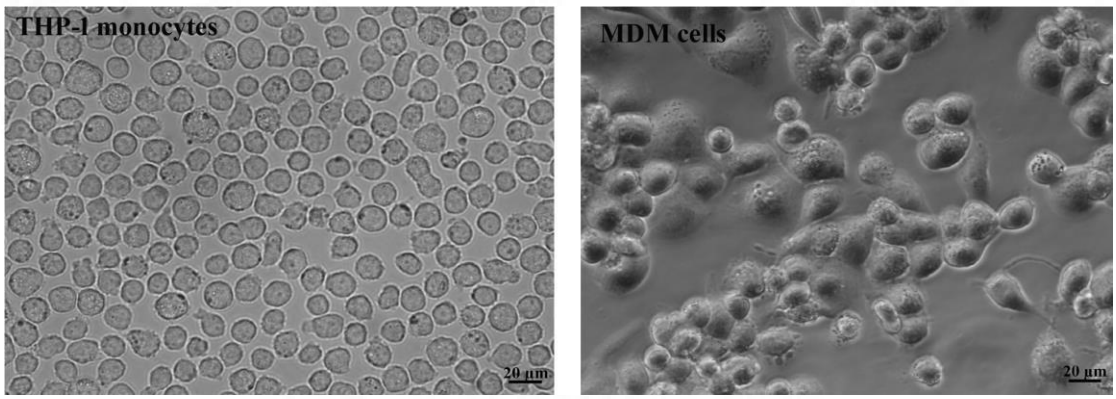

Figure 4.10: Phase contrast image of differentiation of THP-1 cells to MDM cells using PMA for 48h. Scale bar 20µm, Magnification 40x

4.9. CYTOTOXICITY ASSAYS

4.9.1. CYTOSKELETAL INTEGRITY BY RHODAMINE PHALLOIDINE STAINING

Cytoskeletal rearrangement after exposure to LPS, LTA, PHA and TNP was analyzed by using fluorescent probe Rhodamine phalloidine. Rhodamine phalloidine is an actin-binding dye that emits red fluorescence. No cytoskeletal rearrangement of MDM cells was evident after exposure to 5EU/ml LPS for 24h (**Figure 4.11 A**). The slight slandering of cells was visible after exposure to 1000µg/ml of LTA (**Figure 4.11 B**), PHA (**Figure 4.12 A**) and TNP (**Figure 4.12 B**). Macrophage characteristics like pseudopodia were absent in PHA (1000µg/ml) and TNP (1000µg/ml) treated cells. The cellular morphology becomes round due to actin destabilization. Visible reduction in the number of cells was observed following exposure of 1000µg/ml of PHA and TNP for 24h, as the cells were detached from the culture dish.

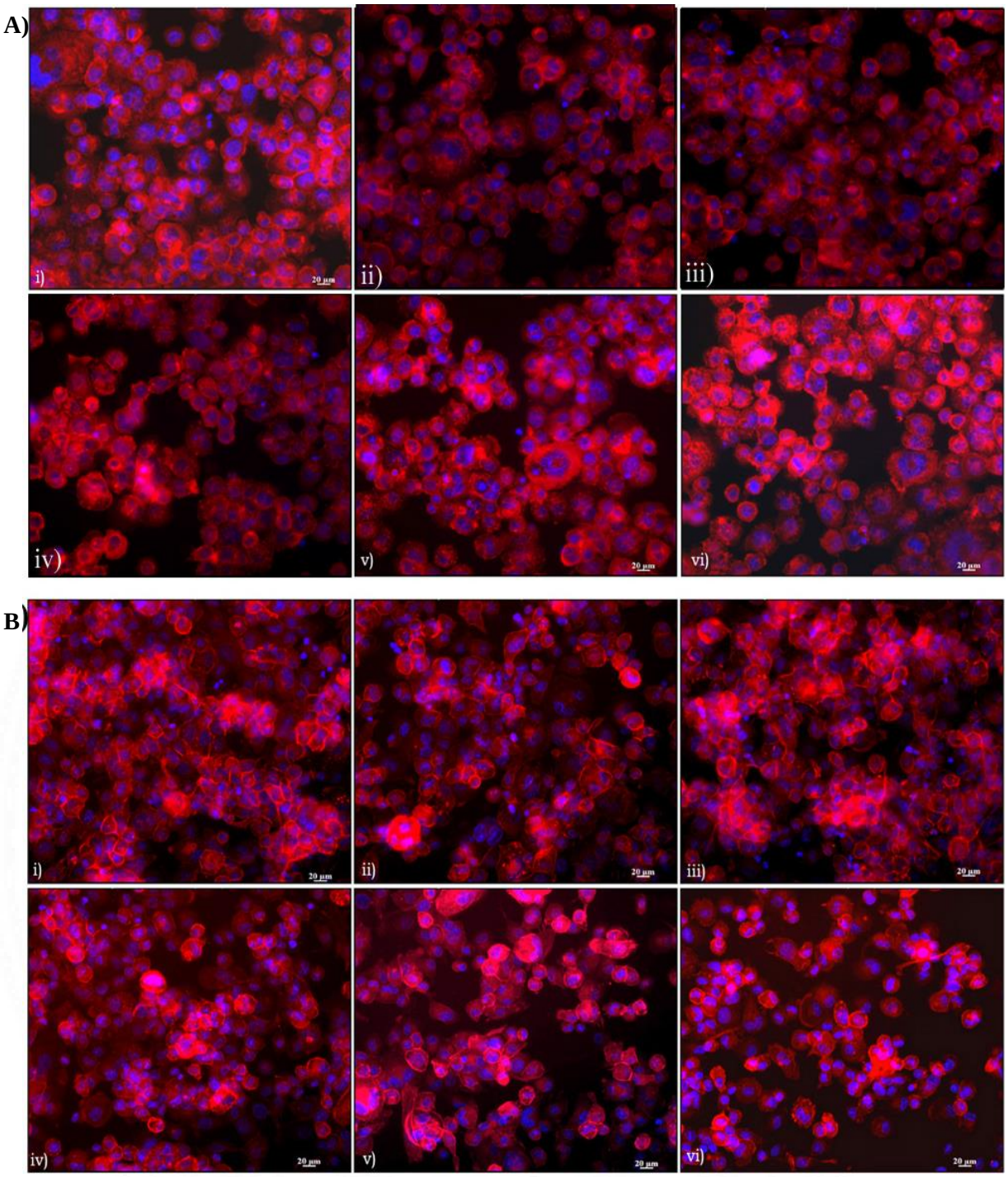

Figure 4.11: Rhodamine phalloidine staining of MDM cells **A)** i) Control ii) LPS 0.25EU/ml iii) LPS 0.5EU/ml iv) LPS 1EU/ml v) LPS 2EU/ml vi) LPS 5EU/ml **B)** i) Control ii) LTA 0.1µg/ml iii) LTA 1µg/ml iv) LTA 10µg/ml v) LTA 100µg/ml vi) LTA 1000µg/ml for 24h. Untreated cells were used as control. The scale bar represents 20µm. Magnification 20x

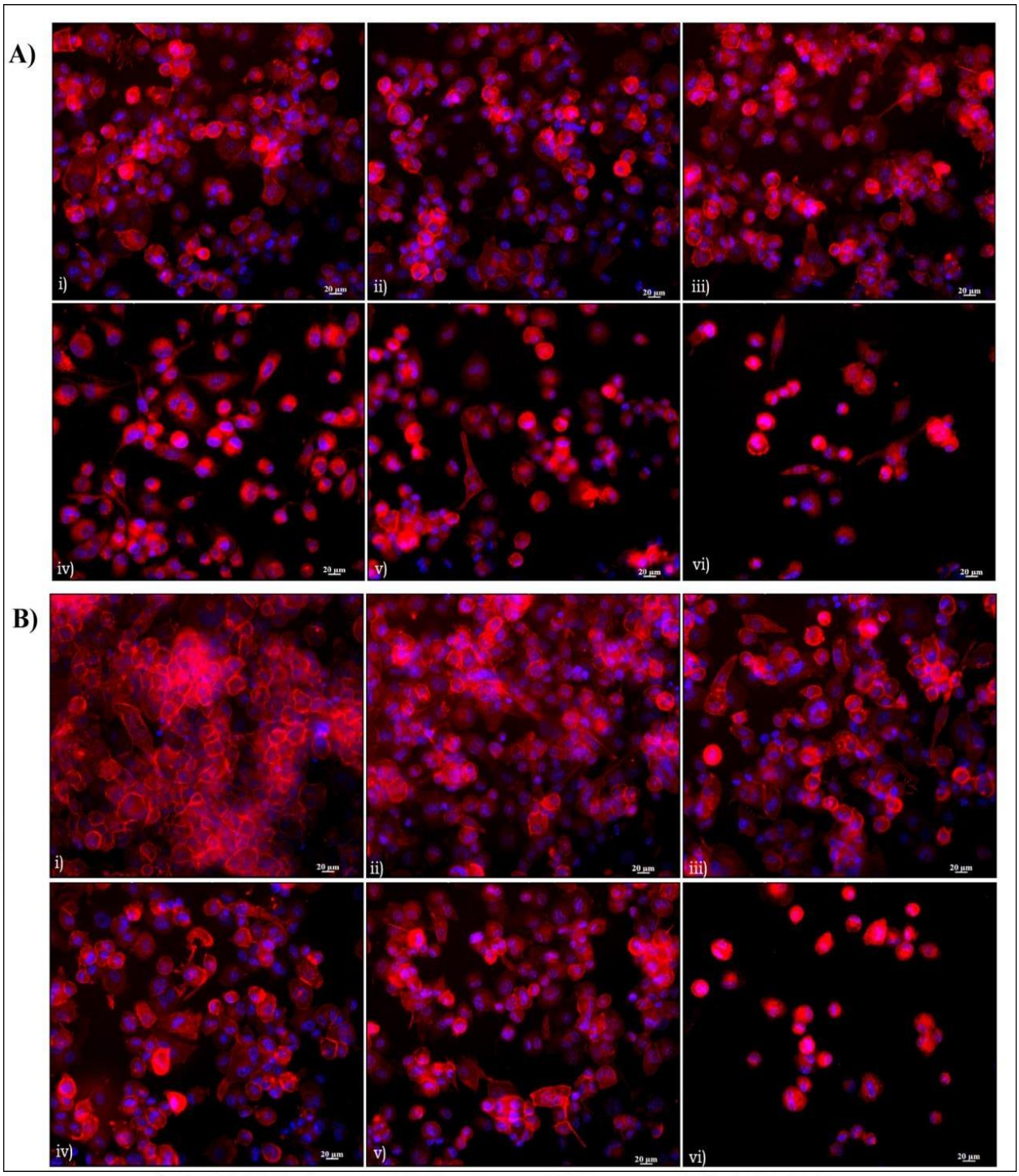

Figure 4.12: Rhodamine phalloidine staining of MDM cells **A)** i) Control ii) PHA 0.1µg/ml iii) PHA 1µg/ml iv) PHA 10µg/ml v) PHA 100µg/ml vi) PHA 1000µg/ml **B)** i) Control ii) TNP 0.1µg/ml iii) TNP 1µg/ml iv) TNP 10µg/ml v) TNP 100µg/ml vi) TNP 1000µg/ml for 24h. Untreated cells were used as control. The scale bar represents 20µm. Magnification 20x

4.9.2. MORPHOLOGY ANALYSIS BY GIEMSA STAINING

Cellular morphology was analyzed after exposure of LPS, LTA, PHA and TNP using Giemsa staining. MDM cells exhibit characteristic cell adhesion and spreading morphology of macrophages. There was no change in morphology and cell adhesion properties following 24h exposure of LPS up to 5EU/ml. It was noted that between 0.1 to 100µg/ml of LTA, PHA and TNP also did not affect the cell adhesion and spreading character of MDM cells. However, exposure of cells at 1000µg/ml of LTA, PHA and TNP caused instability in cell attachment. Loss of membrane integrity resulted in the detachment of cells from the culture flask. There found a reduction in the number of cells and were appeared round in morphology (**Figure 4.13)**.

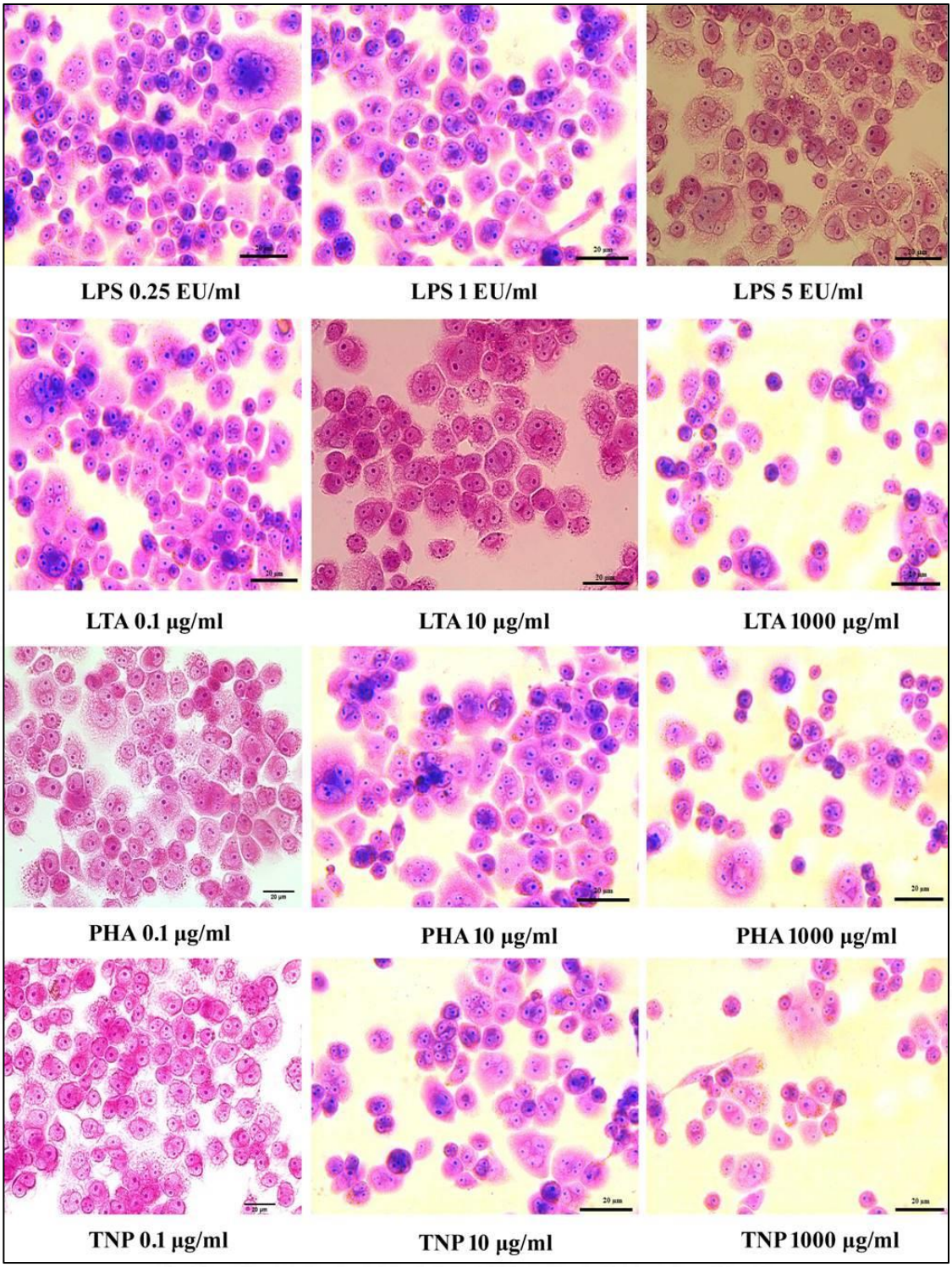

Figure 4.13: Giemsa staining for analysis of cellular morphology following 24h exposure with LPS (0.25EU/ml, 1EU/ml, 5EU/ml), LTA, PHA and TNP (0.1µg/ml, 10µg/ml, 1000µg/ml). Scale bar 20µm, Magnification 40x

4.9.3. MITOCHONDRIAL ACTIVITY BY MTT ASSAY

As shown in **Figure 4.14**, LPS does not affect mitochondrial activity in relation to the dose exposed to MDM cells even after 72h of exposure. The cellular mitochondria were seemed to be active up to 100µg/ml of LTA, PHA and TNP for 24h. Statistically significant decrease in mitochondrial activity was observed in cells exposed to 1000µg/ml of LTA (72.71±0.04), PHA (40.9±0.02) and TNP (57.55± 0.03) compared to untreated cell control. When the time of exposure increased to 48 to 72h, there was a gradual reduction in mitochondrial activity. After 72h, 40- 50% reduction in mitochondrial activity was detected at 100µg/ml of LTA (43.04±0.002), PHA (56.73±0.004) and TNP (64.45±0.004) respectively.

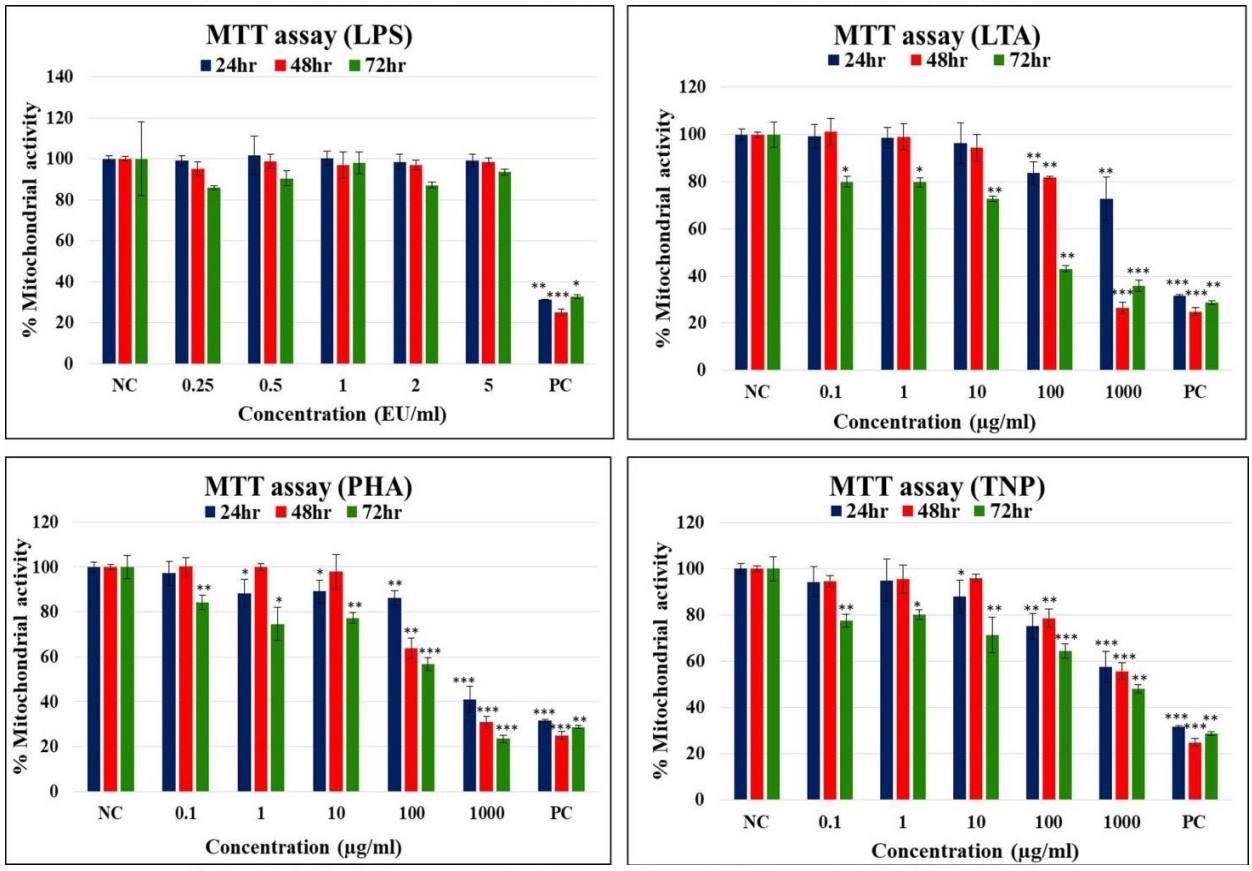

Figure 4.14: MTT assay on MDM cells exposed to LPS, LTA, PHA and TNP for 24h, 48h and 72h. The values are expressed in percentage with respect to control. Phenol treated cells were used as the positive control. The data represent mean ± SD of three independent experiments, n=3. Asterisk above columns denotes statistically significant difference, compared to the control (* $p<0.05$, ** $p< 0.01$ and *** $p<0.001$)

4.9.4. LYSOSOMAL ACTIVITY BY NEUTRAL RED UPTAKE ASSAY

Lysosomal activity in terms of uptake of neutral red dye was analyzed following exposure of LPS, LTA, PHA and TNP. Reduction in the uptake of neutral red dye by the lysosomes indicates loss of functional activity of lysosomes. LPS (0.25 to 5EU/ml) treated cells showed normal lysosomal activity comparable to negative control following 24h, 48h and 72h exposure. Loss of lysosomal activity begins at 24h exposure at the highest concentration of LTA, PHA and TNP (1000 µg/ml). LTA, PHA and TNP induced decline in lysosomal activity increased in a dose and time-dependent manner. Exposure of 100µg/ml LTA, PHA and TNP up to 72h caused a significant reduction in lysosomal activity at a range of 61.84±0.003, 76.59±0.007 and 68.38±0.006, respectively (**Figure 4.15**).

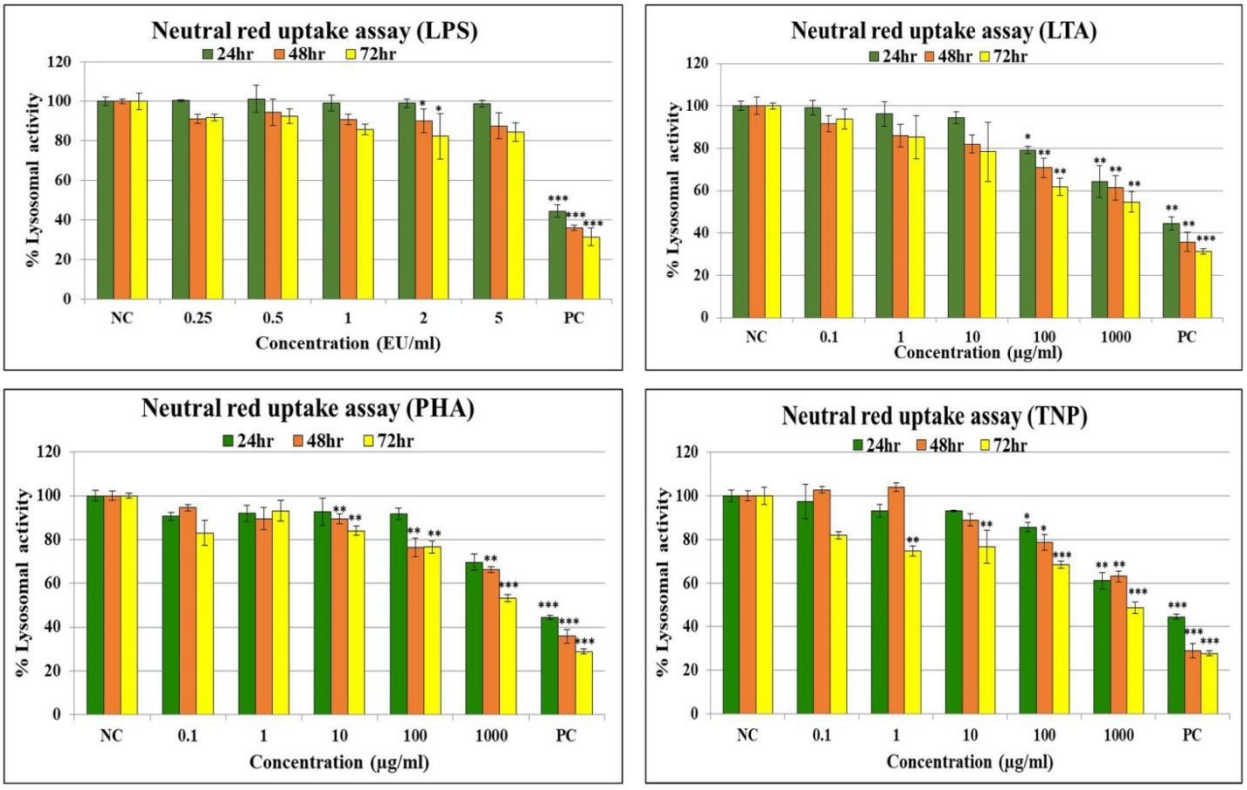

Figure 4.15: Neutral red assay on MDM cells exposed to LTA, PHA and TNP for 24h, 48h and 72h. The values are expressed in percentage with respect to control. SDS treated cells were used as positive control. The data represent mean ± SD of three independent experiments, n=3. Asterisk above columns denotes statistically significant difference, compared to the control (* $p<0.05$, ** $p< 0.01$ and *** $p<0.001$

4.9.5. FREE RADICAL GENERATION BY DCFHDA ASSAY

ROS production in MDM cells after exposure with LPS, LTA, PHA and TNP was analyzed by using the fluorescent probe DCFH-DA. Emission of green fluorescence due to the oxidation of DCFH by reactive hydrogen radical generated on exposure to LPS, LTA, PHA and TNP were recorded at 1h, 3h, 24h, 48h and 72h time intervals (**Figure 4.16**). From the assay, it was observed that LPS and PHA treatment do not induce ROS generation in MDM cells within the time intervals. LTA and TNP exposure have a positive effect on ROS generation in a dose and time-dependent manner. Incubation of MDM cells with the highest concentration of LTA (1000μg/ml) generated ROS within 3h (22986.6 ± 801.30) and 24h (36928 ± 1317.5). An increase in time of exposure (48 and 72h) enhanced ROS generation even at lower concentration of LTA and TNP.

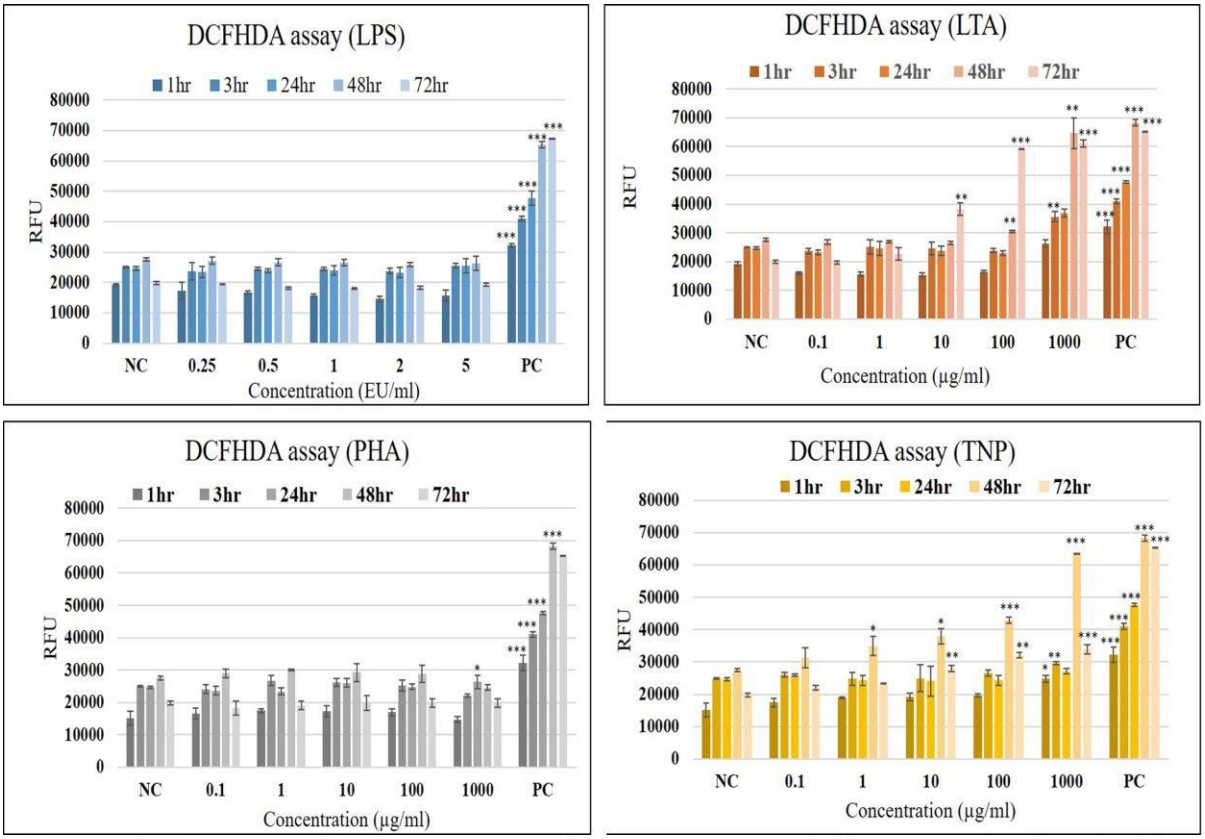

Figure 4.16: Free radical release by DCFH-DA assay after LPS (0.25, 0.5, 1, 2 and 5EU/ml), LTA, PHA and TNP (0.1, 1, 10, 100 and 1000µg/ml) for 24h, 48h and 72h. Data represent the mean ± SD of three independent experiments. Asterisk denotes a statistically significant difference compared to the control group (*p<0.05, **p<0.01 and ***p<0.001).

4.9.6. NITRILE RADICAL RELEASE ASSAY

The induction of nitrile radical release on exposure of toxicants to MDM cells was analyzed by Griess assay. From **Figure 4.18,** it was evident that there was no stress-induced release of nitrile radical after LPS, LTA and PHA exposure for 24h, 48h and 72h. The concentration of NO production was obtained from a standard graph plotted as O.D at 540nm against the concentration of nitrite **Figure 4.17**. An increase in nitrile radical production (0.49±0.006) was observed after exposure to 1000µg/ml of TNP for 24h. It was also found that there is a significant rise in NO following LTA and TNP challenge when the time of exposure extended to 48 and 72h. 48h exposure increased release of NO in 100 µg/ml LTA (14.21±0.0007), 1000µg/ml LTA (26.71±0.096) and 1000µg/ml TNP (21.42±0.038). Following 72h of LTA and

TNP, NO release observed in the following as 100µg/ml LTA (13.57±0.012), 1000µg/ml LTA (32.78±0.08) and 1000µg/ml TNP (20.78±0.0007).

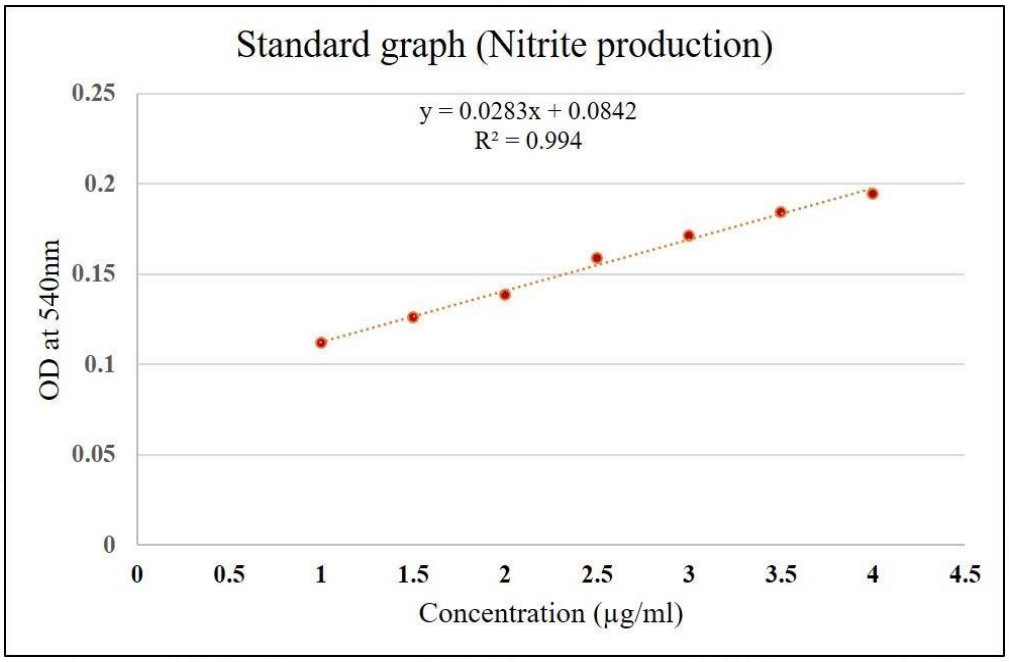

Figure 4.17: Standard graph of Nitrite production

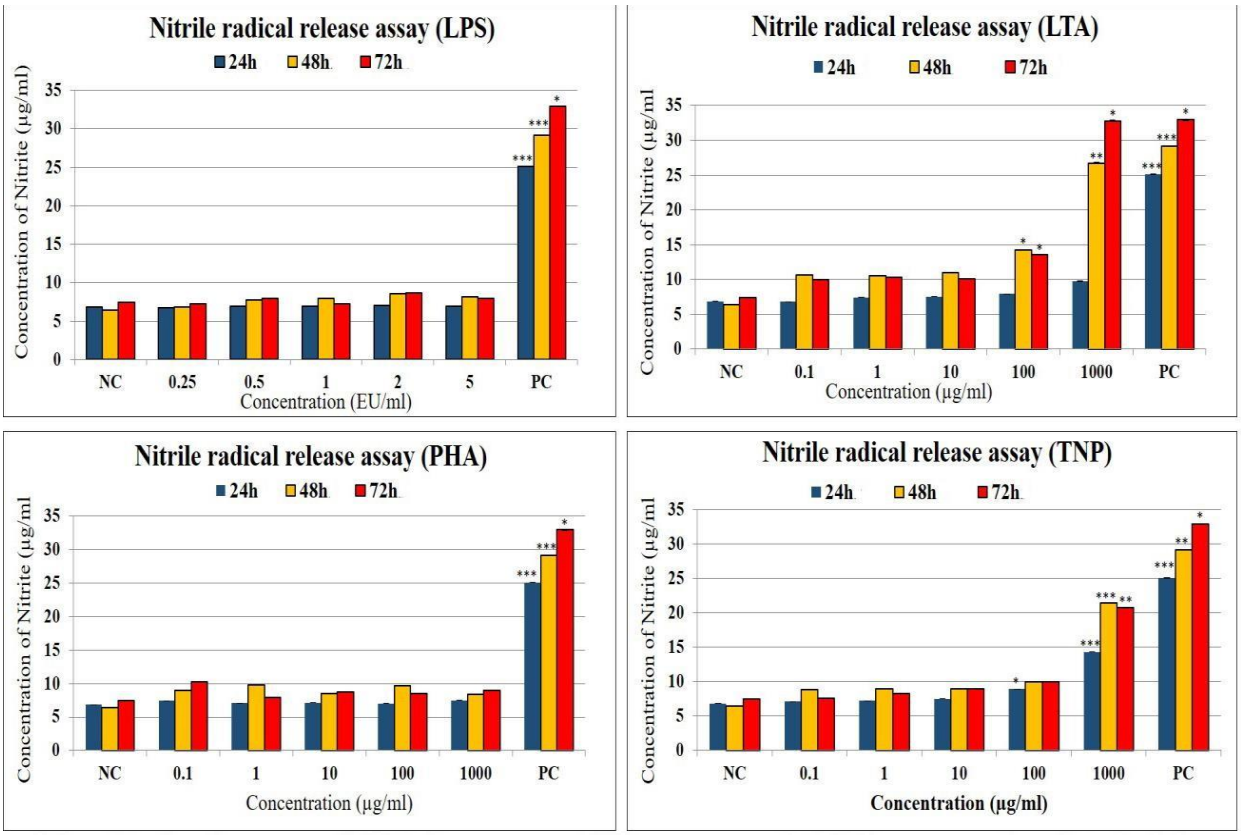

Figure 4.18: Nitrile radical release by Griess assay after LPS (0.25, 0.5, 1, 2 and 5EU/ml), LTA, PHA and TNP (0.1, 1, 10, 100 and 1000µg/ml) for 24h, 48h and 72h. Data represent the mean ± SD of three independent experiments. Asterisk denotes a statistically significant difference compared to the control group (*p<0.05, **p<0.01 and ***p<0.001).

4.9.7. LYSOSOMAL INTEGRITY BY ACRIDINE ORANGE STAINING

Loss of lysosomal integrity after exposure to LPS, LTA, PHA and TNP was evaluated by staining with AO fluorescent probe. AO emits red fluorescence in acidic pH inside the lysosomes. When there is a loss of lysosomal membrane integrity, there is leakage of lysosomal contents into the cytoplasm. The alkaline pH of the cytoplasm shifts the fluorescence of AO from red to green. From **Figure 4.19 A,** it was observed that there was no lysosomal destabilization following exposure to 0.25 to 5EU/ml of LPS in 24h. LTA (**Figure 4.19 B**), PHA (**Figure 4.20 A)** and TNP (**Figure 4.20 B**) showed a dose-dependent reduction in red fluorescence compared to untreated cell control. Reduction in red fluorescence of AO indicates destabilization of lysosomes in treated cells. It is visible in cells exposed to 1000µg/ml of LTA, PHA and TNP.

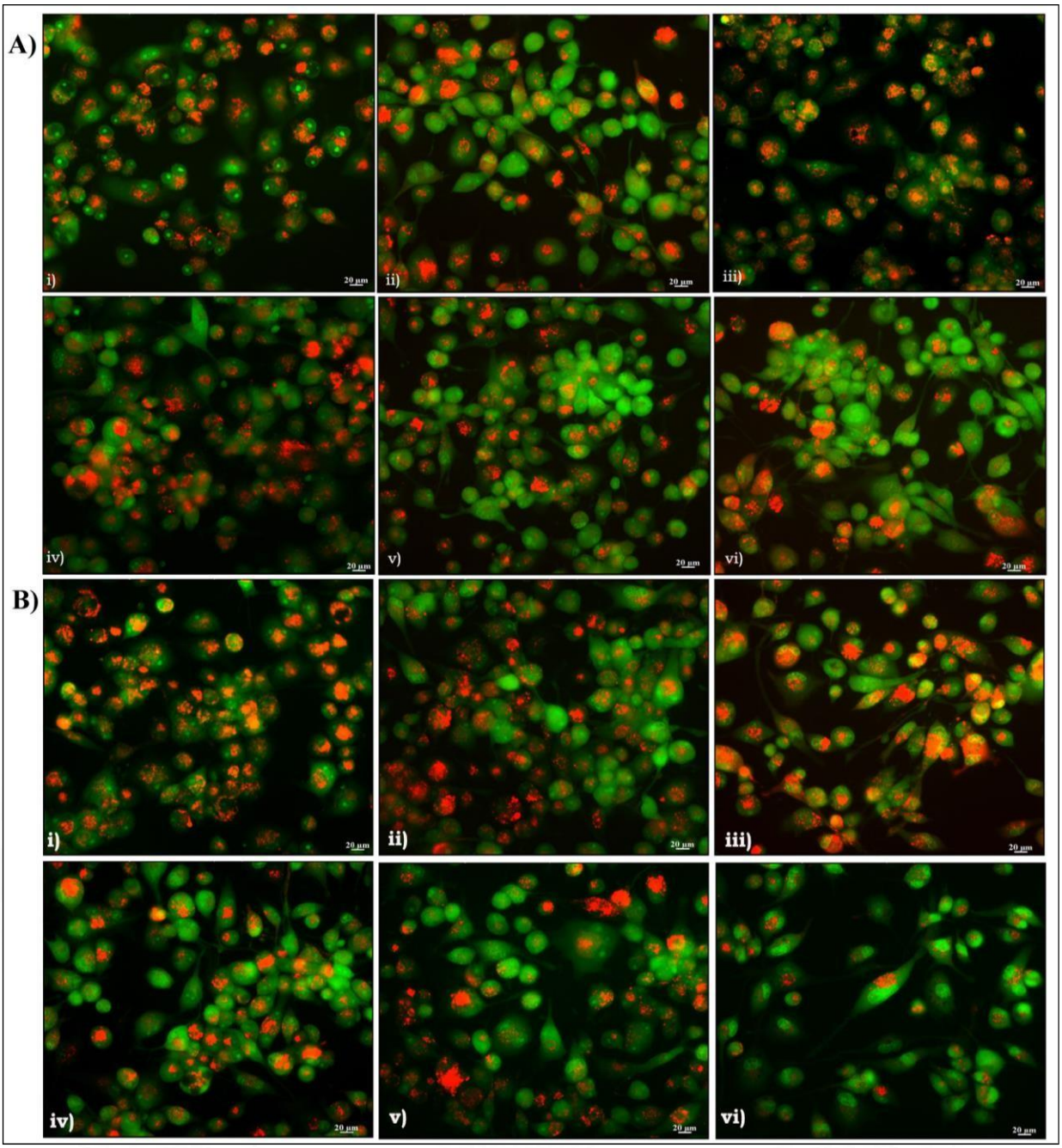

Figure 4.19: AO staining for lysosomal integrity of MDM cells exposed to **A)** i) Control ii) LPS 0.25EU/ml iii) LPS 0.5EU/ml iv) LPS 1EU/ml v) LPS 2EU/ml vi) LPS 5EU/ml **B)** i) Control ii) LTA (0.1µg/ml) iii) LTA 1µg/ml iv) LTA 10µg/ml v) LTA 100µg/ml vi) LTA 1000µg/ml for 24h. The green signal indicates the cytoplasm. Red signal shows the lysosomes. Scalebar 20µm. Magnification 20x

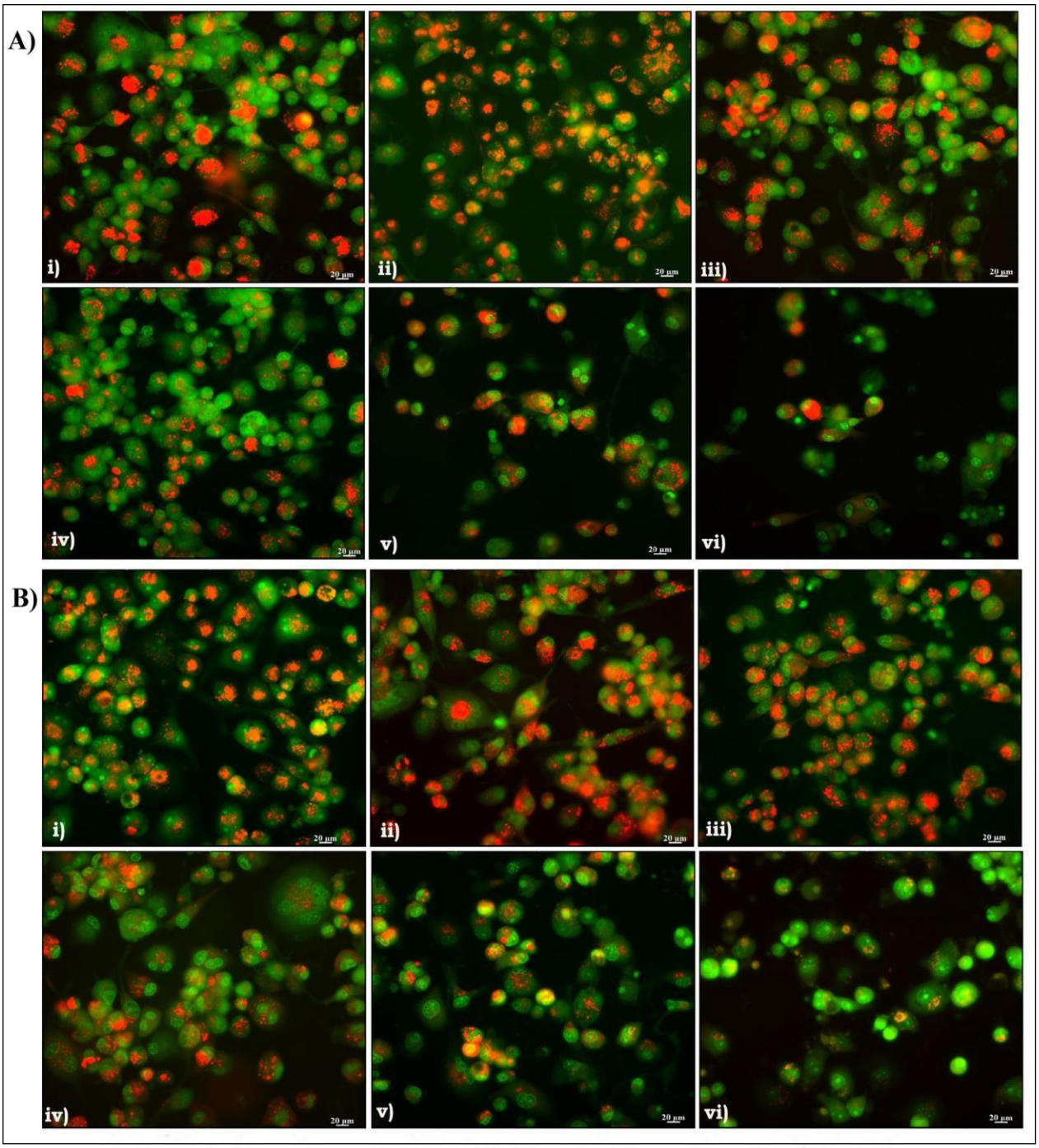

Figure 4.20: AO staining for lysosomal integrity of MDM cells **A)** i) Control ii) PHA (0.1µg/ml) iii) PHA 1µg/ml iv) PHA 10µg/ml v) PHA 100µg/ml vi) PHA 1000µg/ml **B)** i) Control ii) TNP (0.1µg/ml) iii) TNP 1µg/ml iv) TNP 10µg/ml v) TNP 100µg/ml vi) TNP 1000µg/ml for 24h. The green signal indicates the cytoplasm. Red signal shows the lysosomes. Scalebar 20µm. Magnification 20x

4.9.8. FLUORESCENCE IMAGING OF MMP USING JC1 DYE

Loss of mitochondrial membrane potential after exposure to LPS, LTA, PHA and TNP was analyzed by JC1 staining. From **Figure 4.21**, it was observed that there was no change in MMP following 24h exposure with 0.25 to 5EU/ml of LPS. In cells with active MMP, JC1 forms red fluorescence emitting J-aggregates, where the loss of MMP results in the formation of J-monomers, which emits green fluorescence. Exposure to the higher concentration of LTA, PHA and TNP for 24h showed a reduction in red fluorescence and enhanced green fluorescence. Reduction in red fluorescence of JC1 indicates loss of MMP when exposed to 1000µg/ml of LTA, PHA and TNP.

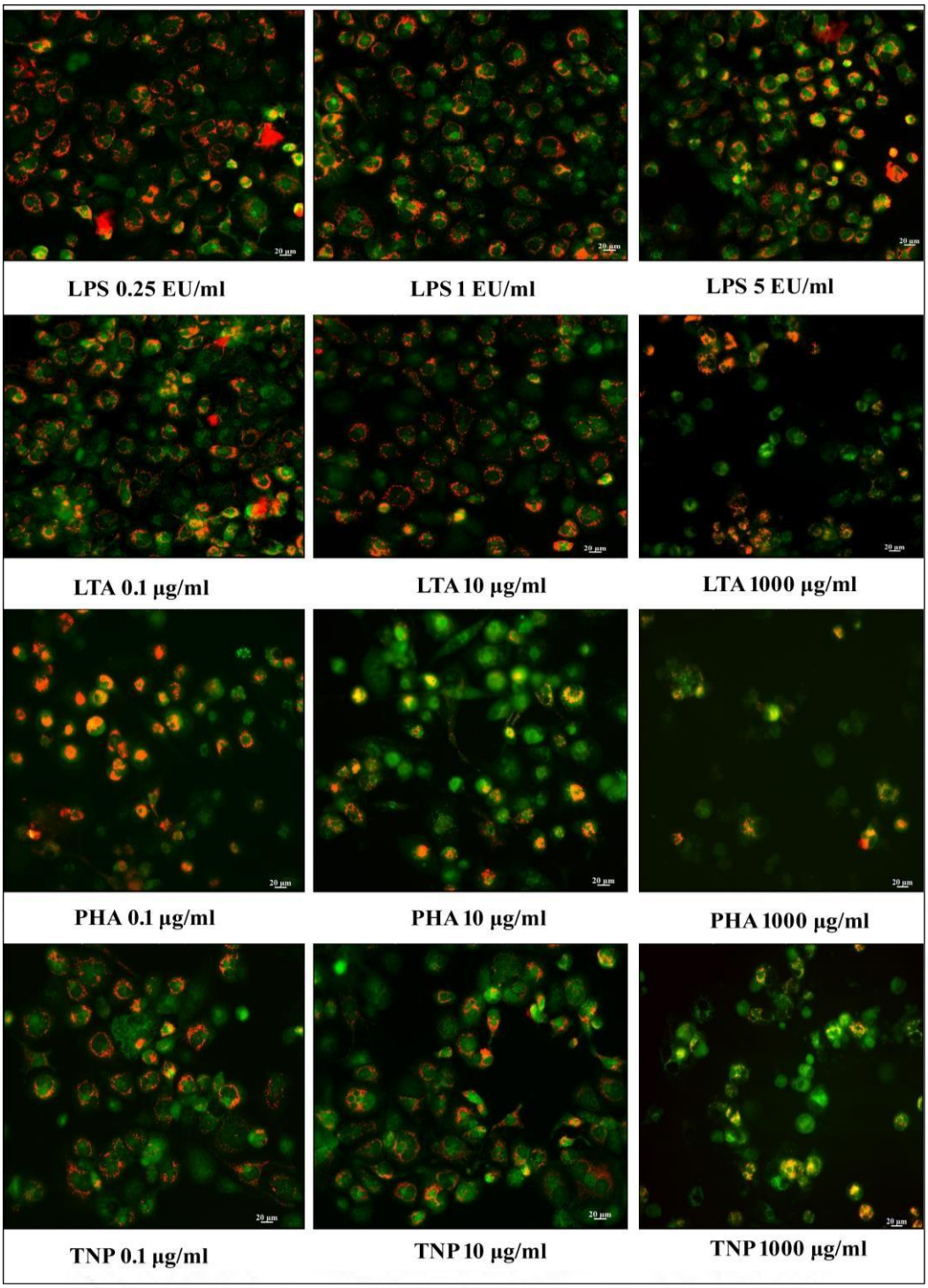

Figure 4.21: MMP analysis in MDM cells using JC1 fluorescence probe after exposure with LPS (0.25EU/ml, 1EU/ml, 5EU/ml), LTA, PHA and TNP (0.1µg/ml, 10µg/ml, 1000µg/ml) for 24h. Red fluorescence indicates J-aggregates. Scale bar 20µm, Magnification 20x

4.9.9. MITOCHONDRIAL MEMBRANE POTENTIAL BY DilC1 (5) ASSAY

The viability of every cell depends on healthy and functional cellular organelles, especially mitochondria and lysosomes. Any damage to the structural and functional integrity of these organelles leads to loss of viability or cell death. In every cell, mitochondria act as machinery for ATP synthesis and are the primary energy source. Maintenance of MMP in a narrow range is necessary for coupling the process of oxidative phosphorylation with an active synthesis of ATP. The present study evaluated the loss of MMP of MDM cells following exposure of LPS, LTA, PHA and TNP for 24h using DilC1 (5) fluorescent probe. It was observed that exposure of LPS up to 5EU/ml did not affect MMP. 1000µg/ml of LTA induced significant loss of MMP and thereby the intensity of DilC1 (5) fluorescence shifted towards the left of untreated control. Similarly, PHA and TNP also induced loss of MMP in a dose-dependent manner (**Figure 4.22**). The FACS analysis results using DilC1 (5) agree with the results of JC1, which also detects loss of MMP. Similar to the present observation, a decrease in the formation of red fluorescence emitting J-aggregates is clearly visible in the image of JC1 analysis.

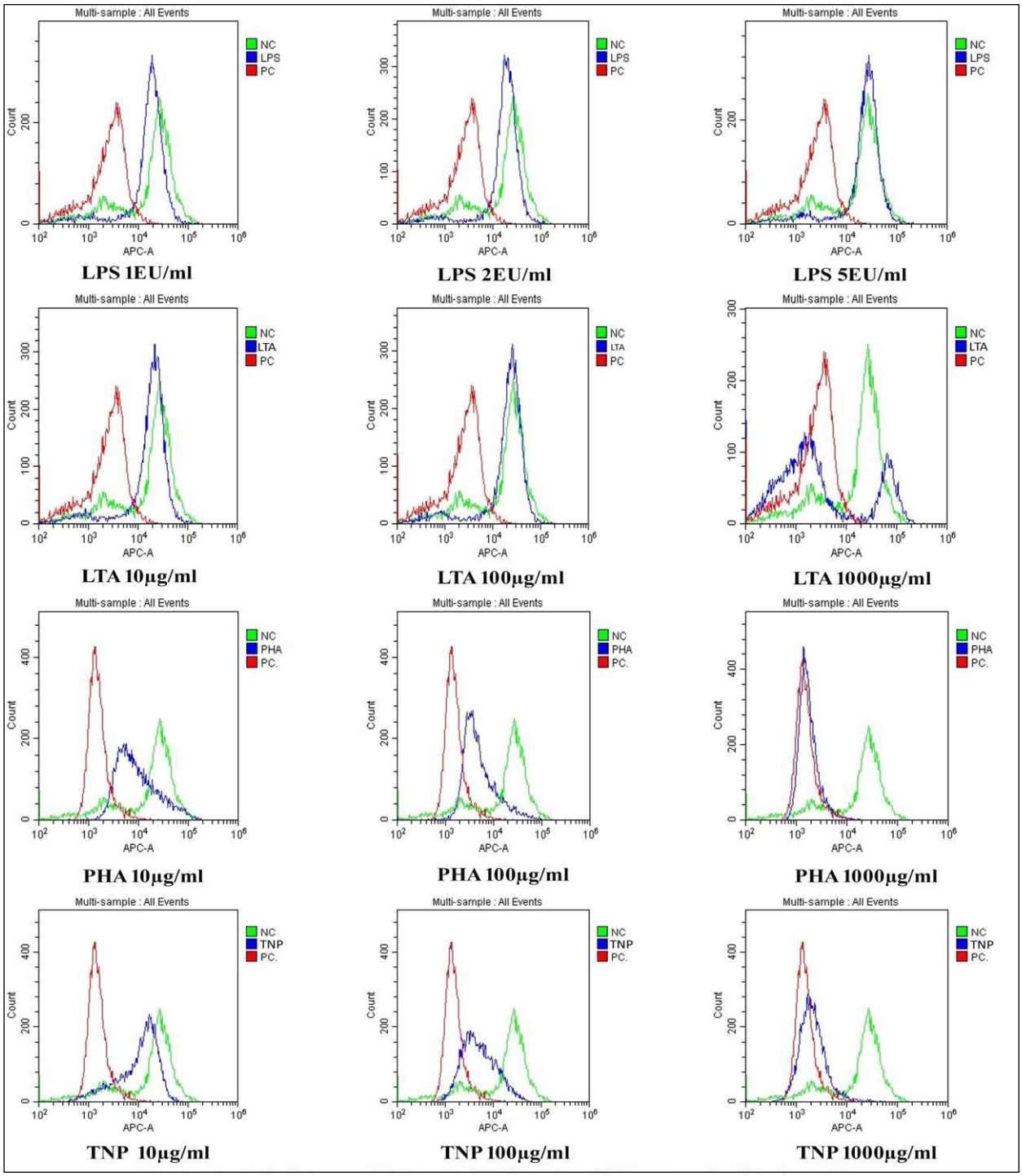

Figure 4.22: MMP analysis in MDM cells using DilC1(5) fluorescence probe following exposure with LPS (1EU/ml, 2EU/ml, 5EU/ml), LTA, PHA and TNP (10µg/ml, 100µg/ml, 1000µg/ml) for 24h.

4.9.10. LDH RELEASE ASSAY

Loss of cell membrane integrity and release of LDH to the extracellular matrix was analyzed by CyCOUNT LDH release assay. No significant loss of membrane integrity and release of LDH were observed after treatment with 0.25 to 5EU/ml LPS for 24h, 48h and 72h. There was a significant loss of cell membrane integrity and LDH release at 100 and 1000µg/ml of LTA, PHA and TNP compared to control (**Figure 4.23**).

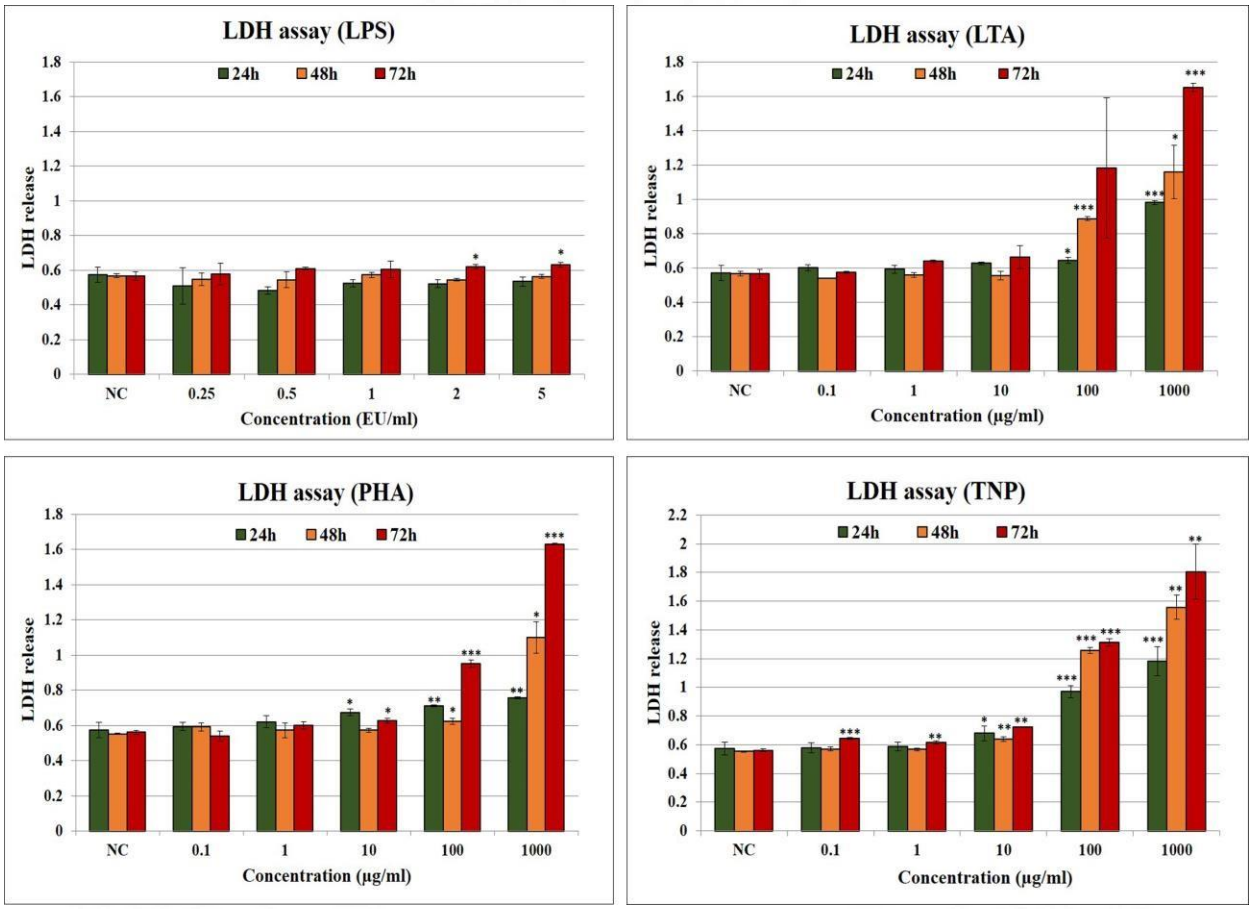

Figure 4.23: LDH release in MDM cells following the exposure of LPS, LTA, PHA and TNP for 24h, 48h and 72h. The data represent the mean ± SD of three independent experiments. The asterisk above columns denotes a statistically significant difference compared to the control group (*p<0.05, **p<0.01 and ***p<0.001).

4.9.11. CALCEIN AM-PI ASSAY

The ratio of live and dead cells was analyzed by dual fluorescent staining with Calcein-AM and PI. Calcein-AM emits green fluorescence inside the live cells, where PI stains cell nuclei and emits red fluorescence. The study shows no significant difference in the fluorescent intensity following LPS compared to control. Exposure of LPS to MDM does not cause cell permeabilization. There was no significant reduction in Calcein-AM signals even after exposure of 5EU/ml at 24h (3.74±0.22), 48h (3.59±0.19) and 72h (3.18±0.12). This again confirmed the non-toxic behaviour of LPS towards MDM cells at the selected concentrations. The result of LTA showed a decrease in the fluorescence ratio with increasing concentration. Contrary to the LPS, LTA was found to be toxic, which was in accordance with the other assay results. The Calcein AM-PI analysis of PHA and TNP also showed a dose-dependent cell death of MDM cells. LTA, PHA and TNP exposure elicited significant membrane permeabilisation in MDM cells at 1000µg/ml (2.82±0.27, 1.31±0.17 and 1.76±0.30 respectively) following 72h. The results suggest an exponential increase in the number of dead cells in all three exposure periods for LTA, PHA and TNP (**Figure 4.24**). Corresponding fluorescent image of Calcein AM-PI staining is shown in **Figure 4.25**

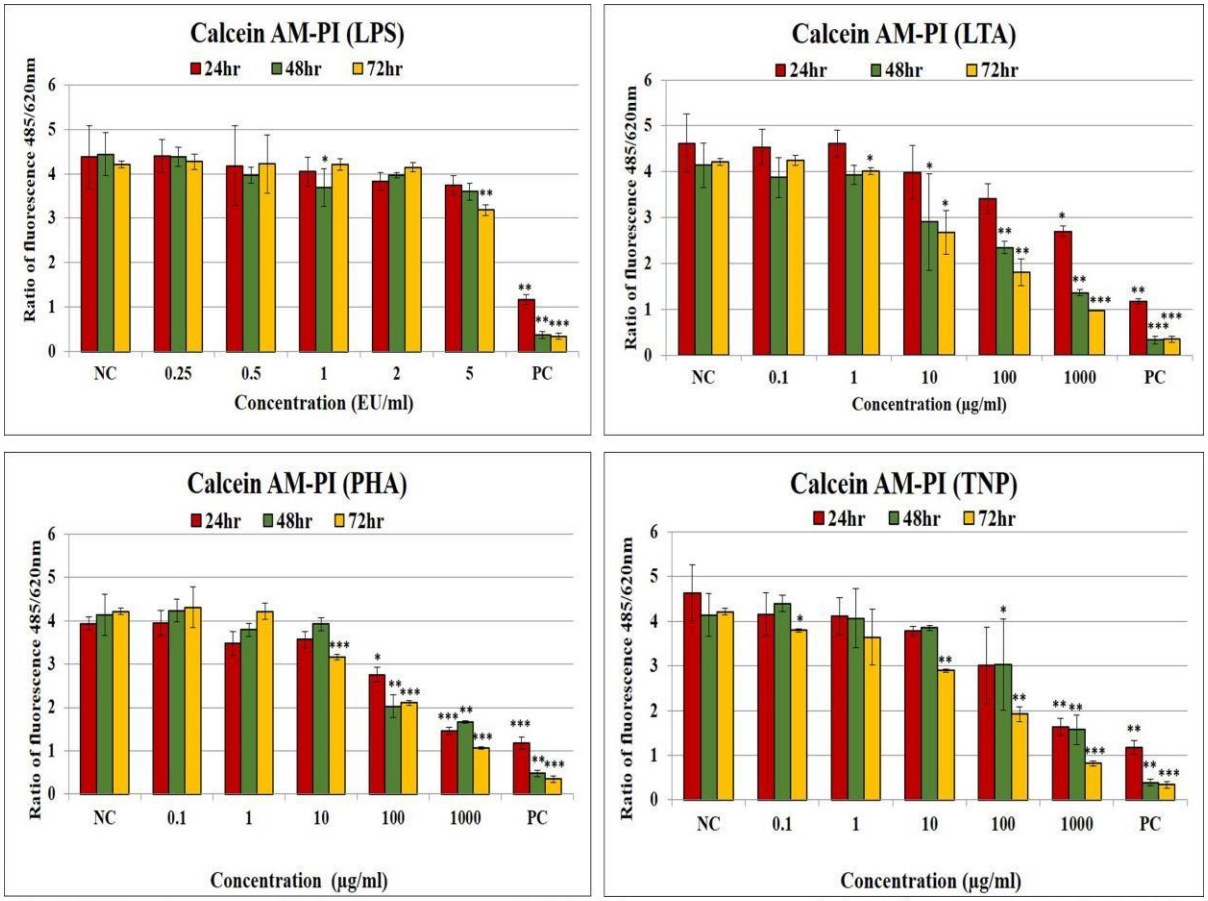

Figure 4.24: Calcein AM-PI assay in MDM cells following exposure with LPS (0.25EU/ml, 0.5EU/ml, 1EU/ml, 2EU/ml, 5EU/ml), LTA, PHA and TNP (0.1µg/ml, 1µg/ml, 10µg/ml, 100µg/ml, 1000µg/ml) for 24h, 48h and 72h. The data represent mean ± SD of three independent experiments. Asterisk above columns denotes statistically significant difference, compared to the control group (*p<0.05, **p<0.01 and ***p<0.001).

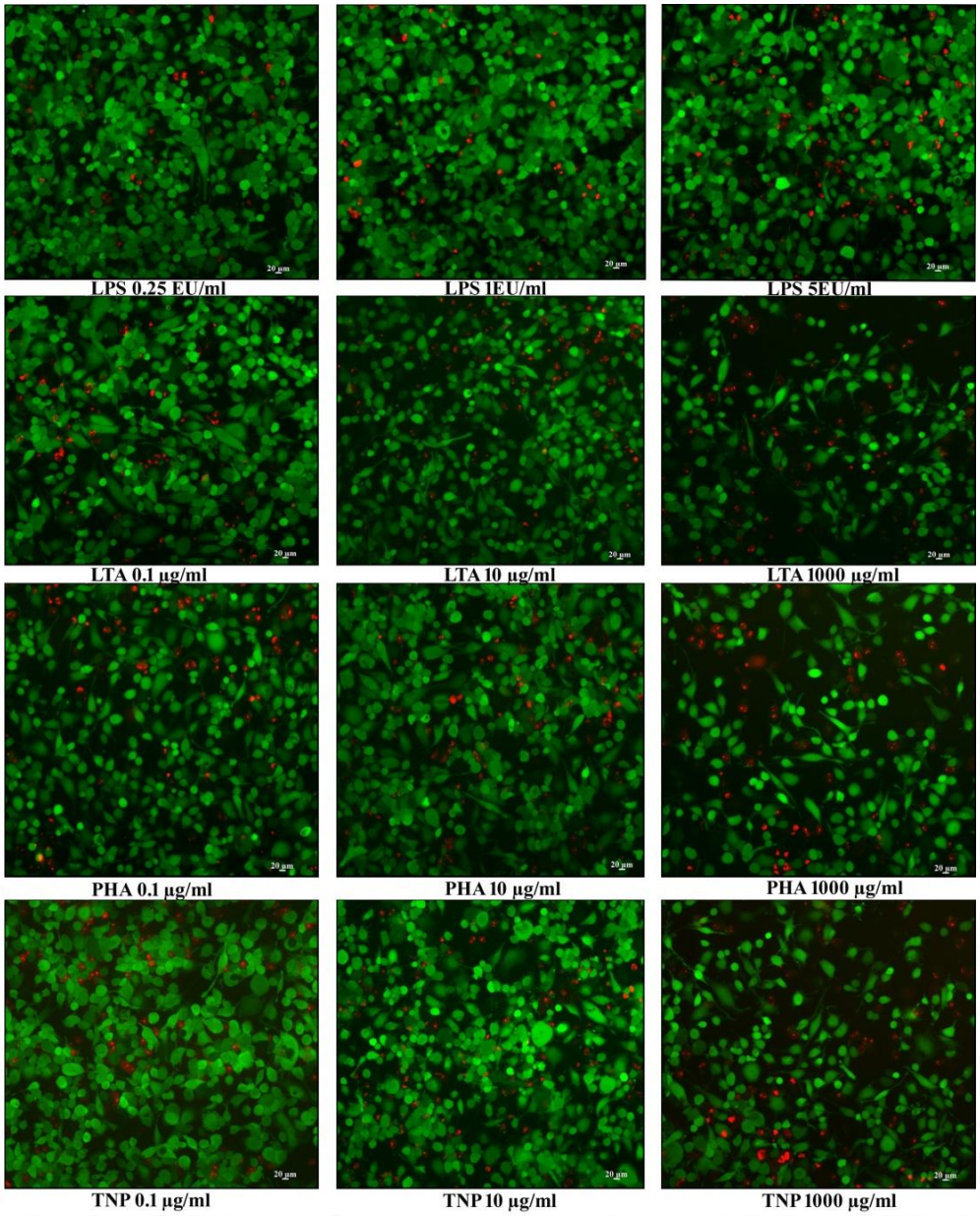

Figure 4.25: Fluorescent image of MDM cells stained with Calcein AM-PI following 24h exposure with LPS, LTA, PHA and TNP. The green signal indicates cells stained with Calcein AM and the red signal indicates cells stained by PI. Scale bar 20µm. Magnification 10x

4.9.12. ANNEXIN V-PI APOPTOSIS ASSAY

The detection of apoptosis in MDM cells was assessed after treating LPS, LTA, PHA, and TNP for 24h. Apoptosis formation was confirmed by assessing the phosphatidylserine externalization using Annexin V in conjunction with FITC using flow cytometric analysis. The flow cytometric

analysis of LPS treated MDM cells at the concentration of 5EU/ml exhibited no evidence of apoptosis in early [Annexin (+) PI (-)] and late [Annexin (+) PI (+)] apoptotic stages but indicated 1.5% necrotic cells [Annexin (-) PI (+)]. In contrast, 16.99% apoptotic [Annexin (+) PI (-)] and 69.52 % viable cells [Annexin (-) PI (-)] were seen after 1000µg/ml LTA treatment. Whereas the control group showed 89.54% viable cells (**Figure 4.26**). The percentage of necrotic cells was found to increase when exposed to 1000µg/ml of PHA. Here, 45.6% of the cells in the treated group existed in the necrotic stage and 40.9 % of cells were in the non-apoptotic stage. A drastic decrease in viability was observed in MDM cells when exposed to the highest concentration of TNP (1000µg/ml). It was noted that 16.3% cells were in early apoptotic [Annexin (+) PI (-)] and 23.7% [Annexin (+) PI (+)] were in late apoptotic stage (**Figure 4.27**). Graphical representation of the percentage of cells undergoing apoptotic and necrotic cell death was shown in **Figure 4.28**

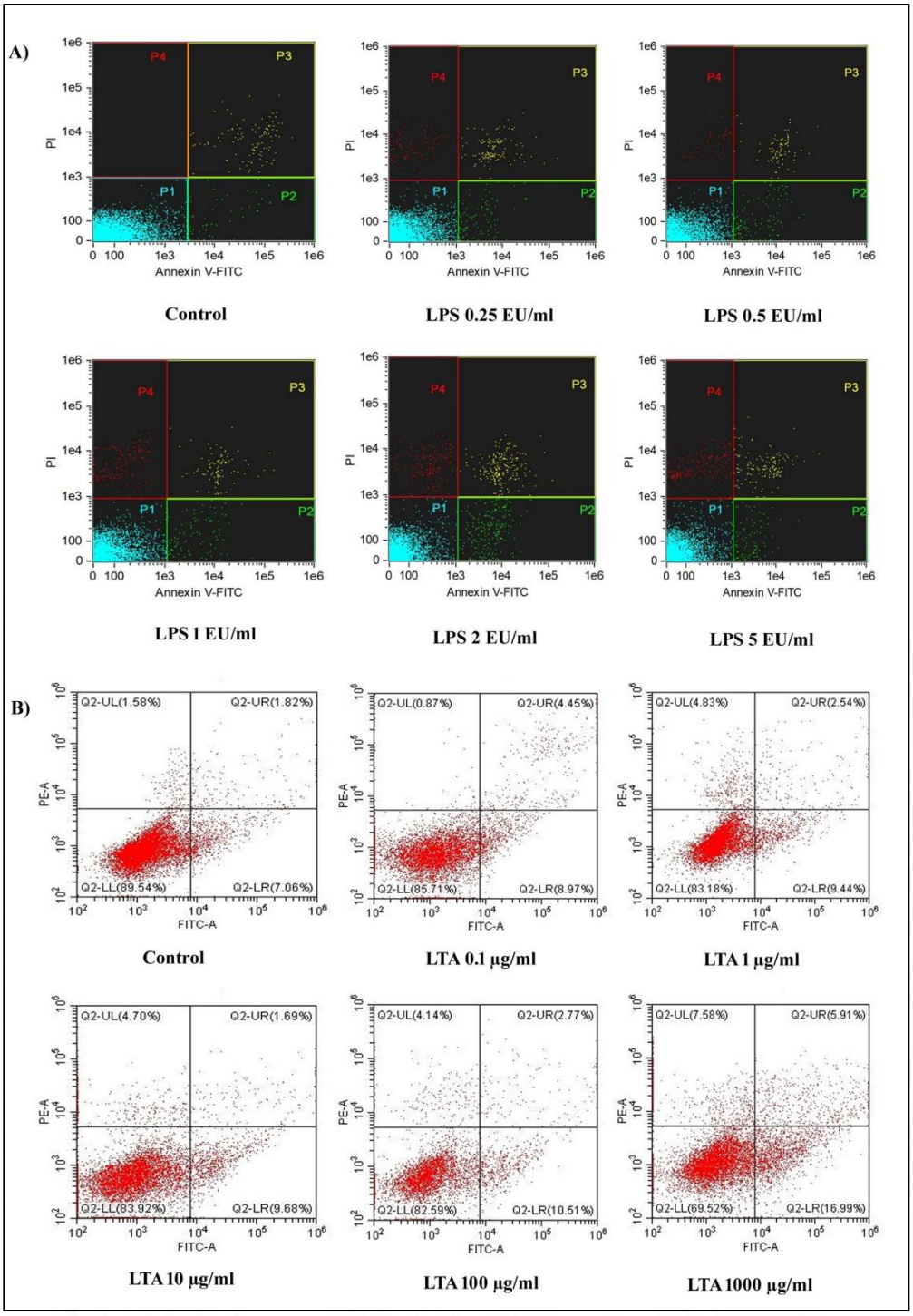

Figure 4.26: Apoptosis by Annexin V/PI staining of MDM cells exposed to **A)** LPS (0.25, 0.5, 1, 2 and 5EU/ml) and **B)** LTA (0.1, 1, 10, 100 and1000µg/ml) for 24h. Quadrant P1 represents non-apoptotic cells, P2 indicates Annexin V positive early apoptotic cells, P3 indicate double-positive late apoptotic cells and P4 indicate PI-positive necrotic cells.

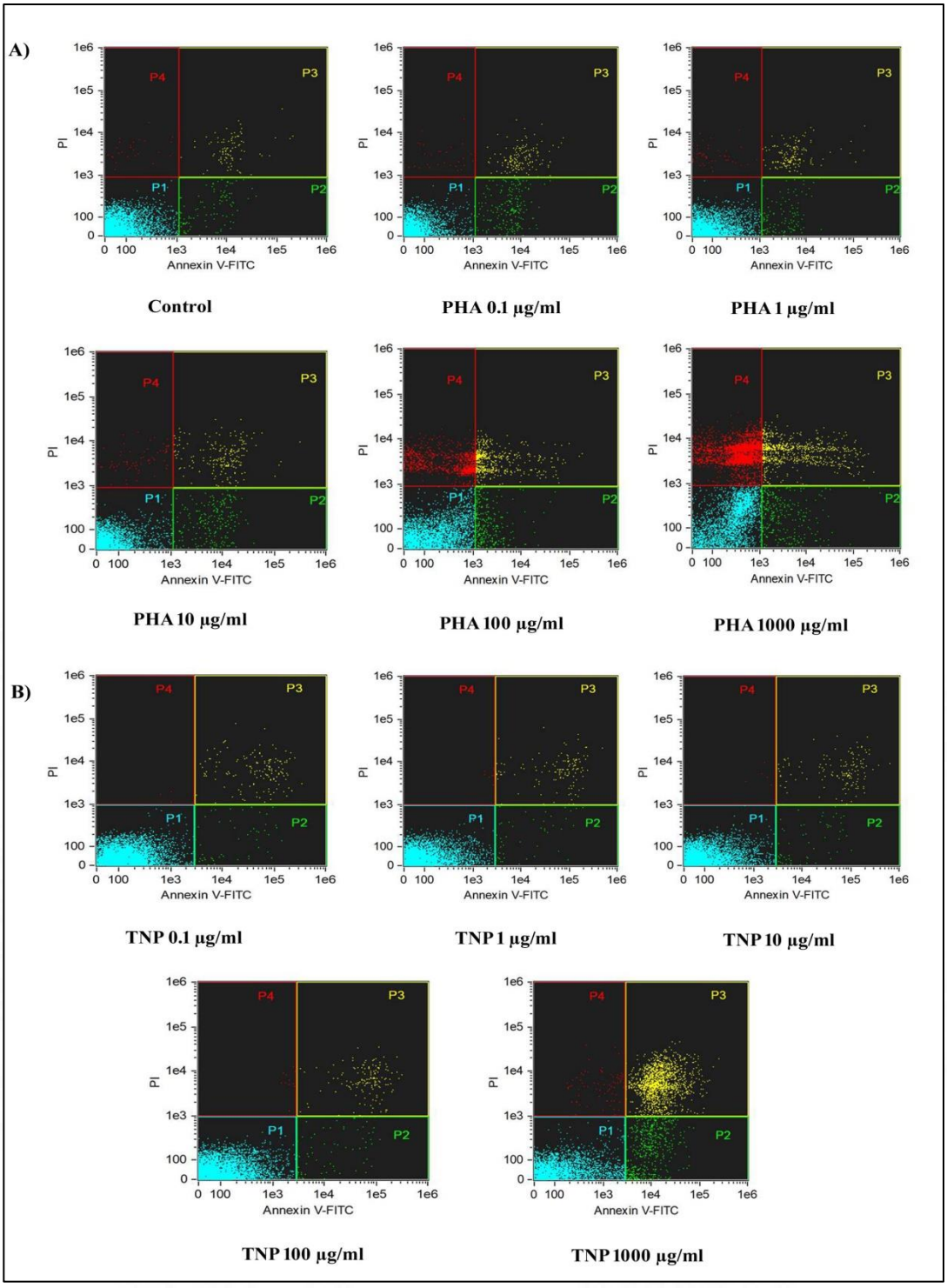

Figure 4.27: Apoptosis by Annexin V/PI staining of MDM cells exposed to **A)** PHA (0.1, 1, 10, 100 and1000µg/ml) and **B)** TNP (0.1, 1, 10, 100 and1000µg/ml) for 24h. Quadrant P1 represents viable cells, P2 indicates Annexin V positive early apoptotic cells, P3 indicate double-positive late apoptotic cells and P4 indicate PI-positive necrotic cells.

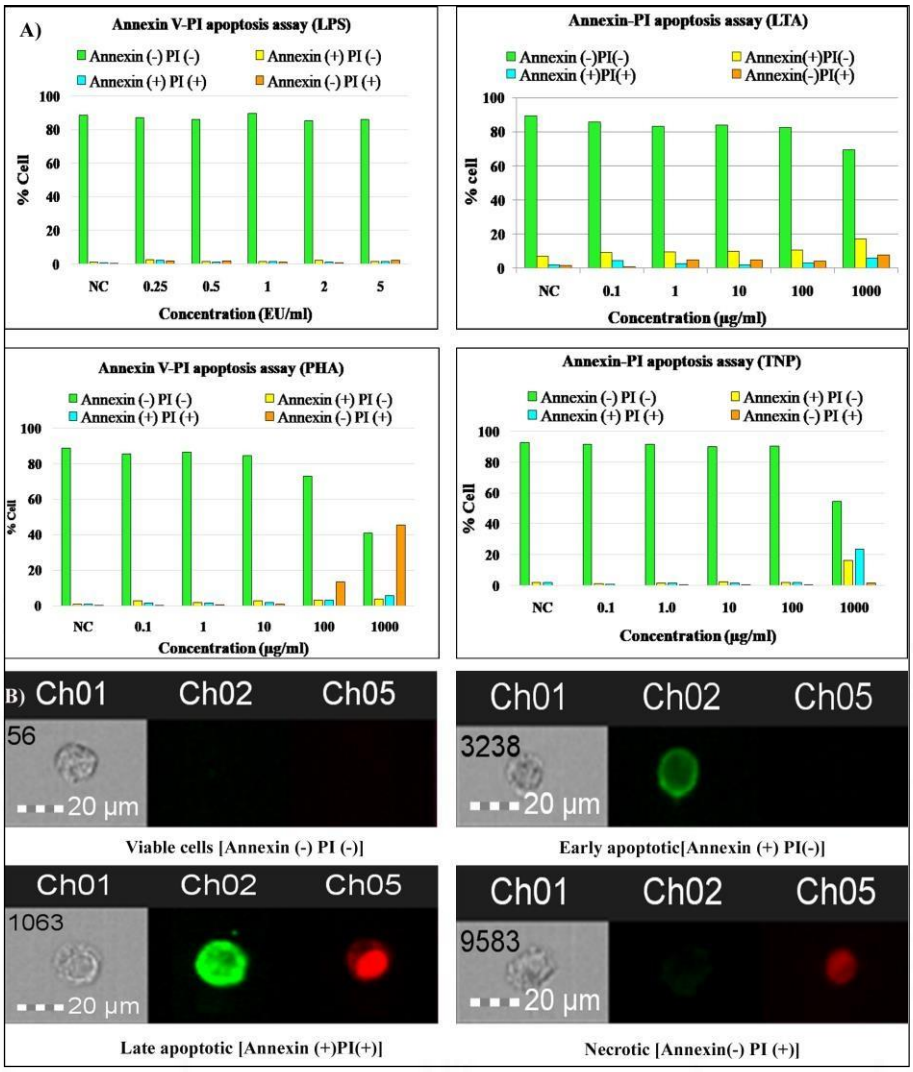

Figure 4.28: A) Graphical representation of apoptosis in MDM cells following LPS (0.25EU/ml, 0.5 EU/ml, 1 EU/ml, 2EU/ml, 5EU/ml), LTA, PHA and TNP (0.1µg/ml, 1µg/ml, 10µg/ml, 100µg/ml, 1000µg/ml) exposure. **B)** Representing image of viable cells, early apoptotic, late apoptotic and necrotic cells. Scale bar 20µm.

4.9.13. GENE EXPRESSION BY qRT-PCR

Immune response against toxicants begins with the inflammation and the release of cytokines. In general, immune signals transmit through various intracellular pathways. NFκB and COX2 signalling have a significant role in pyrogen induced inflammatory pathway. Cytokines are the mediators of inflammatory signals within the network of the immune system. In the present study, expression of genes specific for inflammatory pathways (NFκB and COX2) along with pyrogenic cytokines (IL-1β and TNF-α) was studied in MDM cells before and after exposure to

LPS, LTA, PHA and TNP. It was observed that 24h exposure of selected concentration (1EU/ml, 2EU/ml, 5EU/ml) of LPS caused a dose-dependent increase in expression of NFκB, COX2 and IL-1β in MDM cells. LTA (10µg/ml, 100µg/ml, 1000µg/ml) exposure caused a marked increase in NFκB, COX2, IL-1β and TNF-α expression. In the case of PHA (10µg/ml, 100µg/ml, 1000µg/ml), there was a dose-dependent increase in expression of NFκB, COX2 and IL-1β where TNF-α expression was comparable to control even at the highest concentration. TNP (10µg/ml, 100µg/ml, 1000µg/ml) exposure induce augmented expression of NFκB, COX2, IL-1β and TNF-α compared to control (**Figure 4.29**).

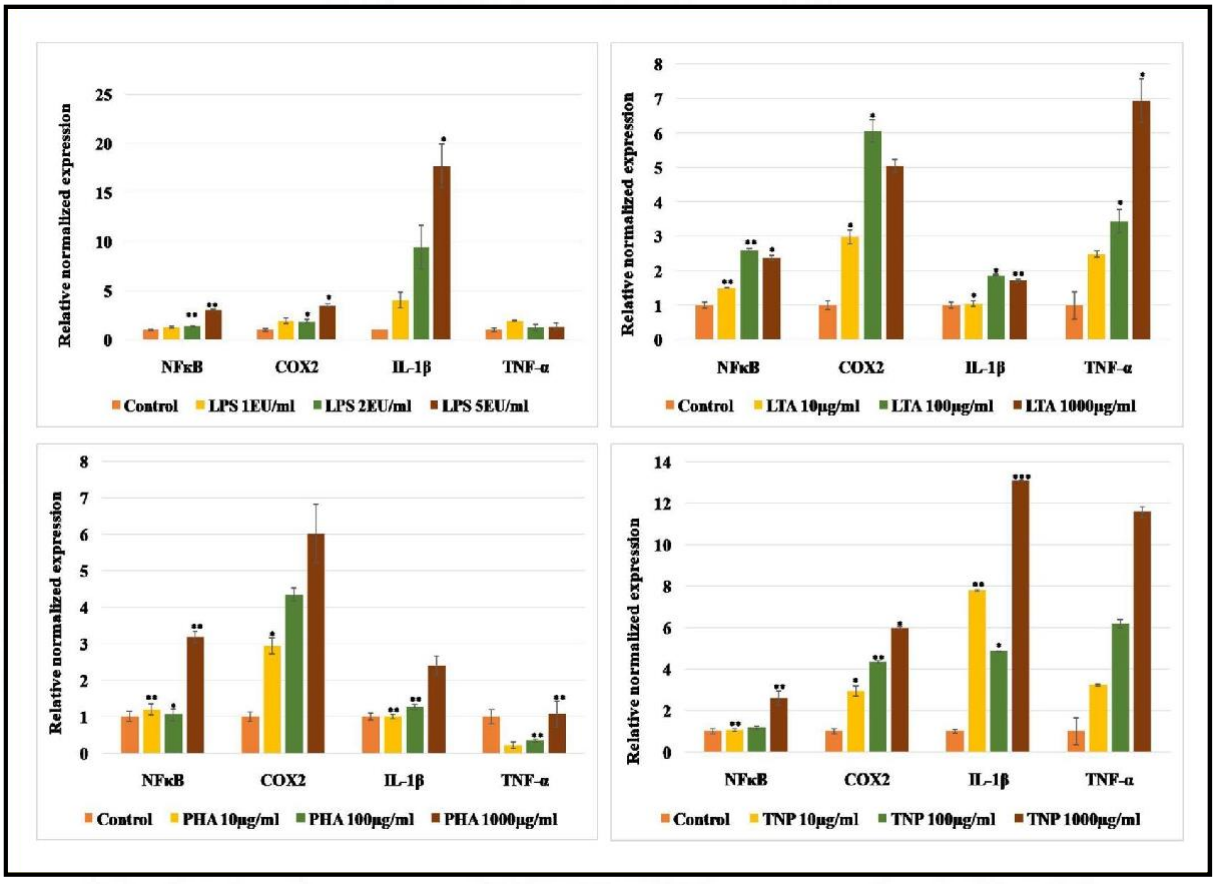

Figure 4.29: Inflammatory gene expression in MDM cells following 24h exposure to LPS (1EU/ml, 2EU/ml, 5EU/ml), LTA (10µg/ml, 100µg/ml, 1000µg/ml), PHA (10µg/ml, 100µg/ml, 1000µg/ml) and TNP (10µg/ml, 100µg/ml, 1000µg/ml) relative to β-actin (housekeeping gene). n=3, Data shown are the means ± SD from two independent biological replications.

4.9.14. FACS FOR NFκB ACTIVATION

Activation of the NFκB pathway is an indicator of inflammatory signals. FACS analysis of NFκB activation in THP-1 cells was done after 24h exposure of LPS, LTA, PHA and TNP. It was found that LPS, LTA, PHA and TNP induced NFκB activation in THP-1 cells in a dose-dependent manner. Following the exposure of 1, 2 and 5EU/ml of LPS, there was activation of NFκB at 3.0, 4.97 and 13.2%, respectively. On exposure of 10, 100 and 1000µg/ml of LTA induced 29.8%, 52.8% and 82.3% activation of NFκB, respectively (**Figure 4.30**). PHA exposure enhanced the activation of NFκB at 0.1% (10µg/ml), 12.3% (100µg/ml) and 33.8% (1000µg/ml). Similarly, 1.71, 4.73 and 5.48% cells showed activation of NFκB following 10, 100 and 1000µg/ml of TNP (**Figure 4.31**).

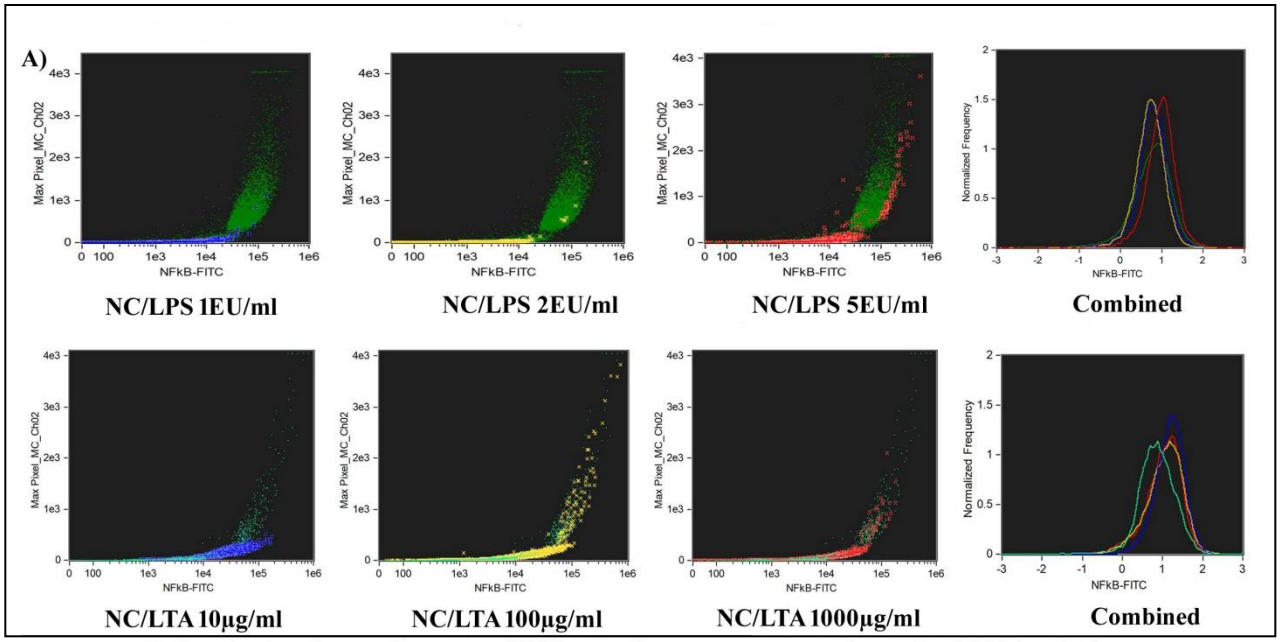

Figure 4.30: A) FACS analysis of NFκB activation in THP-1 cells after LPS and LTA exposure for 24h. Green-NC, Blue 1EU/ml, Yellow 2EU/ml, Red 5EU/ml LPS, Green-NC, Blue 10µg/ml, Yellow 100 µg/ml, Red 1000 µg/ml LTA

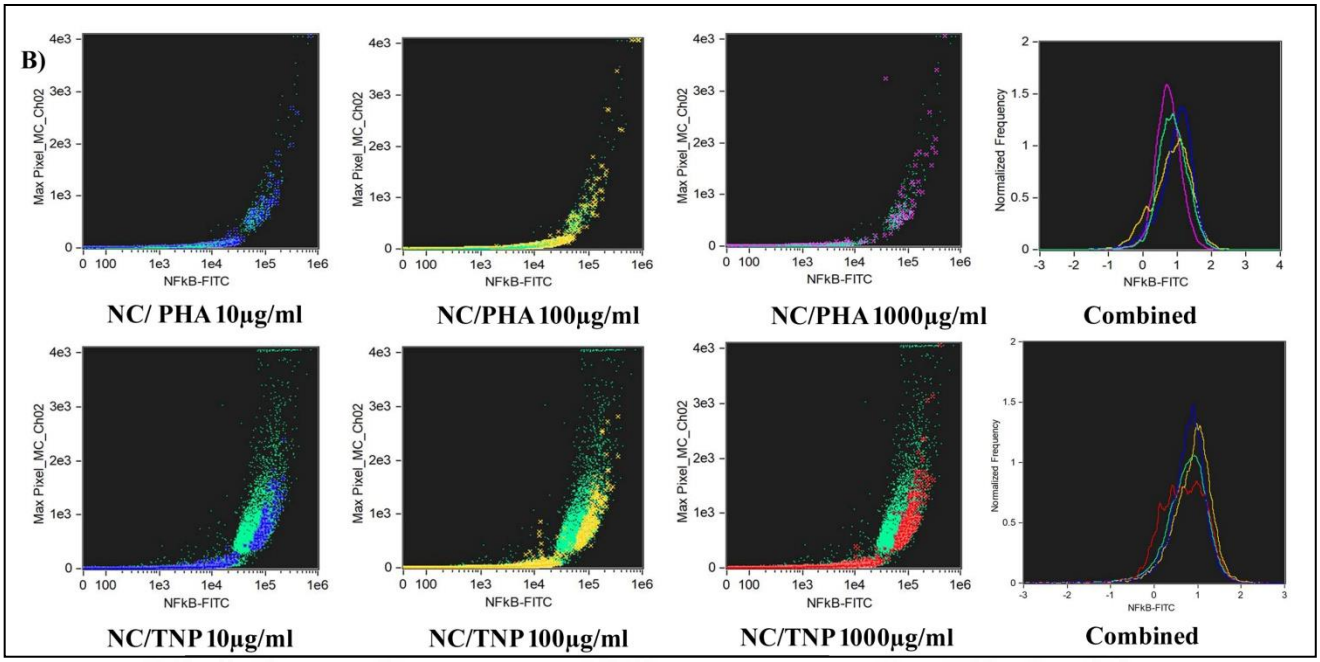

Figure 4.31: A) FACS analysis of NFκB activation in THP-1 cells after PHA and TNP exposure for 24h. Green-NC, Blue 10µg/ml, Yellow 100 µg/ml, Violet 1000 µg/ml PHA, Green-NC, Blue 10µg/ml, Yellow 100 µg/ml, Red 1000 µg/ml TNP

4.9.15. IMMUNOCYTOCHEMISTRY ANALYSIS OF NFκB

Immunocytochemistry analysis (ICC) of LPS induced NFκB activation was studied in MDM cells using a fluorescent microscope (Axio scope). An increase in green fluorescence was observed when exposed to 2EU/ml of LPS. Following exposure of 5EU/ml of LPS, there was the translocation of cytoplasmic NFκB to the nucleus and was observed as green spots in cell nuclei (**Figure 4.32**).

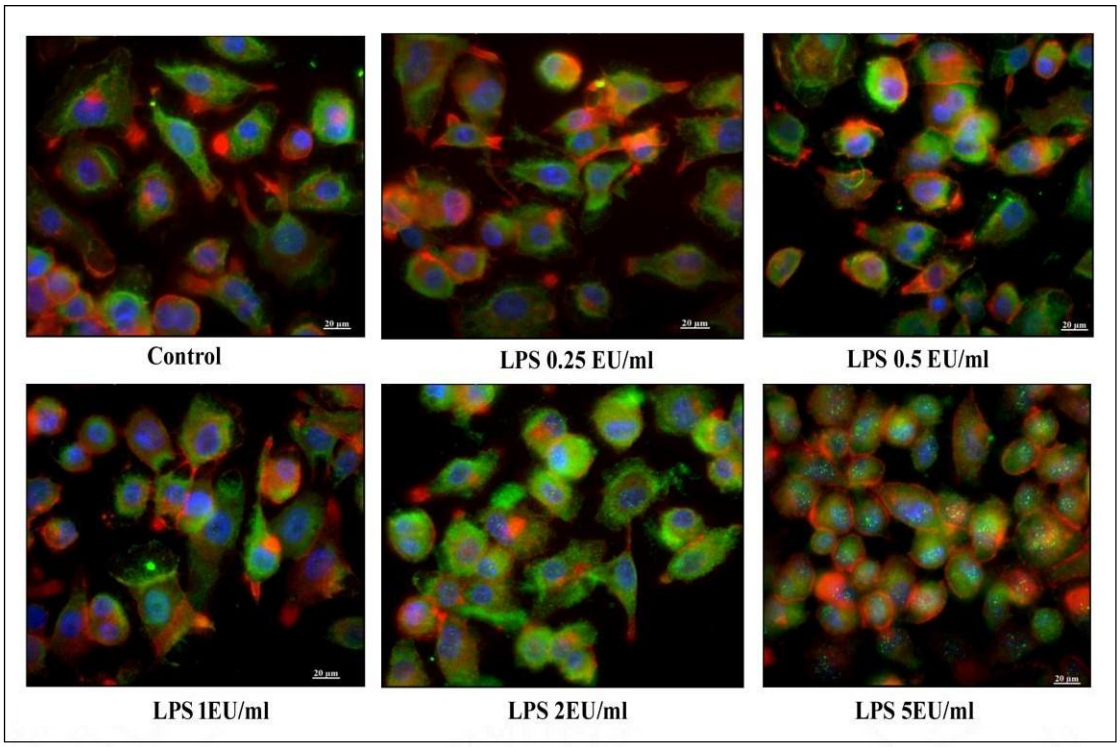

Figure 4.32: Immunocytochemistry analysis of NFκB activation in MDM cells following 24h exposure of LPS. Red: Actin filaments, Green: Anti-NFκB ab, Blue: Nucleus, Scalebar 20µm, Magnification 40x

4.9.16. FACS FOR COX2 ACTIVATION

Activation of COX2 followed by NFκB represents critical signalling for PG synthesis and associated induction of fever. Here, COX2 activation in THP-1 cells after 24h exposure of LPS, LTA, PHA and TNP was analyzed by FACS using anti-COX2 ab tagged with FITC. It was observed that 0.92, 2.8 and 9.79% cells showed COX2 activation following the stimulation of 1, 2 and 5EU/ml LPS. **Figure 4.33 represent** LTA induced activation of COX2 as 7.0% (10µg/ml), 4.09% (100µg/ml) and 9.78% (1000µg/ml). It was noted that 5.91, 11.0 and 16.4% COX2 activation observed when PHA (10, 100 and 1000µg/ml) exposed to THP-1 cells. As shown in **Figure 4.34.** TNP induced COX2 activation at 3.82% (10µg/ml), 8.38% (100µg/ml) and 19.3% (1000µg/ml).

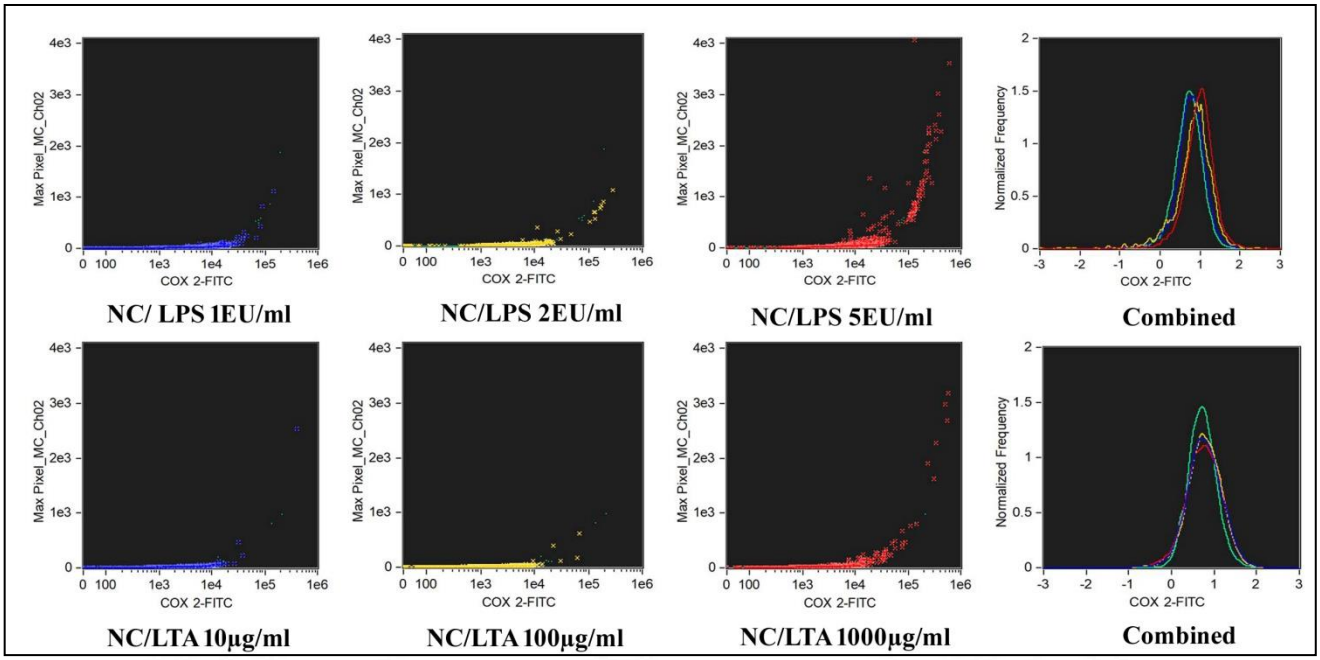

Figure 4.33: A) FACS analysis of COX2 activation in THP-1 cells after LPS (1,2 and 5EU/ml) and LTA (10, 100 and 1000µg/ml) exposure for 24h. Green-NC, Blue 1EU/ml, Yellow 2EU/ml, Red 5EU/ml LPS, Green-NC, Blue 10µg/ml, Yellow 100 µg/ml, Red 1000 µg/ml LTA

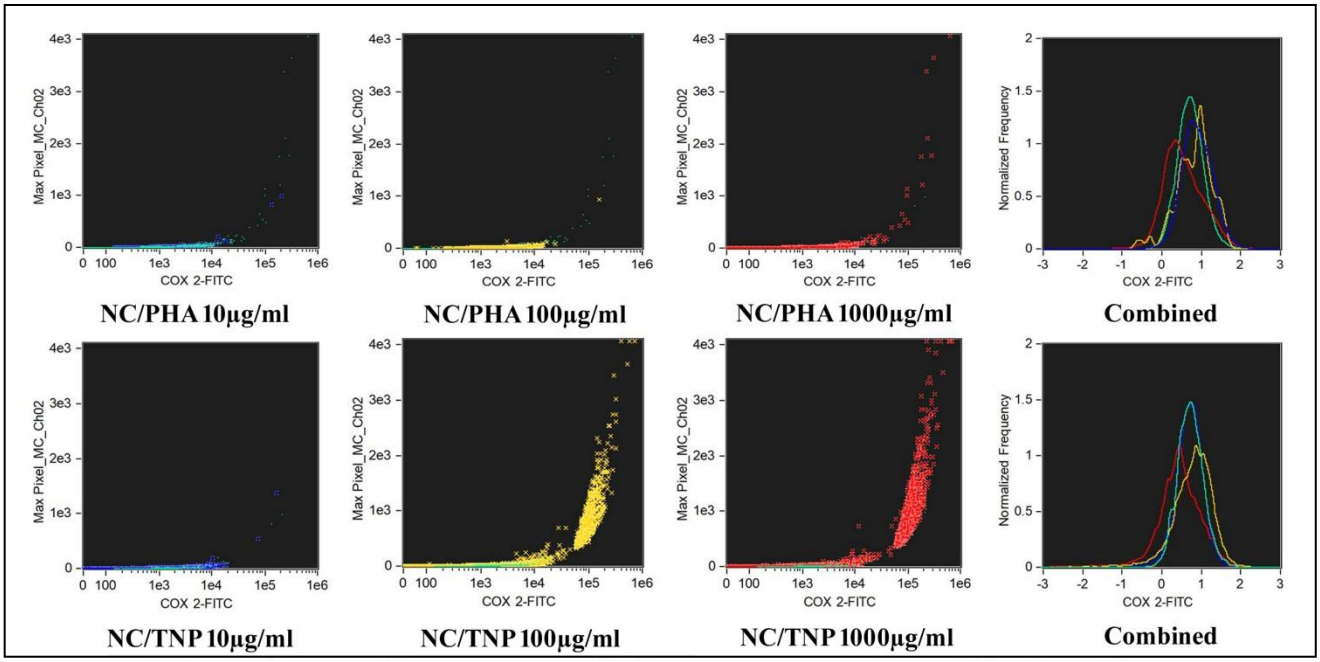

Figure 4.34: FACS analysis of COX2 activation in THP-1 cells after PHA (10, 100 and 1000µg/ml) and TNP (10, 100 and 1000µg/ml) exposure for 24h. Green-NC, Blue 10µg/ml, Yellow 100 µg/ml, Red 1000 µg/ml PHA, Green-NC, Blue 10µg/ml, Yellow 100 µg/ml, Red 1000 µg/ml TNP

4.10. ANALYSIS OF PYROGENICITY

4.10.1. PYROGENIC RESPONSE

All the animals acclimatized before administration of LPS

4.10.1.1. CLINICAL SIGNS

Animals did not show any abnormalities throughout the experiment after injection.

4.10.1.2. BODY WEIGHT

The body weights of the animals were recorded individually on the initiation of acclimatization and the day of the experiment.

4.10.1.3. MORTALITY

No mortality or morbidity was observed following *E. coli* LPS administration

The test sample (physiological saline containing lipopolysaccharides) administrated rabbits showed a rise in temperature of 0.97^0C in animal number 183♂, 0.89^0C in 184♂ and 2.4^0C in animal number 234♂, respectively. All the test sample injected rabbits showed a rise in rectal temperature of 0.5^0C or above the basal temperature during the observation period. The mean value of temperature rise observed in three rabbits is 1.42 ± 0.69^0C (**Table 4.3**). Graphical representation of an increase in body temperature is shown in **Figure 4.35**

Particulars	**183♂**	**184♂**	**234♂**	**Mean ±SD**
Animal body weight (g)	2440	2630	2605	2588±84
Temperature 30' prior to test (^{o}C)	39.23	39.15	38.91	39±0.13
Volume of injection (ml)	24.40	26.30	26.05	25.5±0.84
Temperature, 60' after injection (^{o}C)	40.17	40.02	40.35	40.18±0.13
Temperature, 90' after injection (^{o}C)	40.19	40.04	40.72	40.31±0.29
Temperature, 120' after injection (^{o}C)	40.13	40.02	40.28	40.14±0.10
Temperature, 150' after injection (^{o}C)	40.20	40.00	40.32	40.17±0.13
Temperature, 180' after injection (^{o}C)	40.17	40.03	41.31	40.50±0.57
Rise in temperature (^{o}C)	0.97	0.89	2.40	1.42±0.69

Table 4.3: Rectal temperature of rabbits before and after administration of LPS, n=3, Data represents Mean±SD.

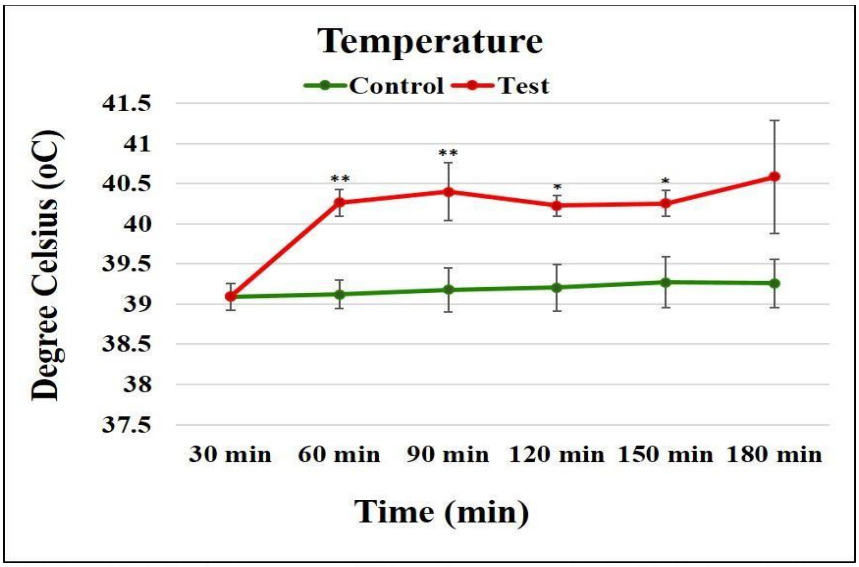

Figure 4.35: Graphical representation body temperature of rabbits before and after administration of LPS, n=3, Data represents Mean±SD.*p<0.05, **p<0.01, ***p<0.001

4.10.2. HEMATOLOGICAL PARAMETERS

Venous blood was collected from marginal ear vein of animals before and after administration of LPS and subjected to haematological analysis. The results of the study indicated that white blood cell count was decreased in LPS treated animals compared to control. RBC count, haemoglobin concentration, blood cell volume, and platelet count remained within normal range and were comparable to that of control. (**Table 4.4**)

S.No	Parameter	Control	Test
1	WBC(10^3/mm^3)	8.3 ± 1.15	4.3± 2.05
2	RBC(10^6/mm^3)	6.32 ± 1.33	5.93 ± 0.83
3	HGB (g/dl)	13.9 ± 2.6	14 ± 2.35
4	HCT (%)	37.6 ± 8.05	36.6 ± 6.17
5	MCV (μm^3)	59.4 ± 1.95	61.5 ± 1.75
6	MCH (pg)	22.06 ± 1.51	23.5 ± 0.7
7	MCHC (g/dl)	37.06 ±1.5	38.2 ± 0.34
8	Platelets (10^3/mm^3)	155 ± 25	230 ± 44

Table 4.4: Haematology parameters of rabbits exposed to LPS. WBC; white blood cells, RBC: red blood cells, HGB; haemoglobin, HCT; haematocrit, MCV; Mean corpuscular volume, MCH; Mean corpuscular haemoglobin, MCHC; mean corpuscular haemoglobin concentration, n=3.The data represent the mean ± SD.

4.10.3. BIOCHEMICAL PARAMETERS

Serum separated from animal blood before and after LPS administration was subjected to biochemical analysis. It was observed that there is a significant rise in the concentration of Glucose, Triglycerides, Alanine transaminase, Aspartate transaminase, Alkaline phosphatase and creatinine in treated animals compared to control. The result indicated a significant change in biochemical parameters and is an indication of malfunctioning of the Liver and kidney (**Table 4.5**)

S.No	Parameter	Control	Test	Reference range (Melillo,2007)
1	Glucose (mg/dl)	137.3 ± 4.66	261± 14.0*	75–155
2	Cholesterol (mg/dl)	21.5 ± 14.84	24 ± 14.14	10-80
3	Triglycerides (mg/dl)	57 ± 8.48	129.5 ± 16.2*	7–205
4	Alanine transaminase (IU/L)	46.85 ± 1.06	182.15 ± 11.8*	45–80
5	Aspartate transaminase (IU/L)	42.3 ± 9.33	226.8 ± 74.9*	35–130
6	Alkaline phosphatase (IU/L)	58 ± 8.48	88.5 ± 48.7	12-96
7	Total Protein (g/dl)	8.65 ± 0.21	8.75 ± 0.07	5.4-7.5
8	Albumin (g/dl)	3.7 ± 0.42	3.6 ± 0.14	2.7–5.0
9	Creatinine (mg/dl)	1.92 ± 0.11	2.835 ± 0.77	0.5–2.5

Table 4.5: Biochemical parameters of rabbits exposed to LPS. The data represent mean ± SD, n=3. The asterisk above values denotes a statistically significant difference compared to the control group (*$p<0.05$)

4.10.4. ANALYSIS OF CYTOKINES FROM RABBIT BLOOD SERUM

The release of pyrogenic cytokines IL-1α, IL-1β and TNF-α from LPS challenged rabbit was analyzed using ELISA. Serum was separated from venous blood collected at 3rd hour following injection. The result indicated a significant increase in serum concentration of IL-1α (2.8-fold), IL-1β (2.7 fold) and TNF-α (4.8 fold) in LPS challenged rabbits compared to control. (**Figure. 4.36)**

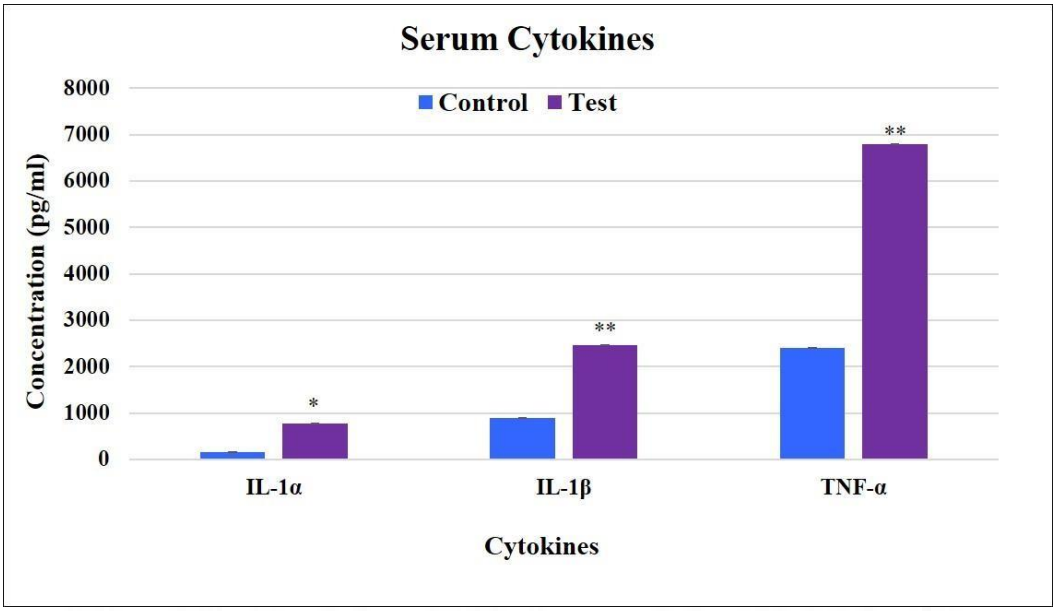

Figure 4.36: Analysis of serum cytokines from rabbits exposed to LPS. n=3. The data represent the mean ± SD. Asterisk denotes statistically significant difference (*p< 0.05 and **p<0.01).

4.10.5. EXPRESSION OF CYTOKINE GENES IN RABBIT PBMC

The expression of IL-1β, IL-8 and NFκB genes in PBMC isolated from LPS challenged rabbits were analyzed using the qRT-PCR technique. PBMC was separated from venous blood collected at the 3rd hour by density gradient centrifugation. **Figure 4.37** indicated an increase in expression of IL-1β, IL-8 and NFκB in LPS challenged rabbits compared to control.

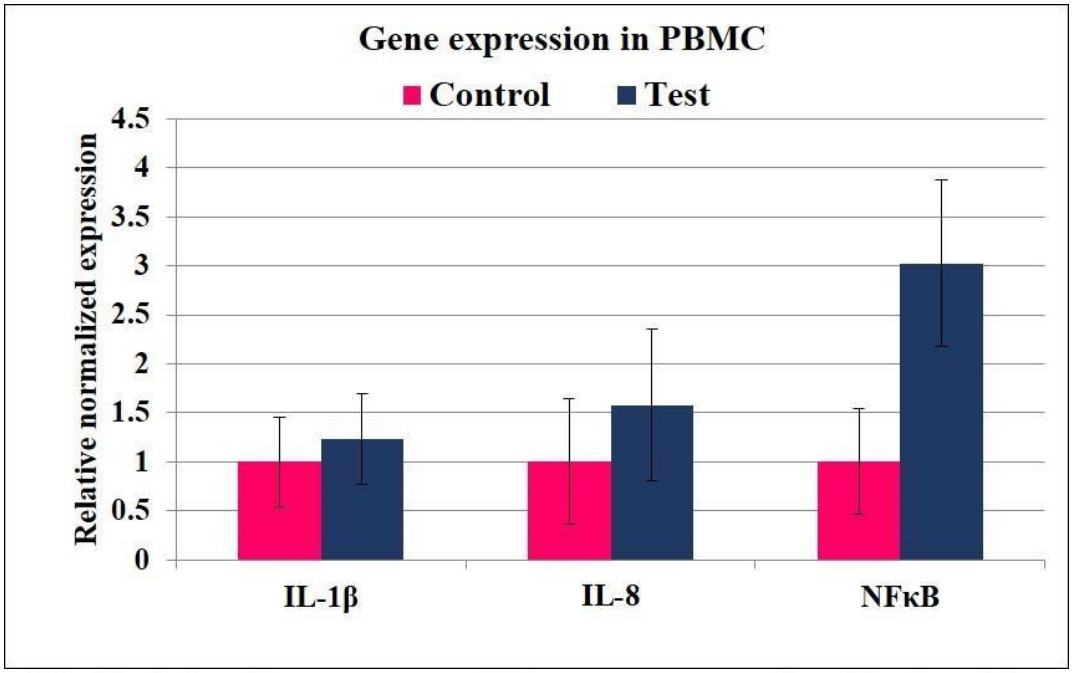

Figure 4.37: Expression of IL-1β, IL-8 and NFκB genes from PBMC following *i.v.* exposure of LPS to rabbits relative to GAPDH (housekeeping gene). n=3, Data represents the mean ± SD

CHAPTER 5: DISCUSSION

5. DISCUSSION

5.1. DETECTION OF IL-1β RELEASED FROM POOLED HUMAN BLOOD USING ELISA

The continuous progress in the field of medical research focused on the safety aspect of the therapeutic products, medical devices, implants and biological materials. Even though the exposure of the medical devices or parenteral drugs may be temporary or permanent, there is a chance of direct or indirect contact of these materials with blood, cells, tissues and organs. The presence of any of these non-self substances can evoke a primary immune response mainly driven by monocytes, macrophages and neutrophils (Dinarello CA, 2000). The accidental contamination of pyrogenic substances in the parenteral drugs, medical devices, tissue-engineered products and biomaterials can induce severe life-threatening febrile reactions leading to septic shock and even death. There are many microbial, organic and inorganic substances of varying origin and composition present in the environment, capable of inducing severe fever/febrile responses in the living system. The known examples of pyrogens are LPS (Beutler and Rietschel, 2003) and LTA (Wang *et al.*, 2003)

European pharmacopoeia recommended three pyrogen assays, namely RPT, LAL and MAT. All these assays are validated and regulated to test pyrogenic contamination in medical products (European Pharmacopoeia, 1997, European Pharmacopoeia, 2010, The United States Pharmacopeia USP 31, NF 26, 2008). Inadequate sensitivity, the requirement of a large number of animals and species variations are the major drawbacks faced by the conventional pyrogen assay. To overcome these shortcomings, an IVPT method was developed by Hartung and Wendel in 1995 to detect pyrogenic cytokines released from human immune cells in response to bacterial and non-bacterial pyrogens. The concentration-dependent release of IL-1β by the monocytes and tissue macrophages after direct contact with exogenous pyrogenic substance makes it a read-out parameter of pyrogenicity (Dinarello *et al.*, 1986).

In the present study, pooled human blood from healthy volunteers after checking their routine haematology and biochemical parameters was used to detect IL-1β. The major problem of using individual blood sample for IVPT is the unusual reaction of individual donors to different

pyrogen stimuli. Pooling of blood samples facilitates easy availability of the required amount of blood for the assay, reduces the inter-individual variation of cytokine release and maintains the requisite number of healthy leukocytes required to release IL-1β. Detection of IL-1β present in the serum separated from pyrogen induced pooled human blood was performed using indigenously developed ELISA plates (Mohanan *et al.,* 2010). The study evaluated the pyrogenic response of pooled human blood after treating with different concentration of LPS, LTA, PHA and TNP. This study also facilitates detection of IL-β release after pyrogenic induction with respect to time and the concentration of pyrogen required to stimulate pooled blood. It was reported that IL-1β is the most suitable readout parameter for IVPT as it is consistently released in a concentration-dependent manner upon exposure to the same minimal pyrogenic stimulation. Measurement of IL-1β is very much feasible with the ELISA technique (Daneshian *et al.,* 2009).

The IL-1β release from pooled human blood after LPS (1EU/ml and 5EU/ml), LTA (0.1µg/ml and 10µg/ml), PHA (15µg/ml and 30µg/ml) and TNP (15µg/ml and 30µg/ml) exposure was analyzed for an incubation period of 8h. Pooled blood without any pyrogenic stimulant was served as negative control and was kept along with every batch of the assay. No significant increase in IL-1β levels was noticed in negative control for the entire period of incubation. The peak concentration of IL-1β was observed within 6h of post-stimulation of 1EU/ml LPS. 5 EU/ml of LPS treatment induced a significant rise of IL-1β early at 2h of incubation. These results are consistent with the earlier report of Banerjee *et al.,* in 2011 using human whole blood assay. It was also noted that IL-1β induced by 1µg/ml LTA reached the maximum at 1h of induction and 0.1µg/ml LTA took a period of 2 to 4h for maximum release. In stimulation with PHA and TNP, a maximum IL-1β release was observed within 0-2h at 30µg/ml concentration. The delayed response of IL-1β release by blood cells on the challenge with a low concentration of pyrogens may be due to the insufficient quantity of toxicants to occupy the TLR receptors on monocytes/ macrophages to activate immune signalling may be due to the slow release of synergistically acting cytokines from immune cells. The detection of chemical toxicant (non-endotoxic pyrogen) demonstrated in the present study is of great advantage over LAL assay as it can detect only Gram-negative endotoxin. The study conducted in the pooled blood reaction strongly correlated with the previous report based on the human whole blood assay (Daneshian *et al.,* 2009). The advantage of human whole blood assay over the other assays relies on the

specific and effective detection of Gram-negative, Gram-positive, biological, and chemical pyrogens present in any type of material. Thus, the indigenously developed ELISA method for pyrogen detection allows a simple, much faster, reliable, cost-effective system for detecting endotoxin and non-endotoxin pyrogens.

5.2. DIFFERENTIATION OF THP-1 CELLS TO MONOCYTE DERIVED MACROPHAGES (MDM)

Inflammations usually induce differentiation of monocytes to macrophages having characteristic M1 phenotype. Similarly, anti-inflammatory signals stimulate the differentiation of monocytes to the M2 phenotype. Differentiation of monocytes to macrophages alters morphological characteristics, adherence property and inflammatory responses (Lund *et al.,* 2016). PMA is a widely used ester, which stimulates the differentiation of monocytes to macrophages. PMA activates protein kinase-mediated pro-inflammatory signalling pathway. The differentiation of monocytes to macrophage image under a phase-contrast microscope depicts the characteristic morphological and adherence capability of macrophages after PMA treatment. Immunocytochemistry using CD68 antibodies were also used to observe PMA triggered differentiation of THP-1 monocytes to macrophage phenotype. CD68 is a transmembrane glycoprotein expressed in cells of the monocyte-macrophage lineage. More specifically, CD68 is a specific marker of macrophages having an M1 phenotype (Kunz-Schughart *et al.,* 2003). In this study, the high intensity of green fluorescence was observed due to the binding of FITC tagged anti-CD68 antibody in MDM cells compared to undifferentiated THP-1 monocyte. This is due to the increased expression of CD68 on MDM, pointing out the active differentiation of THP-1 monocyte to M1 phenotype. Macrophage differentiation is also associated with increased volume of cytoplasm and granularity. An increase in the number of membrane-bound cell organelles (Mitochondria and Lysosomes) at some point of differentiation is the reason behind enhanced granularity in MDM cells. This will increase the side scattering of cells while conducting FACS. In the present experiment, the shift of SSC value confirms the differentiation of THP-1 monocytes to MDM cells (Kradin *et al.,* 1986).

5.3. MOLECULAR AND CYTOTOXICITY

The functioning of the cellular organelles is the essential criteria to be examined during cytotoxicity evaluation of biological and chemical toxicants. Any change in membrane potential and oxidation-reduction pathway may result in loss of cell viability. Accidental contamination of pyrogens in injectables, pharmaceuticals and medical implants can induce febrile reaction to toxic shock syndromes in humans and animals. Immune cells activated by exogenous pyrogens will lead to induction of inflammation and release of pyrogenic cytokines. Release of pyrogenic cytokines IL-1β, IL-6 and TNF-α trigger fever and febrile reaction by releasing prostaglandins (PGs) at the thermoregulatory centre of the hypothalamus (Dinarello.2004). Immune cells, especially monocytes and macrophages, are the key mediators of the innate immune system, involved in phagocytosis and elimination of foreign particles. Depending upon the inflammatory responses, monocytes differentiate into macrophage and migrate to various organs and tissues. Loss of activity or death of these monocytes and macrophages negatively affect the normal homeostasis of the immune system, thereby leading to serious health issues in human. LPS and LTA are the most common bacterial pyrogenic contamination reported in biological preparation. Plant mitogen PHA and chemical agent TNP were also included in this study, in addition to bacterial endotoxins to check the comparative toxicity in monocytes. In the present study, THP-1 cells served as a model for monocytes and MDM cells as macrophages.

5.3.1. EFFECT OF TOXICANTS ON ORGANELLE FUNCTION AND INTEGRITY

MTT and neutral red assays were carried out to check the organelle function in terms of mitochondrial reduction potential and dye uptake by lysosomes. The mitochondria function as the powerhouse of a cell; any change in membrane potential and oxidation-reduction pathway may result in loss of cell viability. It was observed that LPS has no significant effect on mitochondrial oxidation-reduction potential and lysosomal phagocytic potential. Morphological characteristics observed under Rhodamine-phalloidine and Giemsa staining also associate the non-toxic impact of LPS on cell membrane integrity. It was reported that the toxicity of LPS in human airway epithelial cells was found to be non-cytotoxic at lower concentrations. LPS mediated cytotoxicity requires long-term interaction with monocytes for more than 72h (Liu *et al.,* 2018).

Pyrogenic response by Gram-positive bacteria relay on the LTA component present in their cell wall. LTA from *B. subtilis* showed cytotoxicity in a dose and time-dependent manner. There was a significant loss of mitochondrial and lysosomal activity in MDM cells after 48 to 72h of exposure. A higher concentration of LTA disrupted the organization of cellular actin filament and was detached from the culture plate. Rounding up of cells and loss of characteristic macrophagic extensions indicate impairment to actin filament and associated membrane destabilization. The presence of serum in cell culture media has a prominent role in the cytotoxicity of LTA in mouse fibroblast monolayer. At the same time, the absence of serum in media enhanced the toxic effect by inhibiting collagen synthesis and cell membrane destabilization (Leon *et al.,* 1983).

PHA and other plant lectins are widely used as a mitogen for biological, immunological and biochemical research. Short-term exposure of PHA up to 100µg/ml did not induce any toxic response in MDM cells up to 24h. Loss of viability was observed only after exposure to the highest concentration. There is a significant loss of mitochondrial oxidation-reduction potential and lysosomal activity in a time and dose-dependent manner. Incomplete oxidation-reduction reaction in mitochondria will result in electron leakage and associated imbalance in cellular respiration. Phagocytic and proteolytic activities of lysosomes are responsible for the cellular defence and waste excretory mechanism. PHA mediated reduction in the endocytic activity of lysosomes directly affect cellular metabolism and finally lead to cell death. Differentiated macrophages with characteristic stellar shape were observed by Rhodamine staining after exposure with a lower concentration of PHA. Long-term exposure of PHA results in damage to the actin network and thereby, macrophages lost their cell membrane protrusions and appeared round in morphology. Similarly, the cytotoxicity of five isolectins of PHA was studied in human H9 lymphoma cells. It was reported that cellular toxicity was observed only after three or more days of exposure. The toxicity of lectins differs according to their isoforms and strength of binding with the cell surface moiety (Ren *et al.,* 2008).

TNP is a pale yellow odourless solid extensively used in various industries for manufacturing matches, batteries, dyes and textiles. Exposure of human to ammonium picrate (either ingestion or inhalation route) can cause severe poisoning (Sunderman *et al.,* 1945). Mechanism of TNP

mediated toxicity was evaluated in the present study at different time intervals. The mitochondrial and lysosomal activity of MDM cells was significantly affected by the long-term exposure of TNP in a dose-dependent manner. There was a visible cell membrane destabilization on exposure to the higher concentration of TNP. It was reported that TNP induces immune toxicity through activation of inflammatory cytokines in guinea pig and cat. The lethal dose of TNP exposure varies with the type of animal species used for the study (Weeks *et al.,* 1983).

5.3.2. FREE RADICAL GENERATION

Free radicals are highly reactive unstable atomic species with an unpaired electron in their outermost orbit. The generation and propagation of free radicals are linked to various diseases and ageing in human. Several studies are available on the free radical generation and anti-oxidant mechanism in a living system. Even though, their role in cellular toxicity and associated defence mechanisms are still under investigation. The fluorescent dye DCFH-DA is widely used as an indicator for free radical generation inside the cells. Inflammation and free radical generation associated with exogenous pyrogen can lead to apoptotic or stress-induced death of immune cells. Spitzer *et al.,* 2020 reported the induction of oxidative stress in rodent mammary gland after *in vivo* administration of LPS. It is well known that LPS can activate superoxide dismutase 1 (Sod1) and Nqo1 enzymes and thereby increased the release of H_2O_2 inside the cell. In the present, the selected concentration of LPS was insufficient to cause any free radical generation and oxidative stress in MDM cells even after 72h of exposure.

There are reports indicating the time-dependent death of mixed neuronal-glial cells by LTA. The proliferation of glial cells and astrocytes were reported after 72h incubation with LTA. Short-term exposure of LTA was not enough to induce direct cellular toxicity to neuronal cells. Co-ordinate release of inflammatory cytokine from astrocytes and NO radicals from microglia lead to stress-induced neuronal cell death. It was evident from the study that NO radical and associated superoxide anion production has a significant role in LTA mediated toxicity in neuronal cells (Kinsner *et al.,* 2005). In the present study, LTA and TNP induced time and dose-dependent hydroxyl and nitrile radical generation in MDM cells. Long-term exposure of LTA (10 to 1000µg/ml) caused a significant rise in hydroxyl radical production. Oxidative stress ultimately leads to cellular death when ROS production exceeds the resistance offered by the

antioxidant defence mechanism of cells. It was reported that LTA is capable of inducing oxidative stress in heart-derived H9c2 myogenic cells. There was a significant reduction in the expression of antioxidant enzymes super oxide dismutase, catalase and glutathione peroxidase in H9c2 cells. LTA also activates inflammatory pathways through the induction of the p38 and JNK pathway (Gutiérrez-Venegas *et al.,* 2020).

PHA from *Phaseolus vulgaris* is capable of generating oxidative stress in human platelets. PHA stimulates hydroxyl and nitric oxide radical generation by activating $Ca^{2+/}$Calmodulin dependent kinase β (CaMKKβ)/AMPKα pathway. According to the published data, ROS mediated DCFH oxidation depended on the dose and time of PHA exposure. PHA induces maximum DCFH oxidation at a dose of 50µg/ml within 15 min. When the time of exposure increased, there was a gradual reduction in ROS generation. DCFH oxidation in PHA treated platelets become similar to the control (within 30 min) (Signorello *et al.,* 2020). In the present study, the analysis of DCFH oxidation and nitrile radical generation was done after exposure to PHA for 1 to 72h. There was no significant free radical generation in MDM cells when the time of exposure was prolonged and is similar to the findings of Signorello *et al.,* 2020. Lack of ROS generation may be due to the active antioxidant defence mechanism of MDM cells against external stimuli. Differentiated THP-1 cells offer more resistance to exogenous inflammatory agents by activating glutathione, glutathione-related antioxidant enzymes and manganese superoxide dismutase through TLR2 signalling (Karwaciak *et al.,* 2017).

5.3.3. MITOCHONDRIAL MEMBRANE POTENTIAL

Mitochondrial membrane potential (MMP) is maintained in a narrow range to keep the critical functioning of cellular respiration and ATP generation. MMP results from a proton pump operating between inner mitochondrial membranes to store energy formed as a result of oxidative phosphorylation. Loss of MMP will result in a sudden drop in cellular ATP level and cell death. It is evident from the present study that the MMP of THP-1cells was not affected by the exposure of LPS (0.25 to 5EU/ml). There is no shift in DilC1(5) fluorescence in LPS treated THP-1 cells, which indicates the proper maintenance of MMP inside the cells. The orange-red fluorescence observed in JC1 staining also confirms active MMP in MDM cells exposed to LPS. From the present study, it was evident that the selected concentration of LPS was insufficient to

cause mitochondrial dysfunction and a reduction in MMP. However, there were reports of mitochondrial fragmentation, metabolic shift, ROS production and increased MMP in microglial cells after 24h exposure with 50ng/ml of LPS. LPS mediated activation of NFkB and MAPK pathway resulted in metabolic reprogramming of microglial cells. The pro-inflammatory state of microglia cells was associated with mitochondrial fission and increased production of reactive oxygen and nitrogen species inside the cells. (Nair *et al.*, 2019).

Mitochondrial dysfunction associated with bacterial sepsis is a critical event in inflammatory injury and cell death. Bacterial exotoxin (LTA) showed a dose-dependent loss of MMP in THP-1 cells. Decrease in fluorescence of DilC1(5) dye from cells treated with LTA, PHA and TNP pointing towards the lack of active membrane potential. There was a reduction in the formation of J-aggregates associated with loss of MMP when the higher concentration of LTA, PHA and TNP. Change in fluorescence from orange-red to green is visible in JC1 staining. There are no reports available about the action of TNP on lysosomal destabilization. Co-ordinate action of ATP synthase complex, Cytochrome C and membrane anchoring proteins are involved in the regulation of MMP. TLR2 mediated signalling associated with bacterial sepsis had a role in intracellular H_2O_2 and mitochondrial O_2^- production. It was reported that LTA could induce the release of cytochrome C and associated loss of MMP in H9c2 cells (Liu *et al.*, 2018). ATP depletion and associated acidosis, the release of cytochrome C and dysfunction of electron transferring protein complexes can create instability in MMP. PHA mediated activation of cytochrome C and induction of apoptotic pathway was reported in A549 cells. Isoforms of PHA-erythroagglutinin has been used as an anticancer drug because of their apoptotic death induction in cancer cells. Mitochondrial apoptotic pathway initiated via loss of MMP caused caspase activation in A549 cells. PHA-E mediated inhibition of PI3K/Akt and MEK/ERK-mediated survival signalling intern activate apoptosis (Kuo *et al.*, 2011).

5.3.4. LYSOSOMAL MEMBRANE INTEGRITY

Lysosomes mediated autophagy, exocytosis and degradation of cellular components are necessary for the proper functioning of immune cells. Lysosomes are the j of various metabolic and catabolic functions. Lysosomal membrane destabilization and release of hydrolytic enzymes into the cytosol, in turn, activates lysosome dependent death pathway. The present study

observed that LPS up to 5EU/ml does not induce lysosomal membrane destabilization. Orange-red fluorescence of AO dye within the lysosomes is intact in LPS treated cells, as like untreated MDM cells. It was reported that 50ng/ml of LPS was sufficient to induce lysosomal membrane permeabilization. LPS interaction with TLR4 receptors on macrophages activates signalling through TRIF resulted in apoptotic death of primary macrophages and RAW 264.7 macrophage, principally through lysosomal dysfunction (Schilling *et al.,* 2013). In the present study, lysosomal membrane destabilization was observed after LTA, PHA and TNP exposure. Change in AO fluorescence from red to green indicates the destabilization of the lysosomal membrane and leakage of hydrolytic enzymes into the cytosol. The result of the present observation supports the findings of neutral red assay of LTA, PHA and TNP, where the phagocytic potential of lysosomes was significantly affected in a dose and time-dependent manner. Bacterial exotoxin forms membrane pores when exposed to acidic pH, which drives the translocation of these toxins into the lysosomes. Exotoxin mediated oxidative stress also release reactive hydroxyl and nitrile radicals. Diffusion of these ROS to lysosomes will result in lipid peroxidation and membrane destabilization. Lysosomal membrane permeabilization is considered as a primary or secondary route of cell death executed via apoptotic or necrotic mechanisms (Aits *et al.,* 2013).

5.3.5. MECHANISM OF CELL DEATH

The acetoxymethyl ester of Calcein (Calcein-AM) is a highly lipophilic molecule with enhanced cell permeability. The non-fluorescent Calcein -AM entering into the viable cell is converted into a highly green-fluorescent Calcein by the esterases, thereby making it an efficient stain for viable cells (λ_{ex} 490 nm, λ_{em} 515 nm). In contrast, the nuclear staining dye PI enters the nuclei through a dead cell membrane and intercalates with the DNA double helix of the cell to emit red fluorescence (λ_{ex} 535 nm, λ_{em} 617 nm). Calcein AM-PI dual staining thus helps to estimate the percentage of live and dead cells in a population. Annexin V-PI is a well-established technique used to sort cells in the early, late and necrotic stage of cell death. Annexin V specifically binds with phosphatidyl serine residue found on the membrane surface of apoptotic cells. The present study investigated the ability of LPS, LTA, PHA and TNP can induce apoptosis/necrosis in THP-1 modified MDM cells.

Apoptosis is an energy-dependent programmed cell death through activated caspase characterized by condensation and fragmentation of cell nuclei, reducing cytoplasm to nuclear volume and blebbing of the plasma membrane. Apoptotic cells shrank to form small membrane-bound vesicles and are engulfed by phagocytic cells. In contrast to apoptotic death, necrosis is an ATP independent mechanism, where cells undergo immediate membrane lysis and shedding of cell components to the surrounding environment. It was reported that acute and chronic exposure of LPS induced cell cycle reactivation and apoptotic cell death of mouse neural stem cells (NE-4C). It was associated with significant expression of RB family proteins, particularly RBL1/p107 and RB1/p105 in neuronal cells (D'Angelo *et al.,* 2017). Similarly, LPS caused membrane destabilization, detachment, barrier dysfunction and activation of the caspase pathway in endothelial cells. Caspase mediated cleavage of cell junction adherence proteins led to barrier dysfunction and detachment of endothelial cells and resulted in apoptotic cell death (Bannerman *et al.,* 1998). LPS induced ROS generation and fission of mitochondria along with over expression of apoptotic protein Bax and caspase 9 in hepatocytes was reported by Wang *et al.,* 2019. Inflammatory response through MSt1-JNK signalling and mitochondrial apoptosis was said to be the mechanism of LPS mediated hepatic cell death.

Contrary to the above findings, study conducted by Klein and co-workers reported LPS inhibited apoptosis in human neutrophils is through an extracellular regulated kinase pathway (Klein *et al.,* 2001). In the same way, no such significant indication of phosphatidylserine externalization was evidenced in the present study, when 5EU/ml of LPS exposed to THP-1 cells. Meanwhile, the highest concentration of LTA treatment exhibited apoptosis within 24h of exposure. Dose and time-dependent increase in cell death was also observed by Calcein AM-PI dual staining. The study by Wu *et al.,* 2017 supports the result of the present study that in microglia cells, LTA induced apoptosis by increased production of inducible nitric oxide synthase. Reports of DNA fragmentation and enhanced terminal deoxynucleotidyl transferase- mediated dUTP end labelling in dental pulp cells on human deciduous teeth (Wang *et al.,* 2001). The increase in Annexin V positive cells and chromatin condensation, nuclear disintegration, Bax and Caspase 3 activation in human Osteoblast like cells (Tian *et al.,* 2013) also revealed the apoptotic potential of LTA.

PHA and its isolectins were reported (Kochube *et al.,* 2015) to induce apoptotic cell death of human HEp-2 carcinoma cells, where 1μg/ml concentration of PHA showed maximum apoptotic cells compared to 10 and 100μg/ml treated groups. PHA treatment significantly increased the expression of cleaved caspase 3 and Bax 2 in HEp-2 cells, indicating apoptosis-mediated cell death (Kochube *et al.,* 2015). PHA erythroagglutinin was reported to induce mitochondria-mediated apoptosis of human lung cancer cells. The primary mechanism of apoptotic induction in lung cancer cells was similar to that of the above reports, where PHA enhances the release of mitochondrial protein cytochrome C and associated up-regulation of caspase 3, caspase 9 Bax and down regulation of Bcl2 and Bad proteins (Kuo *et al.,* 2011). In the present study, necrotic cell death was prominent after PHA exposure, where apoptosis mediated cell death was detected following TNP in a dose-dependent manner, which is considered as the general mechanism of chemical toxicity. It has been reported that the complex death mechanism pathway depend on the dose of the chemical toxicants exposed to a living system. Lower doses of chemical toxicants trigger autophagy or apoptosis, while higher concentration might cause necrosis (Orrenius *et al.,* 2011). Similar to TNP, an environmental toxicant 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) reported inducing apoptosis in thymocytes (Mc Conkey *et al.,* 1988). The direct or indirect contact/exposure to chemicals due to environmental pollutants generates ROS, cellular organelle damage and associated cell death, which finally disrupts the homeostasis of the tissue/organ system.

5.3.6. GENE EXPRESSION

Gene expression studies direct the way of finding functional activity towards an external or internal stimulus. Expressions of genes lead to the production of specific mRNA that translates into a functional protein and executes characteristic response to stimuli. The present study investigated the expression gene-specific for inflammatory cytokines IL-1β and TNF-α, inflammation-related enzyme COX2 and inflammation-related transcription factor NFκB. It was observed that LPS, LTA, PHA and TNP induced expression of these genes in THP-1 cells, pointing out their potential inflammation-related responses at the protein level. After LPS stimulation, IL-1β exhibited the highest fold change in expression compared to other cytokine genes. NFκB and COX2 expression was enhanced in a dose-dependent manner. Chanput *et al.,*

2010 reported LPS induced expression of NFκB, COX2, IL-1β, IL-6 and TNF-α in monocytes and macrophages in a time-dependent manner. Monocytes showed maximum response to LPS with an early rise in expression of inflammation-related genes compared to macrophages. LPS is a potent stimulator of inflammatory response in monocytes and macrophages. Nuclear translocation of NFκB and transcription of inflammatory cytokines promote an immune response against LPS by monocytes and macrophages. Pathway of LPS mediated inflammation was reported in H292 and THP-1 cells, where TLR4, IκBα, p65, p38 and JUN protein expression was increased in H292 and STAT3, p65, TLR4, IκBα and Janus kinase (JAK) 1 protein expression was also reported to increase in THP-1 cells. In both cells, NFκB mediated downstream signalling to play a major role in inflammation signalling and release of inflammatory cytokines IL-6, IL-8, TNF-α, MMP-9 and TIMP-1(Liu *et al.,* 2018).

Interaction of LTA and TLR-2 receptors activate a cascade of inflammatory signals through various adaptor molecules. Similar to the present result, Chang *et al.,* 2010 reported significant unregulated expression of IL-6 and TNF-α in Raw 264.7 macrophages after exposure of LTA for 1, 6 and 24h. LTA also enhanced phosphorylation of ERK1/2 protein, which is necessary for the degradation of IκB inhibitor from NFκB-IκB complex. The release of NFκB from its inhibitor accelerates nuclear translocation and transcription of NFκB dependent genes. LTA not only activated phosphorylation of ERK1/2 but also enhanced the DNA-binding activity of NFκB. LTA induced expression of TNF-α, IL-1 and COX2 were also reported in mice H9c2 cardiomyocytes in a dose-dependent manner (Bustamante *et al.,* 2013). The rate of expression of inflammation-related genes is not the same for LPS and LTA, although they induce a similar immune response *in vitro* (Bulgari *et al.,* 2017*)* and *in vivo* (Finney *et al.,* 2012). PHA is a plant mitogen, which activates T cells through TLR, CD2 and CD3 receptor interaction. PHA induces proliferation and differentiation of lymphocytes and is an inevitable mechanism to defend against inflammation or injury. It was reported that PHA enhanced the expression of IL-2, IL-2R, IL-6, IL-10, TNF-α and IFN-γ in PBMC within 1 to 4h of stimulation (Fan *et al.,* 1998). The cytokine gene expression profile depends on various factors, including ligand-receptor interaction, the strength of the ligand-receptor complex, activation of intracellular signalling, age and immunity of individual, *etc.* Jeljeli *et al.,* 2019 reported age-dependent expression of 13 different inflammation-related cytokines IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-10, IL-

12(p70), IL-13, IL-17, IFN-γ, MCP-1/CCL2, MIP-1b/CCL4 and TNF-α from PHA exposed whole blood collected from neonates and children. Pyrogen induced activation and expression of inflammation-related genes ultimately result in propagation of immune signals to various organs and tissue and subsequent dyshomeostasis of thermoregulation at the CNS level.

5.3.7. NFκB ACTIVATION

Interaction of LPS with TLR4 present on the surface of monocytes/macrophages initiates various inflammatory pathway. Transduction of TLR4 signalling is dependent on adaptor molecules MyD88 and TRIF-TRAM from cell surface and endosomes, respectively. TLR4 signalling through these adaptor molecules initiates events of pro-inflammatory cytokine expression. NFκB is the most recognized and studied pro-inflammatory transcription factor involved in LPS-TLR4 inflammatory response. Phosphorylation of p65 subunit of NFκB plays a key role in activation, translocation and specificity of downstream signalling by NFκB. Translocation of cytosolic NFκB transcription factor to the nucleus is necessary for active expression of pro-inflammatory cytokine gene. Phosphorylation of p65 and translocation of NFκB depend on the concentration and strength of LPS-TLR4 binding (Bagaev *et al.,* 2019). Immunocytochemistry analysis of MDM cells after LPS treatment showed a dose-dependent increase in NFκB activation. LPS, even in nanogram concentration, is capable of inducing NFκB translocation in MDM cells. Nuclear translocation is readily visible as green spots in the nucleus after exposure to 5EU/ml of LPS. It was reported that LPS use TLR and IL-1 superfamily receptor for transducing signals to immune cells. IL-1 family of cytokines conveys inflammatory signals through MyD88, IRAK, IRAK2, and TRAF6 adaptor molecules for the activation of NFκB. LPS also make use of a similar pathway in THP-1 cells. LPS mediated NFκB activation induces cytokines IL-1β, IL-6 and TNF-α, eventually leading to a pyrogenic response in humans (Zhang *et al.,* 1999).

Inflammation-induced by LTA follows a cascade of phosphorylation and dephosphorylation of kinase enzymes in immune cells. As discussed earlier, LTA can induce nitric oxide synthase (iNOS) and subsequent release of NO radicals in macrophages. Expression of iNOS in macrophages is proved to be linked with protein kinase C mediated NFκB activation (Kuo *et al.,* 2003). Instead of TLR4 in LPS inflammation, LTA transduced inflammatory signals through TLR2 receptors. *In vivo* studies with *S. pneumonia* LTA demonstrated the role of TLR2

mediated activation of neutrophil influx and inflammatory cytokine release in mice model (Dessing *et al.,* 2008). LTA- TLR2 receptor interaction in macrophage cells activates tyrosine kinase, PI3K/Akt and p38 MAPK signalling pathways. Activated kinases can cause a marked increase in NFκB-specific DNA–protein complex formation. This DNA-protein complex is an indicator of translocated NFκB, which promote transcription of iNOS and pro-inflammatory cytokine genes (Kao *et al.,* 2005). Agreed to the above results, the present study also detected dose-dependent activation of NFκB in THP-1 cells exposed to LTA, suggesting the possible immunotoxic potential of bacterial exotoxins in human monocytes.

It was reported that PHA acts as a stimulant of NFκB-DNA binding in human cord blood mononuclear Cells (hCBMC). After 1h incubation with PHA, hCBMC induced a significant rise of NFκB. More specifically, the p65 subunit of NFκB found to interact with nuclear DNA after PHA stimulation (Schroeter *et al.,* 2004). PHA also induces transcription of API2 and MALT1genes. AP12-MALT1 protein is a stable chimeric protein that acts as a constitutive stimulator of NFκB (Seto M., 2004). In the present study, PHA and TNP induced activation of NFκB in THP-1 cells. Since monocytes are highly sensitive and reactive to external stimuli, PHA can trigger NFκB activation and associated differentiation. Similarly, TNP-substituted protein was found to induce inflammatory bowel disease in IL-2 deficient mice and was associated with decreased production of anti-inflammatory cytokine TGF-β (Lúdvíksson *et al.,* 1997).

5.3.8. COX2 ACTIVATION

Pathogenic contamination during clinical procedures leads to multi-organ failure and death in patients due to septic shock. Septicemia is the worst condition, which can be triggered by bacterial toxins, including LPS and LTA. The route of septic shock is initiated preliminarily as inflammation followed by activation of NFκB and COX2. COX is the enzyme that catalyzes the conversion of arachidonic acid to PGs. PGE2 is the potent mediator of pyrogenic fever. There are reports of COX2 activation by LPS in mouse transformed Clara cells (Britt Jr *et al.,* 2012), RAW264.7 macrophages (Eliopoulos *et al.,* 2002), IEC-6 cells (McElroy *et al.,* 2012) and human neutrophils (Nagano *et al.,* 2002). COX2 expression has multiple responses in different cells. Reports of COX2 activation strongly support LPS induced immune activation in various cell types. There are various adaptor molecules for the COX2 activation pathway following the

LPS challenge. The activation pathway involves phosphorylation of MAPK proteins, JNK, p38 and/or ERK, activation of Tpl2-dependent CREB and NFκB. COX2 activation eventually leads to the release of PGE2, thromboxane A2 and pro-inflammatory cytokines. Here, NFκB and COX2 activation by LPS in THP-1 cells are similar to the previous reports. Present results suggest the potential of a low concentration of LPS to activate an immune response, whereas the same concentration does not show a direct cytotoxic effect.

Similar to LPS, LTA induced COX2 activation in THP-1 cells. Increased intensity of green fluorescence after LTA treatment was observed by FACS analysis. LTA can interact with the CD14 receptor and activate NFκB mediated inflammatory response. Neuronal inflammation by bacterial toxins was reported in rat cortical neuronal cells and it was found that LTA activated COX2 and PGE2 synthesis through PTK, PC-PLC, PI-PLC and ERK pathway (Wu *et al.,* 2006). Cytosolic phospholipase A2 (cPLA2), COX-2, PGE2 and IL-6 reported to elevated in LTA exposed human tracheal smooth muscle cells. It was observed that LTA interacts with TLR2 receptors and activate NFκB through MyD88, PI3K, Rac1, Akt and MAPKs adaptor signalling. The activation of NFκB resulted in increased COX2 expression and release of PGE2 in human tracheal smooth muscle cells (Lee *et al.,* 2010). It was known that LTA could induce inflammation through COX2 and PGE2 release from cells. Hence, selective inhibitors of COX2 are helpful for the treatment and prevention of inflammation associated episodes of pain, allergy and carcinogenesis.

PHA can evoke an inflammatory response in human lymphocytes through active production of ROS, MDA and inflammatory cytokines IL-6 and TNF-α. PHA interacts with lectin receptors on immune cells and acts as a mitogen that induces the proliferation of cells (Askari *et al.,* 2020). In THP-1 cells, PHA and TNP found to cause inflammation via NFκB and COX2 activation. PHA was reported to enhance the production of IL-2 from Jurkat cells. IL-2 is a known activator of T cell proliferation and differentiation. Increased phosphorylation of p38-MAPK and COX2 expression in Jurkat cells also indicate the inflammatory response of PHA. COX2 can modulate extend of inflammation through the synthesis and release of prostanoids (Hu *et al.,* 2019).

5.4. ANALYSIS OF PYROGENICITY IN RABBITS

5.4.1. LPS INDUCED PYROGENIC RESPONSE

Pyrogenic effect endotoxin was studied using rabbits. Rabbits are the most suitable model for pyrogenic response. In the present study, *in vivo* response to LPS begins within half an hour and lasts for more than 3h. Rectal temperature is significantly raised from the baseline temperature of 39.12±0.175 to a maximum temperature of 40.58±0.70. LPS is a well-known pyrogen and its activity is principally depending on pyrogenic cytokines released from the immune cells. Elevation in body temperature is associated with variation in the thermoregulatory mechanism of the CNS. There are reports of a time-dependent rise in rectal temperature following the intravenous (*i.v*), intraperitoneal (*i.p*), intramuscular (*i.m*) and subcutaneous (*s.c*) administration of LPS in rabbits. LPS mediated pyrogen response is independent of the route of exposure. The peak value of rectal temperature was reported between 60-210 min (Cartmell *et al.,* 2002).

Endogenous pyrogens (EPs) released following intravenous administration of LPS, poly (I: C) and Newcastle disease virus (NDV) found to induce an immediate rise of rectal temperature in rabbits. Together with PGE2 and cyclic AMP, EPs enhance the magnitude of pyrogen induced fever and are the classical pathway of pyrogen response. Almost all exogenous pyrogens follow the similar pathway of fever induction, where EPs, PGE2 and cAMP lead to variation in the thermoregulatory mechanism of the hypothalamus. Conversely, EPs, PGE2 and cAMP-mediated fever stimulation were not detected in animals with induced hyperthermia. It is informative that hyperthermia associated with environmental or other factors follow the alternate temperature induction pathway compared to exogenous Pyrogens (Siegert *et al.,* 1976). Hyperthermia has shown enhanced fever and febrile reactions in rabbits challenged with LPS. Heat stress facilitates leakage of LPS from intestinal cells into blood circulation, exaggerating pyrogenic fever in stressed animals compared to non-stressed animals. In heat-stressed animals, LPS induced fever is not correlated with the serum concentration of IL-1β and TNF-α. Hence, it was stated that enhanced fever response in the heat-stressed animal purely depends on the amount of endotoxin leaked from the damaged intestinal lining to blood circulation (Shibata *et al.,* 2005).

It was well documented that human monocyte-derived EPs are potent stimulator of fever in rabbits. EPs administrated either *i.v* or intracerebroventricular (*i.c.v*) route can induce pyrogenic fever. However, the animals who received EPs via *i.v* showed more variation than animals who received *i.c.v* challenge. The study depicts the mode of action of EPs at the CNS level, where it interacts with specific receptors and directly affects the thermostatic mechanism. It was suggested that variability in fever response following *i.v* administration is due to the low level of EP-receptor interaction or difference in permeability of EPs through the BBB (Stitt, 1985).

5.4.2. HEMATOLOGICAL CHANGES IN RABBIT BLOOD

The immune system is a wide network of organs and cells continuously monitoring the foreign invaders to prevent their entry into the host system. When an antigenic substance enters the body, there will be a network of immune reactions, which protects the host from adverse reactions. During immune activation, there will be a variation of haematological and biochemical parameters in the host body. In the present study, rabbit blood was analyzed for haematological and biochemical parameters before and after endotoxin administration. White blood cells (WBC) are the defenders, which phagocytose foreign particles or produce antibodies against antigens to neutralize them. In the present study, exposure to LPS in rabbits caused a decrease in WBC count that of control. Burrow reported similar findings in 1979, where lymphocyte count was drastically reduced following the LPS challenge in equine species.

Leucopenia is a common phenomenon following LPS exposure *in vivo*. It was reported that calves challenged with LPS exhibited time-dependent leucopenia, where WBC count was significantly reduced within 30min. Leucopenia persists for 3h and becomes normal within 12h of LPS challenge. Since RBC has no significant role in defence mechanism, the packed cell volume (PCV) was unchanged following exposure of 0.2-20μg/kg LPS (Gerros *et al.*, 1995). The present study agrees with the above findings, where RBC, Hb, HCT, MCV, MCH and MCHC parameters are well comparable to that of control. On the contrary, increased Hb and hematocrit values were reported in the goat following LPS administration along with leucopenia, lymphopenia and tachycardia (Koot *et al.*, 1989).

Lymphocytes and monocytes recruited at the site of inflammation propagate signals and attribute various cellular defence mechanisms, including chemotaxis, adhesion, invasion and phagocytosis. It was reported that LPS exposure leads to a significant reduction of lymphocytes and monocytes in rabbits, where the neutrophil count was found to be normal or somewhat increased after 4h. Even though primary exposure to LPS can induce leucopenia, repeated exposure to the same endotoxin will give rise to leukocytosis due to immune tolerance (Peñailillo *et al.,* 2016).

5.4.3. BIOCHEMICAL CHANGES IN RABBIT BLOOD

Endotoxemia is a condition where bacterial toxins circulate in the blood and negatively affect the function of vital organs. Endotoxemia related dysfunction of the liver, kidney, heart, lungs and brain potentially lead to a fatal condition. In most mammalian species, alanine transaminase (ALT), aspartate transaminase (AST) and Gamma-glutamyltransferase (GGT) are liver-specific enzymes; hence, its elevation indicates damage to the hepatic system. The present study evaluated the biochemical parameters such as blood glucose, cholesterol, protein, liver enzymes and creatinine following the LPS challenges to investigate the change in cellular metabolism and potential damage to the organs. Significant increase in blood glucose, triglycerides, AST, ALT and creatinine are observed in rabbits following the LPS challenge. Dyshomeostasis of glucose metabolism is a prominent feature of endotoxemia. As per the report of Nagaraja *et al.,* in1979, *i.v* administration of endotoxin significantly affected glucose metabolism, liver functions and haematological parameters in calves. It was noted that endotoxin shock-induced immediate hyperglycemia within 15min and persisted for 3h. An elevated level of AST and lysosomal enzymes (acid phosphatase and /3-glucuronidase) were also reported. Severe haemorrhage in the brain, lungs, liver and kidney indicated disseminated intravascular coagulation induced by LPS. It was noted that biochemical changes following endotoxemic shock in calves are similar to those observed in the present study.

There are reports of a significant increase in the concentration of AST, ALT, GGT, triglycerides, urea and creatinine in rabbits on the challenge with a high dose of LPS. It was inferred that LPS is capable of causing lipid metabolism disorders and damage to vital organs (liver and kidney) (Elmas *et al.,* 2006). However, these results are different to those found by Koot *et al.,* 2016 and

Peñailillo *et al.,* 2016, where AST, bilirubin, creatinine and GGT values in endotoxemic rabbits were found to be comparable to that of control. Similar results were reported in piglets challenged with LPS, where AST and ALT values were significantly increased within 4h following administration. Proteins synthesized from the liver, especially α1-globulins and sialic acid, significantly increased and are the potent indicator of acute-phase inflammatory reaction. Extrarenal damage was noted with increased blood urea level. However, creatinine values were comparable to that of normal in the initial phase of infection. Dyshomeostasis of glucose represented as hyperglycemia, followed by hypoglycemia in piglets (Borissov and Andonova, 2000) agrees with the observations of the present study. However, these results are different to those found by Koot *et al.,* 2016 and Peñailillo *et al.,* 2016, where AST, GGT, bilirubin and creatinine values in endotoxemic rabbits are within the reference range and comparable to control.

5.4.4. CYTOKINE GENE EXPRESSION IN PBMC ISOLATED FROM RABBIT BLOOD

Expression of transcription factor NFκB and pro-inflammatory cytokines (IL-1β and IL-8) genes were analyzed in PBMC isolated from rabbit blood. NFκB is the major transcription factor responsible for the active transcription and expression of inflammatory cytokine genes. Here, LPS is a stimulator of immune response in rabbits. Since PBMC, especially monocytes and macrophages are the active producers of cytokines, expression of NFκB, IL-1β and IL-8 were studied in PBMC isolated from rabbit blood before and after administration of LPS. The present study found increased expression of NFκB, IL-1β and IL-8 following LPS administration. These observations suggest LPS induced immune activation in PBMC.

Typically, NFκB is found in the cell cytoplasm as an inactive form bound with IκB. When there is an interaction of foreign substance or PAMPs with TLR, there will be the initiation of inflammatory signals and the release of active NFκB from the NFκB-IκB complex. Translocation of released NFκB into the nucleus will results in the binding of these transcription factors to the promoter region of genes specific for cytokines, chemokines and coagulation factors. NFκB expression has a direct role in immune activation, cell differentiation and cell survival. It was reported that blood samples collected from rabbits, intravenously administrated with staphylococcal enterotoxins A showed a significant increase in NFκB expression compared to

untreated control. Staphylococcus super antigens are pyrogenic in nature. They interact principally with T cells and activate the release of TNF-α, IL-1 and IFNs. Release and interaction of pro-inflammatory cytokines, in turn, activates NFκB translocation and propagation of immune response (Mahmood *et al.,* 2020). NFκB also exhibits a critical role in the generation of cerebral vasospasm with respect to inflammatory cerebral haemorrhage in rabbits. Inflammation involves a series of events, and its magnitude depends on cellular interaction, cytokine production and intracellular signalling. Sub arachnoid haemorrhage is a stimulus of vascular inflammation and was associated with increased expression of NFκB, IL-1β and TNF-α (Zhou *et al.,* 2007).

Cytokine family is an integrated system with a network of multiple cytokines, which mediates immune response against infection or inflammation. In the IL-1 family, IL-1β is the potent pyrogenic cytokine that acts on CNS to induce fever/febrile reaction. Clinton *et al.,* 1991 demonstrate the pathological effect of IL-1β in inflammatory atherosclerosis, where increased expression of the IL-1β gene was found in vascular tissues of LPS challenged rabbits. IL-1β having molecular weight 17kDa, is the biologically active form and was found to be elevated in LPS challenged animals directs its substantial role in infection and vascular injury.

5.4.5. CYTOKINES RELEASED IN RABBIT SERUM

Cytokines released from immune cells act as initiators of inflammation and subsequent immune reactions. The present study investigated the release of pro-inflammatory cytokines IL-1α, IL-1β and TNF-α following *i.v.* administration of LPS in rabbits. LPS challenge significantly increased cytokine release from immune cells. Present study observed 2.7 fold increases in IL-1α, 2.8 fold in IL-1β and 4.8 fold in TNF-α concentration in LPS exposed rabbits compared to control. It was reported that the circulating level of IL-1β and TNF-α have a role in febrile response as well as tolerance to LPS administration in rabbits. Fever response is found in rabbits following increased production of IL-1β and TNF-α, where tolerance is associated with a low level of these cytokines in circulation. It was noted that PBMCs are the major secretors of IL-1β and are responsible for febrile hyper responsiveness in rabbits, 7days post-LPS administration. A rise in serum level of IL-1β and TNF-α follow independent periods despite their similarities in fever induction potential. The peak value of TNF-α observed within 60 min, where IL-1β reached peak rise only after 180 min of LPS challenge (Wakabayashi *et al.,* 1994).

LPS interaction with TLR-4 and CD14 activate inflammatory signals through co-ordinate action of various pro-inflammatory cytokines, including TNF-α. Reports suggest that LPS challenge in rabbits elevated TNF-α within 60 min. However, pretreatment with IL-1 reduce LPS induced TNF-α production even it enhanced TNF-α in non-treated rabbits. This observation was explained in such a way that IL-1 could induce the release of corticosteroids that intern down-regulate the expression of TNF-α mRNA along with IL-1 (Kaplan *et al.,* 1993). TNF-α is a pleiotropic cytokine that activates the synthesis and release of other cytokines from cells and organs. The release of pro-inflammatory cytokines has a role in the regulation of hormone secretion and organ functions. A recent study conducted by Qin et al., 2008 reported that *i.p* administration of LPS in mice showed a profound increase in IL-1β, TNF-α and MCP-1 level in serum and vital organs such as the liver and brain. Kupffer cells in the liver secrete a major part of IL-1β and release it into the blood circulation. These cytokines cross BBB and elicit an inflammatory response at the CNS level. It was also reported that endogenous leukaemia inhibitory factor (LIF) has a protective role in endotoxemic shock. It was noted that *i.p* exposure of LPS significantly increased the serum concentration of TNF-α and IL-6 in mice where the concentration of IL-10 was diminished considerably. These effects were prominent in mice lacks LIF compared to the wild type. Unresolved increase in TNF-α, IL-1β, IL-6 *etc.,* may result in endotoxemic shock and death (Weber *et al.,* 2005).

Anti-inflammatory cytokines or other molecules, which suppress the production and action of pyrogenic cytokines, can attenuate fever and febrile reactions following endotoxin challenge. LPS administration is a stimulator of IL-1β and eicosanoids production from immune cells, peripheral organs and the brain. Ledeboer *et al.,* 2002 found significant elevation of IL-1β following LPS administration in rat blood, liver, spleen and pituitary gland. In addition to IL-1β, a positive correlation of hyperthermic reaction and elevation of IL-6 and TNF-α was found in rats, that underwent LPS challenge. It was noted that peripheral administration of anti-inflammatory cytokine IL-10 markedly attenuated the production of pyrogenic cytokines. IL-10 mediated attenuation of fever is found to mediate by interfering with the humoral and neural signalling of IL-1β and other pyrogenic cytokines to the brain.

Reports indicate that an acute inflammatory reaction initiated by LPS is progressed quickly in mice and resolve within 72h. LPS administration induced a rapid rise in serum TNF-α and IL-6, which play a pivotal role in liver dysfunction (Seemann *et al.,* 2017). Correlation with our findings, it was reported that TNF-α plays a role in dyshomeostasis of glucose metabolism following LPS challenge (Raetzsch *et al.,* 2009). Inflammatory cytokines also have a role in mitochondrial activity and the associated generation of ROS inside the cells. LPS is a potent activator of systemic inflammation, which induces cytokines, oxidative stress, immune cell infiltration, and apoptotic death in vital organs.

CHAPTER 6: SUMMARY AND CONCLUSION

6. SUMMARY AND CONCLUSION

6.1. SUMMARY

The human immune system is well-organized consists of cells, tissues and organs. The immune system acts as the guardian of an organism that fights against all sorts of foreign invaders and injury. The wide network of immune cells, antibodies, complement system, inflammatory mediators and organs preconfigured to protect the body from harmful stimuli. The immediate response to a foreign substance begins with a series of inflammatory reactions. Pro-inflammatory cytokines act as a mediator of inflammation and associated cellular activation. Various classes of cytokines and chemokines are secreted from different type of immune cells and they work in a redundant, pleiotropic, synergetic or antagonistic way. The study of action and production of cytokines in response to an external signal thus helps to analyze the health status of an individual. Because of the extensive applicability, researchers are focusing on cytokines and their receptors as targets for immunotherapy to treat deadly diseases worldwide.

The new era of medical sciences deals with sophisticated diagnostic and therapeutic inventions to ensure the well-being of the human population. It is necessary to ensure the safety of individuals who receive medical care as part of the diagnosis and treatment of disease. Hence, any medical products intended to use in a living system should be free of possible bacterial, biological or chemical contaminations. Any agents that induce fever or febrile reactions in the living system are called pyrogens and their presence in medical products will create mild to severe complications. As a precaution, the regulatory agencies are strictly monitoring the safety of all medical products before their clinical application. The ‘pyrogen free’ clearance is an inevitable criterion for manufacturing and distributing medical products worldwide.

For the past fifty years, the detection of pyrogenic contamination in medical products are employed through rabbit pyrogen test and is considered as the gold standard for pyrogen analysis. While considering animal ethics and laborious procedures, research focused on developing an *in vitro* system to detect pyrogens. As a result, LAL test was invented in the 1980s and replaced 80% of animal use for RPT. Even though LAL test is an excellent alternate to RPT,

it has a lot of limitations. LAL test is highly sensitive to detect Gram-negative bacterial endotoxins but was not suitable for Gram-positive bacterial toxins, herbal and chemical products.

The *in vitro* system consider to serve best for human pyrogen response is the monocyte activation test, where the test system is the human whole blood or blood cells isolated from human blood. Monocytes and macrophages are the cells that respond vigorously to exogenous pyrogens. In the *in vitro* pyrogen test, the whole blood collected from healthy volunteers mimic the *in vivo* immune environment of the human system so that the response against external pyrogen will be similar to that of an individual. All the cellular and non-cellular components of blood collected from volunteers are distributed in their natural environment; they react specifically and sensitively with exogenous pyrogens and release endogenous pyrogens. Detection of endogenous pyrogen, especially IL-1β, provides insight into the potential pyrogenic contamination in a sample. The present study successfully detected IL-1β released from pooled human blood following challenge with bacterial (both Gram-negative and Gram-positive), biological and chemical pyrogens including LPS, LTA, PHA and TNP.

Assessment of direct cellular and molecular toxicity of different classes of pyrogens was carried out using human monocytic leukemic cells, namely THP-1 cells. Differentiation, cellular organelle function, cytoskeletal arrangement, morphology changes, ROS production, lysosomal stability, MMP, mechanism of cell death, cytokine gene expression, NFκB and COX2 activation were investigated by various toxicity assays. Pyrogenic and immunologic response of endotoxin was studied by intravenous administration of LPS to rabbits. All the animals were assessed for rise in body temperature, haematological and biochemical changes before and after exposure to endotoxin. Expression of cytokine gene was analysed in PBMC isolated from rabbit blood and serum concentration of functionally active cytokine was estimated using ELISA.

6.2. METHODOLOGY ADAPTED FOR THE STUDY

- Immunization of rabbits using recombinant human IL-1β
- Collection of venous blood from rabbit after booster doses
- Purification of anti-human IL-1β from rabbit serum using Affinity Chromatography
- Dialysis of purified fraction of IgG against PBS for overnight at 4^{o}C
- Estimation of protein concentration by Micro-Bradford assay
- Conjugation of horseradish peroxidase enzyme on anti-human IL-1β antibody
- Coating of ELISA plate with anti-human IL-1β antibody
- Collection of blood from human volunteers and analysis of haematological and biochemical parameters
- Induction of IL-1β release from pooled human blood using LPS, LTA, PHA and TNP
- Detection of the time-dependent release of IL-1β using indigenously developed ELISA
- THP-1 cells were cultured in RPMI medium with 10% FBS and differentiated to MDM cells using PMA
- Cellular interaction of LPS, LTA, PHA and TNP was analysed using various assays, including
    - Differentiation of THP-1 to MDM cells: Phase contrast microscopy, ICC, FACS
    - Organelle function: MTT assay, NRU assay
    - Morphology: Giemsa staining
    - Cytoskeletal integrity: Rhodamine phalloidine staining
    - Free radical generation: DCFHDA, Griess assay
    - Mitochondrial membrane integrity: JC1 staining, DilC1(5) FACS analysis

- Lysosomal membrane integrity: Acridine orange staining
- Cell membrane integrity: Calcein AM-PI, LDH release assay
- Apoptotic and necrotic cell death: Annexin V-PI FACS analysis
- Immunotoxicity: NFκB and COX2 activation by FACS, ICC analysis of NFκB
- Gene Expression: qRT-PCR

❖ Pyrogenic and immune response was studied by administration of LPS to Newzealand White rabbits.

❖ At the end of the observation period, blood was collected from each animal and were subjected to haematological and biochemical analysis.

❖ Serum separated from blood was analyzed for cytokine IL-1 (α and β) and TNF-α

❖ PBMC isolated from blood and was subjected to gene expression studies.

6.3. MAJOR FINDINGS OF THE STUDY

➢ A single peak of IgG was observed in affinity chromatographic purification of serum collected from immunized rabbits

➢ The required concentration of protein was obtained to prepare antibody-HRP conjugate and ELISA plate coating

➢ Haematological and biochemical parameters of all volunteers were in the normal range

➢ Dose and time-dependent release of IL-1β were observed using indigenously developed ELISA following LPS, LTA, PHA and TNP, mediated stimulation of pooled blood.

➢ LPS at selected concentration do not induce mitochondrial and lysosomal dysfunction, oxidative stress and apoptotic cell death of THP-1/MDM cells.

➢ LTA, PHA and TNP exhibited dose and time-dependent toxicity to THP-1 cells

- LTA, PHA and TNP caused the morphological change, actin destabilization, mitochondrial and lysosomal dysfunction, oxidative stress, mitochondrial and lysosomal hyperpolarization, apoptotic and necrotic cell death.
- LPS, LTA, PHA and TNP, caused an immune response in THP-1 cells with increased expression and activation of NFκB and COX2.
- LPS, LTA, PHA and TNP induced expression of inflammation-related cytokine genes in THP-1 cells
- Single exposure of LPS induced a significant rise in body temperature of rabbits within 30min.
- Change in haematological and biochemical parameters are observed
- LPS exposure resulted in the release of inflammatory cytokines in rabbit blood
- LPS exposure enhanced the expression of inflammation-related genes in PBMC isolated from rabbit blood.

6.4. CONCLUSION

The dose-dependent release of pyrogenic cytokine IL-1β was observed with pooled human blood on challenge with LPS, LTA, PHA and TNP. Pooled human blood served as an alternate test medium for detecting various classes of pyrogens with high sensitivity. IL-1β released from immune cells was detected using indigenously developed ELISA after LPS, LTA, PHA and TNP exposure. The kit was suitable for detecting pictogram level of IL-1β present in the supernatant of pooled blood reaction. Molecular toxicity analysis using the human monocytic cell line model THP-1 shown a non-cytotoxic effect of LPS at a selected concentration where it induces immune activation even at a lower concentration. However, LTA, PHA and TNP showed dose and time-dependent toxicity to THP-1 cells. LTA, PHA and TNP induced loss of mitochondrial and lysosomal activity, membrane destabilization, morphological change, actin destabilization and oxidative stress in a dose and time-dependent manner. An increase in expression of inflammation-related genes including NFκB, COX2, IL-1β and TNF-α along with activation of NFκB and COX2 in THP-1 cells following exposure with LPS, LTA, PHA and TNP confirms potential immunotoxicity. LPS do not induce apoptotic or necrotic cell death. Exposure to the highest concentration of LTA and TNP induce apoptotic cell death, where PHA caused necrotic cell death. Administration of LPS induced pyrogenic response in rabbits within 30min and showed an increase in body temperature of 1.42±0.69^{o}C. Release and expression of inflammatory cytokines were enhanced following LPS administration indicate potential immune activation of endotoxin in rabbits.

6.5. FUTURE PERSPECTIVES

- Isolation of lymphocytes from pooled human blood and to detect the release of IL-1β on challenge with LPS, LTA, PHA and TNP
- Isolation of lymphocytes from pooled blood and cryopreserve to detect the release of IL-1β
- Gene expression of PBMC isolated from pooled blood on challenge with LPS, LTA, PHA and TNP

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ANNEXURE

LIST OF PUBLICATIONS

1. **Prajitha N**, Athira SS, Mohanan PV (2018) Pyrogens, a polypeptide produces fever by metabolic changes in hypothalamus: mechanisms and detections. Immunology letters 204,38-46, 2018

2. **Prajitha N**, Athira SS, Mohanan PV (2018) Bio-interactions and risks of engineered nanoparticles. Environmental Research 172 ,98–108

3. Biby T E, **Prajitha N**, Mohanan PV (2018) Cytoskeletal synchronization of CHO cells with polymer functionalized Fullerene C60. Biointerphases 14(2)

4. Athira SS, **Prajitha N**, Mohanan PV (2018) Interaction of nanoparticles with central nervous system and its consequences. American Journal of Research in Medical Sciences 4(1) 12–32
5. **Prajitha N,** Athira SS, Mohanan PV (2019) Comprehensive biology of antipyretic pathways. Cytokines 116,120–127

6. **Prajitha N**, Athira SS, Mohanan PV (2019) Zinc Oxide Nanoparticles Enhance Oxidative Stress in CHO Cells. Reactive Oxygen Species 8(24):341–357

7. Biby TE, **Prajitha N**, Sakthikumar D, Maekawa T, Mohanan PV (2019) Cellular toxicity on C6 Glial cells induced by dextran stabilized fullerene C60. Brain Research Bulletin. Nov 28.

8. Anju S, **Prajitha N**, Sukanya VS, Mohanan PV (2020) Complicity of degradable polymers in health-care applications. Materials Today Chemistry. (16):-100236

9. **Prajitha N**, Mohanan PV (2021) Cellular and Immunological Response of THP-1 Cells in Response to Lipopolysaccharides and Lipoteichoic Acid Exposure. Biomedical Research and Therapy. 8(9):4562-82

10. Joseph T, Sreejith S, Joseph X, Sangeetha V.P, **Prajitha N**, Vandana U, Jayaprakas C.A, Mohanan P.V (2021) Effect of cyanide ions (CN-) extracted from cassava (Manihotesculenta Crantz) on Alveolar Epithelial Cells (A549 cells). Toxicology,

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11. **Prajitha N**, Mohanan PV (2021) Intracellular inflammatory signalling cascades in human monocytic cells on challenge with Phytohemagglutinin and 2, 4, 6, -Trinitrophenol. Molecular and Cellular Biochemistry (Press)

12. **Prajitha N** , Megha KB , Mohanan P V (2022) Assessment of inflammatory response on pyrogenic induction by in vitro and in vivo methods. Biointerface Research in Applied Chemistry. 12 (6), 7668-7684

ONLINE PUBLICATIONS

13. **Prajitha N**, Mohanan P V (2019) Surface Stabilization Assures Safety Of Buckminster Fullerene, Science Trends, June 19,2019

14. Athira SS, **Prajitha N**, Mohanan PV (2019) Can nanoparticles form an Alliance with Central Nervous System, Science Trends, April 29, 2019

15. Sukanya VS, **Prajitha N**, Mohanan PV (2018) Stem cells accept the degradation products of Poly (ε-caprolactone) Atlas of Science, October 18, 2018

BOOK CHAPTERS

16. Anju S., **Prajitha N**., Reshma V.G., Mohanan P.V. (2020) Black Phosphorous Quantum Dots. In: Inamuddin, Boddula R., Asiri A. (eds) Black Phosphorus. Engineering Materials. Springer, Cham

PRESENTATIONS AT THE CONFERENCES

1. **Prajitha N,** Mohanan PV (2021) 'Cytotoxic potential of lipopolysaccharide, lipoteichoic acid, Phytohemagglutinin and 2,4,6-trinitrophenol using human monocytic leukaemia cells' (Prajitha N, Mohanan PV) at online conference STOX-2021, Pharmacology & Toxicology in view of Covid-19 on January 2021, jointly organized by PGIMER Chandigarh and SCTIMST, Trivandrum
2. **Prajitha N,** Mohanan P.V (2019) 'Zinc Oxide nanoparticle: Toxic impacts on Chinese Hamster Ovary Cells'. Presented at the International Conference on Emerging Advancement in Science & Technology (IC EAST 2019) at Manekshaw Centre, Delhi, India jointly organised by Indian JSPS Alumni Association (IJAA) and Solid State Physics Laboratory (SSPL), DRDO, Delhi during 5-6th September 2019.
3. **Prajitha N**, Athira SS, Syama S, Mohanan PV (2018) 'Cytotoxicity analysis of Graphene Oxide nanoparticles using CHO cell lines'. Presented at the International Conference on the Role of Toxicology in Public Health & 38th Annual Conference of Society of Toxicology, India, Sri Balaji Vidyapeeth, Puducherry during 12-14 December 2018
4. **Prajitha N,** Athira SS, Mohanan P V (2018) 'Determination of ZnO Nanoparticles Induced Toxicity to Chinese Hamster Ovary (CHO) Cell Lines'. Presented at 28th Swadeshi Science Congress, 7-9, November 2018, National Institute for Interdisciplinary Sciences and Technology, Thiruvananthapuram, Kerala, India
5. Athira SS, **Prajitha N**, Syama S, Mohanan PV (2018) 'Consequences of graphene oxide nanoparticles in HEK293 cells'. Presented at the International Conference on the Role of Toxicology in Public Health & 38th Annual Conference of Society of Toxicology, India, Sri Balaji Vidyapeeth, Puducherry during 12-14 December 2018
6. Biby T Edwin, **Prajitha N**, Mohanan PV (2018) 'Toxicity of dextran stabilized Buckminster fullerene in C6 glial cells'. Presented at the International Conference on the Role of Toxicology in Public Health & 38th Annual Conference of Society of Toxicology, India, Sri Balaji Vidyapeeth, Puducherry during 12-14 December 2018
7. Athira SS, **Prajitha N**, Mohanan PV (2018) 'Adverse effect of graphene oxide nanoparticles in HEK293 cells'. Presented at 28th Swadeshi Science Congress, 7-9,

November 2018, National Institute for Interdisciplinary Sciences and Technology, Thiruvananthapuram, Kerala, India

WORKSHOPS ATTENDED

1. Workshop on Image Analysis in Biomedical Research"12- September-2018 at SCTIMST
2. Participated in "Hands-On Workshop on Flow cytometry" scheduled from 5th to 7th December 2019 at SCTIMST.

APPENDICES

A.1. BUFFER PREPARATION FOR AFFINITY CHROMATOGRAPHY

Binding buffer: 20mM sodium phosphate buffer (pH 7.0)

Mix 423ml of **Solution A** and 577 ml of **Solution B.** pH of the solution was adjusted to 7.0 and stored at 4^{o}C.

1. **Solution A: 1M $NaH_2PO_4.H2O$:** Dissolve 138g of NaH_2PO_4 in 1000ml deionized water
2. **Solution B: 1M Na_2HPO_4:** Dissolve 142g of Na_2HPO_4 in 1000ml deionized water

Elution buffer: 0.1M Citric acid (pH 3.0)

Dissolve 10.507g citric acid in 500ml deionized water, adjust the pH to 3.0 and filter sterilize. Store at 4^{o}C.

Neutralizing buffer: 1M Tris-Cl (pH.9.0)

Dissolve 6.057g of Tris-Cl in 50ml deionized water, adjust the pH to 9.0 and filter sterilize.

A.2. BUFFER PREPARATION FOR ELISA PLATE DEVELOPMENT

Coating buffer: 50mM Carbonate-bicarbonate buffer (pH 9.6)

Dissolve 1.59g Na_2CO_3 and 2.93g $NaHCO_3$ in 1000ml deionized water. Adjust the pH to 9.6. Autoclave and store at 4^{o}C

1xPhosphate Buffered Saline (PBS): Dissolve 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 in 800ml deionized water. Adjust the pH to 7.4 using HCl and make up the volume to 1000ml using deionized water. Store at 4^{o}C

Blocking buffer: 1% BSA in 1x PBS

Dissolve 1g BSA in 1000ml 1xPBS, Store at 4^{o}C.