EFFECT OF PMA ON AUTOPHAGY IN HUMAN MONOCYTE CELL-LINE, THP-1

THESIS SUBMITTED TO

NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA

FOR THE PARTIAL FULFILMENT

OF THE MASTER OF SCIENCE DEGREE IN LIFE SCIENCE

Submitted by
ASSIRBAD BEHURA
ROLL NO – 413LS2024

Under the guidance of
Dr. ROHAN DHIMAN
ASSISTANT PROFESSOR

DEPARTMENT OF LIFE SCIENCE
NATIONAL INSTITUTE OF TECHNOLOGY
ROURKELA-769008, ODISHA, INDIA

DEPARTMENT OF LIFE SCIENCE
NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA-769008

Dr. Rohan Dhiman
Assistant Professor.

Ref. No.

Date: 11/5/15

CERTIFICATE

This is to certify that the thesis entitled **"EFFECT OF PMA ON AUTOPHAGY IN HUMAN MONOCYTE CELL LINE, THP-1"** which is being submitted by **Assirbad Behura**, Roll No. **413LS2024**, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by him under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

Dr. Rohan Dhiman
Assistant Professor,
Department of Life Science
National Institute of Technology
Rourkela – 769008, Odisha, India.

Phone no: 91-661- 2462780

Email: dhimanr@nitrkl.ac.in

ACKNOWLEDGEMENT

This project is by far the most significant accomplishment in my life and it would have been impossible without people who supported me and believed in my calibre.

I would like to extend my gratitude and sincere thanks to my honourable supervisor **Dr. Rohan Dhiman,** Assistant Professor, Department of Life Science. He is not only a great lecturer with deep vision but also most importantly a kind person. I sincerely thank him for his exemplary guidance and encouragement. His trust and support inspired me in the most important moments of making right decisions and I am glad to work under his supervision.

I express my sincere thanks to Head of the Dept. **Dr. S. K. Bhutia**, and all other faculties of Department of Life Science, NIT Rourkela for showing sustained interest and providing help throughout the period of my work.

I express my heartfelt thanks to **Miss Shradha Mawatwal**, PhD scholar, Department of life science, for her active cooperation and sincere help.

I am genuinely appreciative of my friends **Uttam Chetan Muni, Pratibha Kumari, Subhashree Priyadarshini** and my entire batch mates for their suggestions and moral support during my work.

Last, but not the least, I would thank the Almighty and my parents, whose dedicated and untiring efforts towards me has brought me at this stage of my life.

Assirbad Behura
413LS2024

DECLARATION

I do hereby declare that the Project Work entitled "**Effect of PMA on Autophagy in Human Monocyte Cell line, THP-1"**, submitted to the Department of Life Science, National Institute of Technology, Rourkela is a faithful record of bonafied and original research work carried out by me under the guidance and supervision of Dr. Rohan Dhiman, Asst Professor Department of Life Science, NIT Rourkela

Date:

Place: **Assirbad Behura**

CONTENTS

LIST OF FIGURES

List of abbreviations

7-IFN- 7 interferon

Atg- Autophagy genes

cAMP- Cyclic AMP

CLR- C-type lectin receptors

DAG- Diacylglycerol

IL-1- Interleukin 1

LPS- Lipopolysaccharide

M.tb- Mycobacterium tuberculosis

MAP1LC3- Microtubule associated protein 1 Light Chain 3

MDR TB- Multi Drug Resistant TB

MR- Mannose Receptor

NLRs- Nod like receptors

PGE_2-Prostaglandin E2

PI3K- Phospho inositole 3 kinase

PKC- Protein Kinase C

PMA-Phorbol 12-Myristate 13-Acetate

RNI- Reactive Nitrogen Intermediates

ROI- Reactive oxygen intermediate

STING- Stimulator for Interferon Genes

TB- Tuberculosis

Th1- T helper- 1 type

TLR- Toll like receptors

TNF-α-tumour necrosis factor-a

XDR TB- Extensively drug resistant TB

1. ABSTRACT

THP-1 cells are human monocyte like cell line that is blocked at certain steps of the differentiation process and its differentiation into macrophages can be induced by addition of PMA. PMA activates Protein Kinase C (PKC) that mimics the physiological activator diacylglycerol (DAG) thus differentiation into macrophage occurs. These cells mimic the alveolar macrophages. So, is a good model to study tuberculosis. According to W.H.O. around 1.3 million people die of this disease every year. *Mycobacterium tuberculosis* the causative organism of this disease enters into the body through the airway passages. The alveolar macrophages phagocyte the bacteria. Once inside they escape degradation by preventing the phagosomes-lysosome fusion. Thus, they survive inside the phagosomes by inhibiting autophagy. To curtail this bacterial load, various host defence mechanisms like apoptosis, reactive oxygen and nitrogen species, phagosome-lysosome fusion and autophagy play a very important role. Autophagy in the cells is induced by ATP, vitamin D, cytokines. ATP is known to increase the intracellular calcium level that facilitates autophagy. Calcimycin, an important calcium ionophore increases the intracellular calcium level thus facilitates autophagy. Since autophagy plays an important role in curtailing bacterial load so we attempted to study the role of PMA in inducing autophagy in THP-1 cells.

Keywords: THP-1, PMA, Autophagy, Tuberculosis, Macrophages, Phagosome-lysosome

2. INTRODUCTION:-

THP-1 cells are human monocyte leukemia cells that were obtained from a one year old leukaemia patient (Tsuchiya et al., 1980). The differentiation of these cells is obstructed at specific steps and the differentiation of these cells can only be brought about by several stimuli that are available (Collins, 1987; Koeffler, 1988). They are morphologically similar, express the same membrane antigens, have similar secretory products with human monocytes. These cells express complement, Fc receptors, lack surface and cytoplasmic immunoglobulin and are phagocytic in nature (Auwerx, 1990). They are not responsive towards TLR antagonists in their undifferentiated stage but become highly responsive upon getting differentiated. This is one of the broadly used cell line to study the regulation of monocytes and macrophages, upon discovery 35 years back.

The differentiation of THP-1 monocytic cells into macrophages is mediated by Phorbol 12 Myristate 13-Acetate (PMA). This is a type of phorbol easter that mimics the activity of diacylglycerol (DAG). Thus they artificially activate Protein Kinase C (PKC). On activation of PKC monocyte to macrophage transition occurs. This differentiation occurs due to the changes in the expression of PKC isozymes. Upon differentiation they actively mimic the human alveolar macrophages thus, are a good model system for studying tuberculosis.

Tuberculosis (TB) is the single largest infectious disease causing nearly 1.3 million deaths per year (WHO, 2013). This is caused by the pathogen *Mycobacterium tuberculosis* (*M. tb).* The uniqueness of this pathogen is that it is able to survive *in vivo* for years before the activation/reactivation of the disease occurs. Although a lot of people are exposed to this pathogen, but the disease occurs only in few of them. This is because of a compromised immune system. The pathogen enters into the body through the airway passages i.e. upon inhaling aerosol droplets containing *M. tb.* When a person gets infected, the macrophages, dendritic cells and lymphocytes are recruited to the site of infection. The accumulation of these cells at the site of infection leads to the formation of granuloma (Ulrichs and Kaufmann, 2006). On formation of granuloma, the occurrence of active infection is prevented in most healthy individuals, but the infection is not completely eradicated (Saunders et al., 1999). This phase is called the phase of latent TB where the host does not shows any symptoms of infection but they still have *M. tb* inside their body that can reactivate into its active form upon compromise of the host immune defence. Thus, complete eradication of the pathogen from the body is very difficult and the appearance of the Multidrug resistant (MDR)

and Extensively drug resistant (XDR) TB has further aggravated the problem. To curtail this bacterial load, various host defence mechanisms like apoptosis, reactive oxygen and nitrogen species, phagosome-lysosome fusion and autophagy play a very important role.

Autophagy is predominantly a cytoprotective process (Kroemer and Levine, 2008). In this particular cytoplasmic molecules are targeted and are separated from the rest of the cytoplasm by development of a double membrane structure around them. This double membraned structure called autophagosome fuse with lysosome on maturation. This leads to the degradation of the sequestered molecules. Autophagy helps in generating substrates for energy metabolism on nutrient scarcity. This is done by non-selective consumption of cellular components (Deretic and Levine, 2009). Autophagy helps in the elimination of the intracellular microbes that enters cytosol. Some intracellular pathogens like *M. tb* avoid degradation by the host by preventing autophagosome lysosome fusion.

As THP-1 cells are monocytes, PMA treatment is necessary for the differentiation of these cells. Differentiation of the cells increases the phagocytic index ultimately leading to efficient internalization of mycobacteria. Since autophagy plays an important role in curtailing bacterial load so we attempted to study the role of PMA in inducing autophagy in THP-1 cells.

3. REVIEW OF LITERATURE:-

3.1. PMA

On treatment of the THP-1 cells with PMA, the proliferation of the cells stop and they start to differentiate into macrophages. This leads to the alternation of the cell morphology as they acquire a variety of cell shapes, many phagocytic vacuoles arise in their cytoplasm and the nucleus becomes more irregular. Upon differentiation the nucleus to cytoplasm ratio decreases as the cytoplasmic volume increases (Sokol et al., 1987). Thus, due to the above mentioned changes that takes place the cells shows adherence property, as they stick to the surface of the culture flask. The flow cytometry results confirmed that the cell volume decreases as these cells show a decrease in the forward scattering of the light. In macrophages the number of membrane bound organelles increases, thus the granularity increases (Sokol et al., 1987; Kradin et al., 1986). Its molecular formula of PMA is $C_{36}H_{56}O_8$ (Figure 1). PMA is an artificial stimulant of macrophages. Vitamin D3 (VD3) that is known to be a natural stimulant of macrophages induces only partial differentiation (Auwerx, et al., 1990).

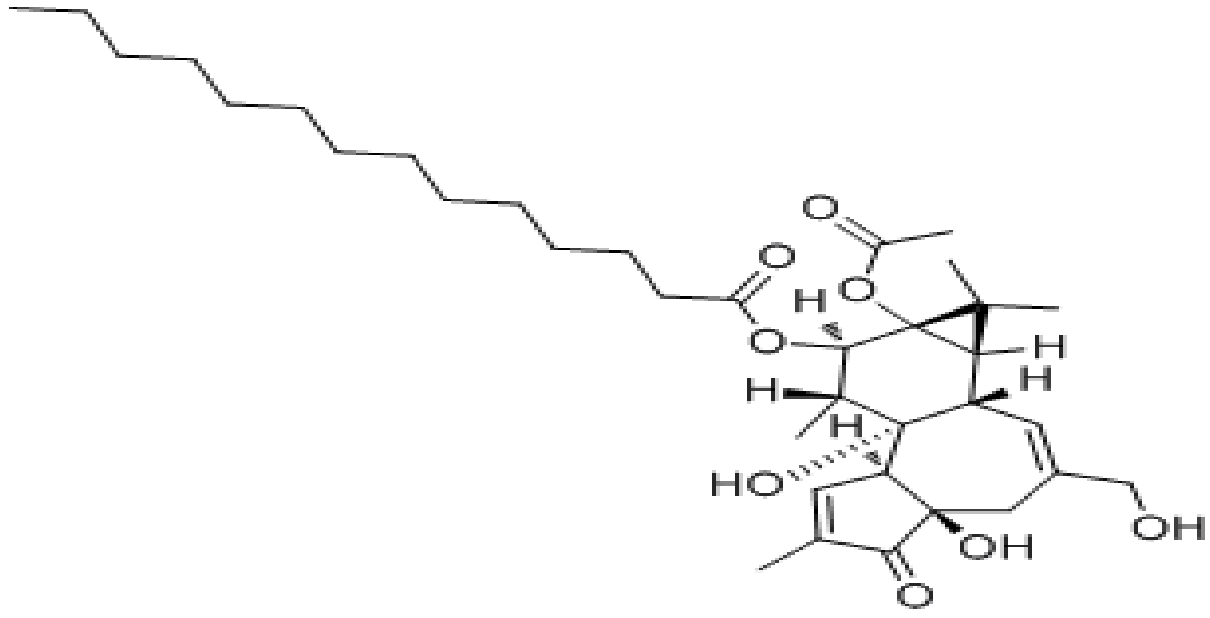

Figure 1: **The structure of PMA** that differentiates THP-1 cells into macrophages. (Adapted from: www.wikipedia.com)

The differentiation of THP-1 cells is induced by both PMA and VD3, by an increase in release of oxygen free radical and an enhanced phagocytic activity. In case of VD3 induced differentiation, the cells do not adhere to the surface, they don't lose their proliferation

capability, don't release Prostaglandin E2 (PGE_2) and release very small amount of tumour necrosis factor-α (TNF-α). Whereas in contrast to VD3, the PMA differentiated cells adhere to the surface, stop proliferating and produce a lot of PGE_2 and TNF-α. The PMA differentiated cells has an enhanced expression of CD11 while CD14 was more expressed in the cells differentiated by VD3 (Schwende et al., 1996).

Not a single cytokine or growth factors that have been tested till date (TNF, IL-2, GM-CSF, IL-1) can induce the differentiation of the monocytes by themselves. But all of them together might mimic the activity of PMA. It has also been reported that the colony formation in THP-1 cells can be inhibited by 7-IFN and TNF (Lubbert and Koeffier, 1988).

3.2. THP-1 (Monocytes and Macrophages)

Various substances like reactive oxygen species, hormones, enzymes etc. are secreted by macrophages and the THP-1 cells are used to study the functions of the various secretions of the macrophages. It has been reported that the several proteins that are secreted from the mature macrophages are also secreted by the THP-1 cells (Johnston, 1988 and Nathan, 1987). The THP-1 cells are mostly used to study the production of peptide hormones and cytokines. The THP-1 cells activate IL-1β as they possess enzymatic activity (Kostura, 1989). The IL-1β mRNA levels in THP-1 cells are induced by both PMA and Lipopolysaccharide (LPS) (Fenton, 1988 and Turner, 1988). It has been reported that the level IL-1 is increased significantly on infection with HIV (Molina et al., 1989).

The THP-1 cells are also used to study the production of apolipoprotein E (apoE) (the lipid binding protein) and lipoprotein lipase (LPL). Various cells synthesize the glycoprotein enzyme LPL that hydrolyzes the core triglycerides into triglyceride-rich lipoproteins to glycerol and free fatty acids (Olivecrona and Olivecrona, 1987). LPL is only produced by the THP-1 cells when they differentiate into macrophage that is on addition of PMA (Sudhof et al., 1987; Auwerx et al., 1988, 1989). Amongst all the human leukemia cell line this property is unique to THP-1 cells.

On monocyte to macrophage transition, various changes in the expression of several genes involved in lipid metabolism occurs. THP-1 cells differentiated by PMA express receptors for acetylated low density lipoproteins (LDL/scavenger receptors), apoE and LPL (Auwerx et al., 1990a, 1990b; Basu et al., 1981) but in macrophages the LDL receptor expression is absent (Figure 2) (Auwerx et al., 1989a, 1989b; Hara et al., 1987).

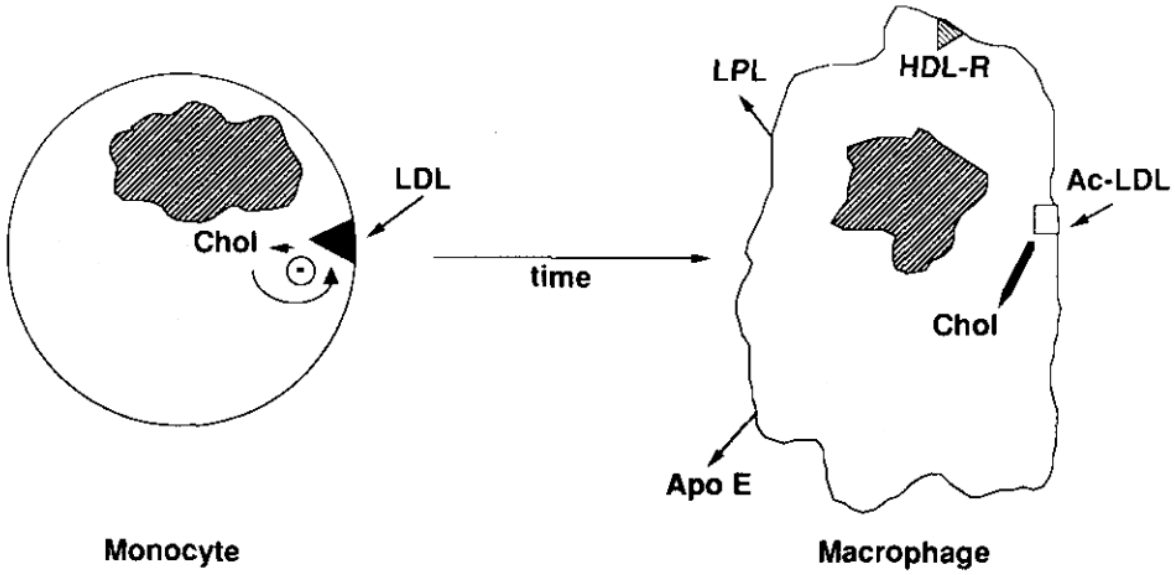

Figure 2: **Differentiation of the Monocytes: -** The expression of genes for lipid metabolism changes on the differentiation of monocytes to macrophages. The expression of receptor for LDL is absent on differentiation of monocytes. The differentiated cells rather possess a receptor for acetyl LDL, HDL, apoE and LPL. (Adapted from: Auwerx, 1991)

On differentiation of the monocytes into macrophages, they acquire the property of accumulating cholesterol inside them. This leads to the formation of foam cells and atherosclerosis. For studying these changes, THP-1 cells are used as a model (Auwerx, 1991).

3.3. Macrophage Receptors Involved in Tuberculosis Pathogenesis

M. tb is detected by the innate immune system only when it binds to the receptors that are available on the surface of macrophages and other myeloid cells. The receptors to which *M. tb* binds are Toll like receptors (TLRs), Nod like receptors (NLRs), C-type lectin receptors (CLRs) and Mannose receptors (MR). After this *M. tb* are engulfed by the macrophages. Inside the phagosomes the pathogen prevents the phagosomes lysosome fusion. Through this way it escapes from the immune system of our body. But when the activation of macrophage occurs, the phagosome and lysosome fusion takes place. Along with that secretion of a variety of cytokines, production of antimicrobial reactive nitrogen intermediates (RNI) and ROI also occurs. This leads to the death of the *M. tb* present inside the body.

CD14, a cell surface glycoprotein induces a signalling cascade on detection of LPS (lipopolysaccharide) by acting as a co-receptor for the TLR-4 (Tavera et al., 2006). In TB, CD14 interacts with many *M. tb* surface components that mediates uptake of the bacteria and

releases the proinflamatory cytokines (Ponta et al., 2003; Drage et al., 2009). Another surface glycoprotein CD44 similarly mediates mycobacterial phagocytosis and induces protective immunity against *M. tb* (Leemans et al., 2003). CD44 is a receptor for glycoprotein and is found in many immune cells where it activates lymphocytes. CD44 plays an important role during the granuloma formation in the lungs infected with *M. tb* (Ponta et al., 2003). Another receptor involved in the uptake of *M. tb* is MR (Mannose receptor). These binds to ManLAM component present on the surface of *M. tb* and initiates the internalization of the pathogen (Schlesinger, 1993).

3.4. Autophagy

Autophagy is a greek word meaning self eating (auto "self" and phagein "to eat"). This word was coined by Dr. Christian de Duve, a Belgian biochemist in 1963. This involves the degradation of unnecessary cellular components by the help of lysosome (Lin et al., 2012). This degradation of cellular components is essential for survival during starvation conditions as it maintains the cellular energy level. Initially the autophagosome formation starts that are double membrane structure and isolate the targeted molecule from the rest of the cytoplasm. The autophagosome mature once they fuse with the lysosome. On fusion the molecules present inside the autophagosome gets degraded due to the acidic environment of lysosome (Patel et al., 2012).

Autophagy is of three different types: macroautophagy, microautophagy and chaperone-mediated autophagy (Peracchio et al., 2012).

Macroautophagy occurs mainly to remove the damaged cell organelles from the cell (Levine et al., 2011). Autophagosome formation takes that acquire the proteases required for degradation of the separated material by fusion with lysosome.

Microautophagy is poorly understood in mammalian cells. Unlike macroautophagy, in microautophagy the lysosomal membrane itself protrudes to sequester the cytosolic components (Russell et al., 2013). The entire cytosolic region constitutes the cargo for microautophagy. This pathway was important for the survival of cells under starvation conditions. It regulates the composition of the lysosomal membrane by degrading the lipids incorporated into the membrane (Li et al., 2012).

Chaperone-mediated autophagy (CMA), is a complex and specific pathway. This requires the unfolding of the protein cargo before it gets internalized into the lysosome. It's very

selective and occurs only for the soluble cytosolic proteins (Levine et al., 2011; Matsushita et al., 2007). This is mostly activated when the cell is under stress. It transfers the protein cargo one after another thus is very different from the other types of autophagy. It only allows certain proteins to cross the lysosomal membrane barrier, thus are characterized as selective (Levine et al., 2011).

It has been seen in neutrophils under different clinical conditions, that they are able to form vacuoles. When neutophils are stimulated with PMA many cytoplasmic vacuoles were observed. This effect of PMA was dose and time dependant. MDC staining was done to identify the nature of the vacuoles formed and it confirmed the existence of acidified autophagosomes. This confirmed the existence of autophagy. On incubation of cells with 3-MA an autophagy inhibitor prior to addition of PMA, the vacuole formation was not seen. The expression of ATG3 and LC3B mRNA increases after the treatment of the neutrophils with PMA (Remijsen et al., 2011).

3.5. Autophagy and *M.tb*

Upon the entry of *M. tb*, macrophages form phagosomes. These phagosomes then fuse with lysosome to form autolysosomes and thus *M. tb* gets degraded.

It has been observed that the *M. tb* growth is reduced in the THP-1 cells when AKT1 and AKT2 are knocked down. SLRs (Sequestasome like receptors) target the microbes to autophagosome (Semba, 1999).

In case of TB infection, the process of autophagy enhances the host resistance by encircling the *M. tb* induced phagosomes maturation arrest (Jagannath et al., 2009; Gutierrez et al., 2004). The immature phagosomes containing *M. tb* are tagged with the autophagy proteins (Atg). First Atg6 i.e. Beclin-1 binds to the phagosomes. With the attachment of belcin-1, many other Atg proteins bind to the membrane making a complex. Finally, the characteristic feature of autophagy i.e. autophagosome formation starts. Then microtubule associated protein 1 light chain 3 (LC3-I) gets degraded and is converted to LC3-II. The LC3-II then binds to the membrane of autophagosome and fusion with lysosome occurs. (Figure-3) (Stromhaug and Klionsky, 2001).

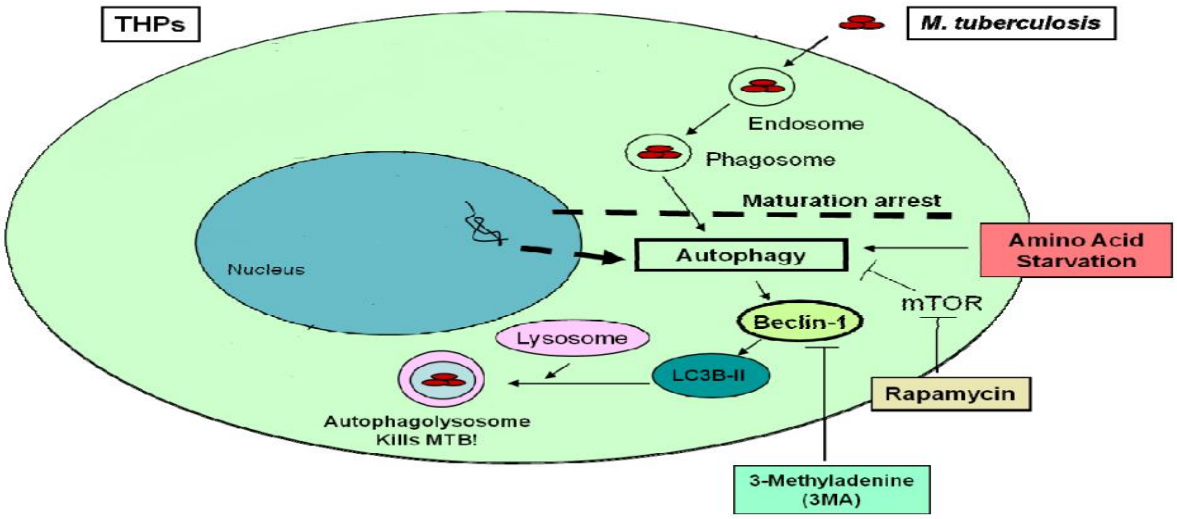

Figure 3: The degradation of *M. tb* due to autophagy (Adapted from: Estrella, 2011)

4. Objective

1. To study the effect of PMA on cell viability of THP-1 cells.
2. To check the effect of PMA on autophagy of PMA differentiated THP-1 cells.
3. To check if sub optimal dose of PMA interferes with the activity of Rapamycin (autophagy inducer).

5. Materials and Methods

5.1. CELL LINES

THP-1 cell line that was used in the study was a kind gift from Dr. Vivek Rai, Institute of Life Science (Bhubaneswar, India). RPMI-1640 growth medium was used to maintain the cells. The contents of the media are; 10% heat inactivated Fetal Bovine Serum (FBS) and antibiotic cocktail like streptomycin and penicillin. The cells were stored at 37°C with 5% of CO_2 .

5.2. CHEMICALS

The chemicals used were of analytical grade and were purchased from commercial sources.

- The cell culture media RPMI-1640 and FBS were purchased from GIBCO (Grand Island, NY)
- The reagents used for detection and analysis during western blotting were purchased from GE healthcare (UK).
- The MTT cell assay kit was purchased from HiMedia Laboratory (Mumbai, India).
- The BCA kit was purchased from Sigma Sigma-Aldrich.
- Following reagents were purchased from HiMedia Laboratory (Mumbai, India): Sodium Chloride (NaCl), Sodium Dodecyl Sulphate (SDS), Acrylamide, Dimethyl Sulfoxide (DMSO), Hydrochloric acid (HCL), Tris base, Glycine, Potassium Chloride (KCl), Potassium Dihydrogen Phosphate (KH_2PO_4), Disodium Phosphate (Na_2HPO_4), Ethylenediaminetetraacetic acid (EDTA), Triton X-100, Glycerol, 2-mercaptoehtanol, Bromophenol Blue, Sodium Hydroxide (NaOH), Phorbol-Myristate-Acetate (PMA) and Bovine Serum Albumin (BSA). Following reagents were purchased from Sigma (St. Louis, Mo, USA): Bisacrylamide, Sodium Deoxycholate. All media and reagents used were endotoxin-free.

5.2.1. CELL CULUTURE MEDIA

Complete RPMI-1640 (with antibiotic)

To prepare complete RPMI-1640 media we need the following chemicals:-

500 ml of RPMI media

50 ml of FBS

5.5 ml of (1X) Penicillin- Streptomycin

To the 500 ml RPMI medium, 50 ml of heat inactivated FBS was added. This was followed by addition of 5.5 ml of Penicillin-Streptomycin. Then this was stored at 4°C by preparing aliquots of 100 ml each.

5.2.2 BUFFER/ SOLUTIONS

10 X Phosphate Buffered Saline (pH 7.4)

To prepare 10X Phosphate Buffer Saline (PBS) we need the following chemicals:-

80 g of NaCl

2 g of KCl

14.4 g of Na_2HPO_4

2.4 g of KH_2PO_4

In 800ml of distilled water, the components listed above were dissolved. Using HCl the pH was adjusted to 7.4. The final volume was adjusted to 1 litre using distilled water.

1 M Tris (pH 6.8 or 7.4 or 8.0 or 9.0)

To prepare 1M Tris we need the following chemicals:-

121.1 g of Tris base

In 800ml of distilled water 121.1g of tris base was dissolved and the pH was adjusted by using HCl. Finally the volume was adjusted to 1 litre by adding distilled water.

Reservoir Buffer 10X (For SDS-PAGE electrophoresis)

To prepare Reservoir Buffer 10X we need the following chemicals:-

3 g of Tris base

14.4 g of Glycine

10 ml of 10% SDS

In 990ml of distilled water the above listed components except 10% SDS were dissolved. And finally 10% SDS was added.

4 X Protein Sample buffer (For SDS-PAGE electrophoresis)

The 4X Protein Sample Buffer was prepared using the following chemicals:-

2.4 ml of Upper Tris (1M, pH-6.8)

0.8 g of SDS

4.0 ml of Glycerol

0.5 ml of β-Mercaptoethanol

4 mg of Bromophenol blue

In 3.3 ml of distilled water the above listed components were added and the final volume was made to 10 ml by adding distilled water. The solution was stored at -20°C after dispencing into aliquots.

Transfer Buffer10X (For Western blotting)

The transfer Buffer 10X was prepared by using the following chemicals:-

10.8 g of Tris base

50.7 g of Glycine

In 250 ml of distilled water the above listed components were dissolved and the final volume was adjusted to 360 ml by adding distilled water. Then 300 ml of methanol was added. The solution was autoclaved.

Acrylamide - bis-Acrylamide mixture

The Acrylamide-bis-Acrylamide mixture was prepared by using the following chemicals:-

30 g of Acrylamide

0.8 g of Bis-Acrylamide

Both the components were dissolved in 70 ml o distilled water. The final volume was adjusted to 100 ml. Then the solution was filtered and was stored at 4°C.

10% Sodium dodecyl sulfate (SDS)

10% SDS was prepared using the following chemicals:-

5 g of SDS

In 40 ml of distilled water 5 g of SDS was dissolved. The volume was then adjusted to 50 ml by adding distilled water.

10% Ammonium Persulfate (APS)

10% APS was prepared using the following chemicals:-

0.1 g of APS

In 1 ml of distilled water 0.1 g of APS was dissolved.

PBS-T (For Western blotting)

PBS-T was prepared using the following chemicals:-

50 ml of 10 X PBS

2.5 ml of Tween-20

450 ml of Distilled water

These were mixed with distilled water.

Lower Tris (4 X) pH 8.8 (For SDS-PAGE electrophoresis)

Lower Tris was prepared by using the following chemicals:-

18.17 g of Tris base

4 ml of 10% SDS

In 80 ml of distilled water tris base was dissolved and the pH was adjusted to 8.8 by using HCl. Finally 4 ml of 10% SDS was added and the final volume was made to 100 ml by adding distilled water.

Upper Tris (4 X) pH 6.8 (For SDS-PAGE electrophoresis)

Upper Tris was prepared by using the following chemicals:-

6.06 g of Tris base

4 ml of 10% SDS

In 25 ml of distilled water, Tris base was dissolved and the pH was adjusted to 6.8 by using HCl. Finally 4 ml of 10% SDS was added and the volume was adjusted to 100 ml by adding distilled water.

RIPA buffer

RIPA buffer was prepared by using the following chemicals:-

1 ml of 1% Triton X – 100

1.5 ml of 150 mMNaCl

0.5 ml of 0.5% Sodium deoxycholate

0.5 ml of 50 mMTris, pH – 8.0

0.1 ml of 0.1 % SDS

0.1 ml of Protease inhibitor cocktail (1X) (PIC)

6.3 ml of Distilled water

All the components except PIC were dissolved in the distilled water and the final volume was adjusted to 9.9 ml by adding distilled water. These were dispensed into aliquots and were stored at -20°C. PIC was freshly added to the buffer when it was used.

5% SKIM MILK (For Western blotting)

Skim Milk was prepared by using the following chemicals:-

5 g of Skim milk

100 ml of PBS-T

In 100 ml of PBS-T 5 g of skim milk was dissolved.

5.3. METHODS

5.3.1. Cell culture

The THP-1 cells were cultured in RPMI-1640 media. The cells were stored at 37°C in presence of 5% CO_2. The media was changed periodically as the cell density increased and the pH of the media became acidic. This was done by centrifuging the media at 1000 rpm for 5 minutes. The supernatant was discarded and the pellet was retained. The pellet was made to dissolve in 5 ml of fresh culture media and was transferred to the culture flask. And the flask was placed back in the CO_2 incubator.

5.3.2. Cell viability

The MTT cell viability kit was used to check the cell viability. The procedure followed was according to the protocol provided by the manufacturer.

2.5×10^4 cells were seeded into 39 wells of 96 well plates. The cells were seeded in triplicates and were incubated for 24 h in an incubator. After 24 h, different concentrations of PMA were added to the wells. The plates were then kept in the incubator for varying time points (24, 48, 72 h). At indicated time points, MTT reagent (10 µl, 5 mg/ml) was added to the wells. The plate was gently swirled to mix the contents and was incubated for 2-4 h at 37°C. Then the formation of Formazan crystals was checked and 100 µl lysis solution was added to all wells except negative control well. The absorbance was taken under 570nm. And the viability graph was plotted according to formula % Cell viability = (OD of Test sample/OD of Control) x 100.

5.3.3. Western blotting

After protein estimation of the cell lysates was done by BCA assay. Electrophoresis (15% SDS-PAGE) was done with 50 µg of protein. The gel was then removed from the electrophoresis unit and was then used in western blotting. The gel was placed carefully in the blotting cassette such that no bubbles were formed. In the blotting cassette sponge/paper/gel/membrane/paper/sponge were arranged accordingly. The nitrocellulose membrane used was treated skim milk to block the non-specific binding site of antibodies. After western blotting the bands got transferred from the gel into the membrane. Then the membrane was probed with anti LC3 antibody (primary antibody) that recognises the presence of LC3-I and LC3-II bands in the membrane. After overnight incubation the

membrane was washed 3 times with PBS-T and secondary antibody conjugated with HRP was added. After 45 minuites the membrane was washed 3 times with PBS-T. The protein bands were visualized using enhanced chemiluminescence kit (Amersham, USA) as per manufacturer's instructions. The membrane was then stripped and was reprobed with anti β-actin antibody following same protocol as above.

Cell lysis

Fresh cell lysis buffer was made by adding 10 µl of PIC to 1 ml of RIPA buffer and was mixed gently. The supernatant of the cultured cells were removed at appropriate time interval and the adhered cells were washed twice with 1X PBS. Then the lysis buffer was added to the cells in the well. The lysis buffer was gently mixed with the cells and the cell lysates were transferred to pre chilled micro centrifuge tubes. Then it was vortexed 3 times at a constant interval of 10 minutes. Then the lysates were centrifuged at 15000 rpm for 15 min at 4°C. The supernatant was made into aliquots and were store at -20°C.

Protein estimation by BSA reagent

First BCA working solution was prepared by mixing 50:1 part of solution A & B. Then 5 µl of BSA standard and the cell lysates were added to each well of a 96 well plate. Then, 200 µl of BCA working solution was added to each well. The plate was incubated at 37° C for 10-15 min till purple colour developed. Then the absorbance was taken at 562 nm. The standard curve for BSA was prepared and from that graph we calculated the concentration of protein present in the unknown sample i.e. cell lysates according to the regression equation.

6. RESULT

6.1. PMA does not kill THP-1 cells at 24, 48 and 72 h of treatment

The THP-1 cells were treated with different concentration of PMA (0.001-1.0µM) for 24, 48 and 72 h. Cell viability stayed the same as in control and did not change even after adding varying concentrations of PMA to the cells. The viability was checked by MTT assay (Figure 4, 5 and 6).

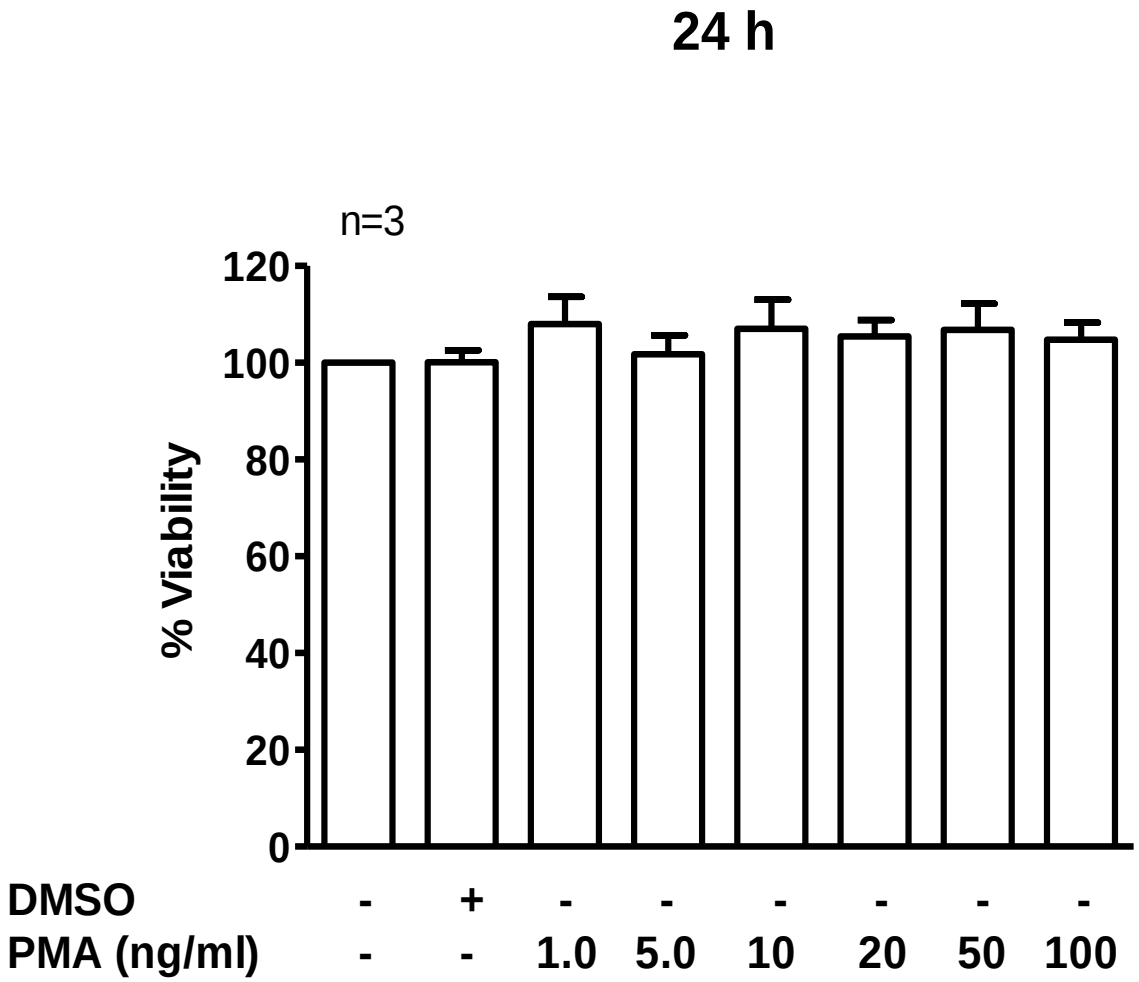

Figure 4: Effect of varying concentration of PMA on cell viability of THP-1 cells after 24 h of treatment. The cytotoxicity was checked by MTT assay as described in materials and methods. THP-1 cells were seeded in triplicate in 96 well plate. Next day, cells were treated with different concentration of PMA as shown in the figure. After 24 h of treatment, MTT was added and cells were incubated for 3 h. After incubation, cell lysis buffer was

added and OD was taken at 562 nm. Data are expressed as percentage cell viability over control cells.

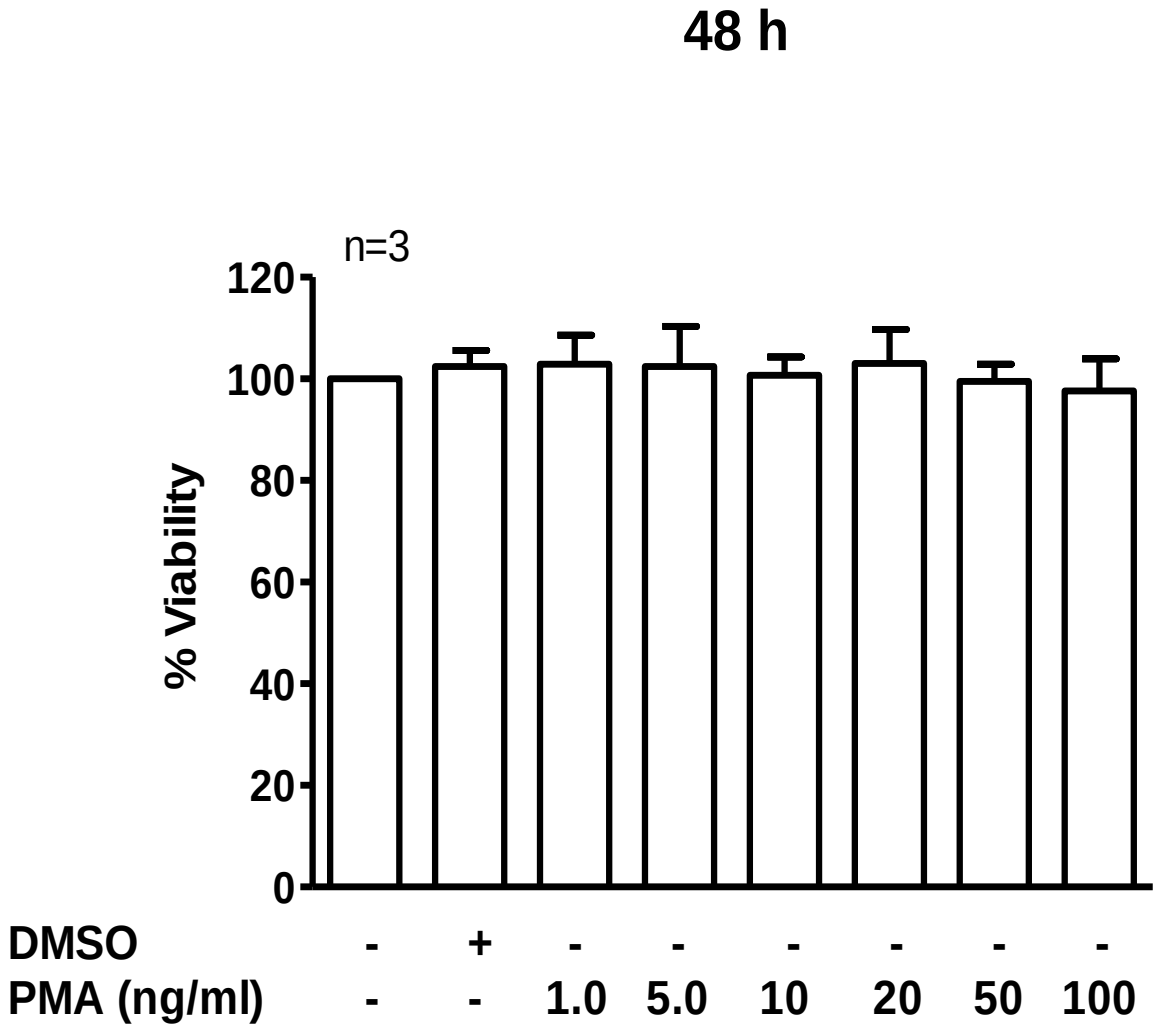

Figure 5: Effect of varying concentration of PMA on cell viability of THP-1 cells after 48 h of treatment. The cytotoxicity was checked by MTT assay as described in materials and methods. THP-1 cells were seeded in triplicate in 96 well plate. Next day, cells were treated with different concentration of PMA as shown in the figure. After 48 h of treatment, MTT was added and cells were incubated for 3 h. After incubation, cell lysis buffer was added and OD was taken at 562 nm. Data are expressed as percentage cell viability over control cells.

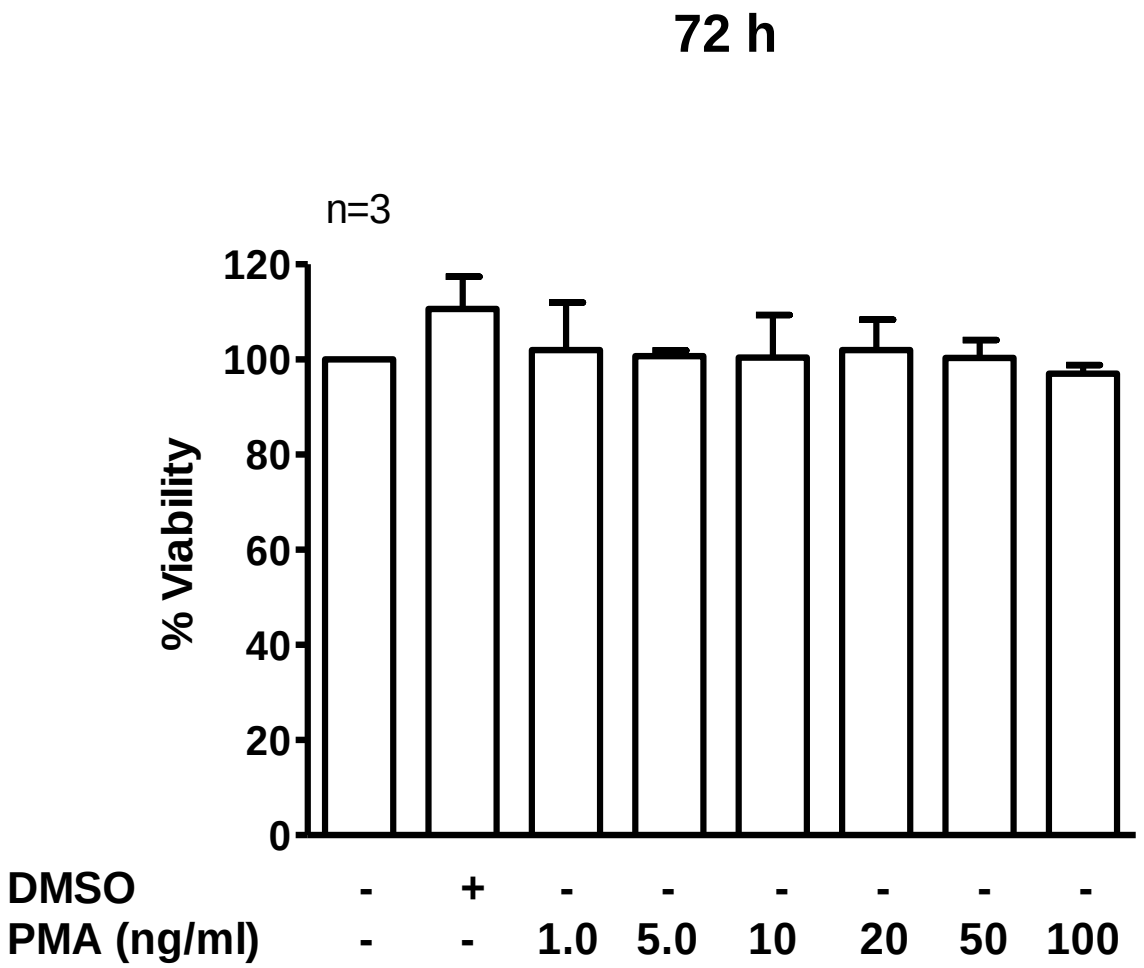

Figure 6: Effect of varying concentration of PMA on cell viability of THP-1 cells after 72 h of treatment. The cytotoxicity was checked by MTT assay as described in materials and methods. THP-1 cells were seeded in triplicate in 96 well plate. Next day cells were treated with different concentration of PMA as shown in the figure after 72 h of treatment, MTT was added and cells were incubated for 3 h. After incubation, cell lysis buffer was added and OD was taken at 562 nm. Data are expressed as percentage cell viability over control cells.

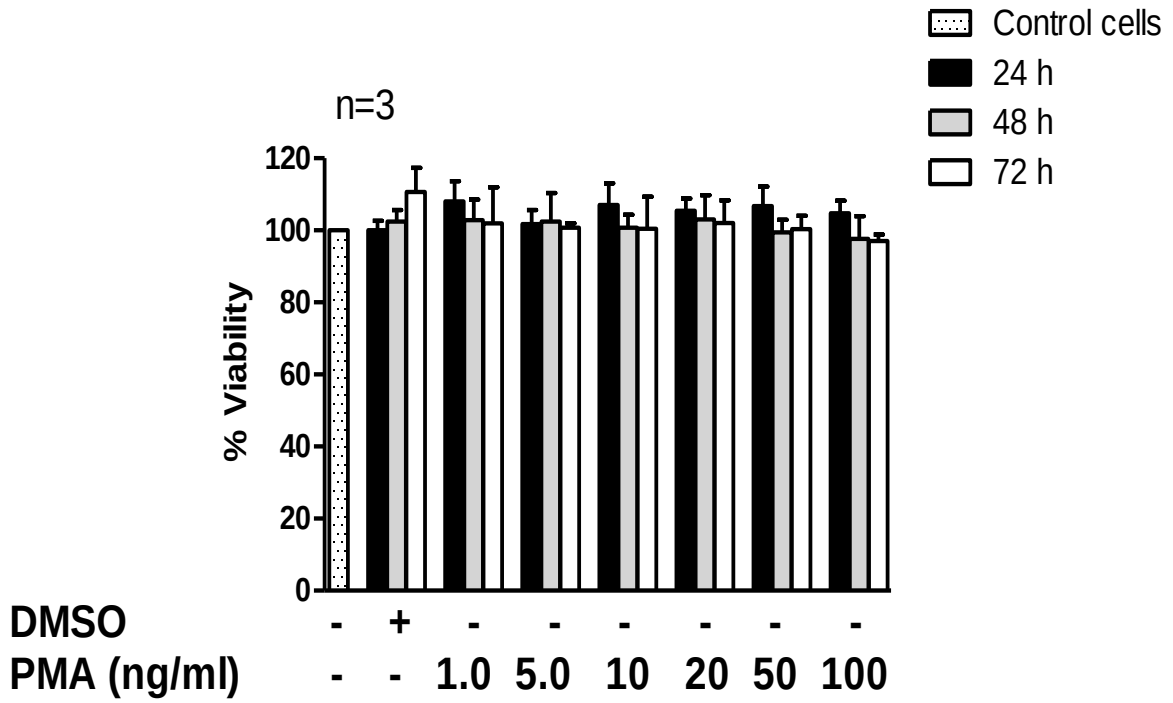

Figure 7: Effect of varying concentration of PMA on cell viability of THP-1 cells after 24, 48 and 72 h of treatment. MTT assay was employed to check the cell viability. Briefly, as written in pervious legend the THP-1 cells were treated with various concentration of PMA for 24, 48 and 72 h. The cumulative % viability at different concentration of PMA are shown here. OD was taken at 562 nm. Data are expressed as percentage cell viability over control cells treatment.

6.2. PMA induces autophagy in THP-1 cells

The effect of PMA on autophagy in differentiated THP-1 cells was studied by western blotting. We found that PMA treatment at the concentration 100 ng/ml and not lower than that leads to accumulation of LC-3-II (an autophagy marker) in THP-1 cells compared to untreated cells. This result suggests that higher concentration of PMA is a potent inducer of autophagy in THP-1 cells (Figure 8 and 9).

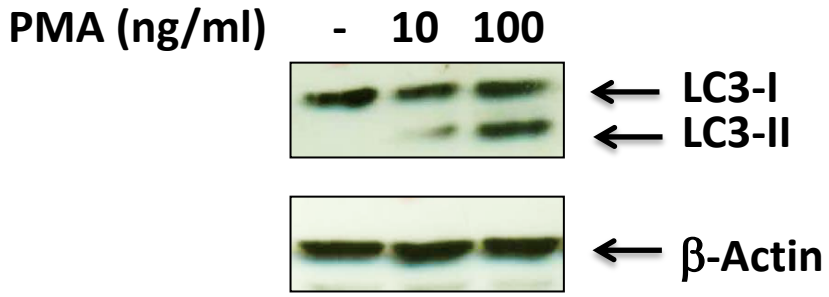

Figure 8: Effect of PMA (10ng/ml and 100ng/ml) on LC-3 degradation in differentiated THP-1 cells after 48 h of treatment. Differentiated THP-1 cells were treated with 10 and 100 ng/ml of PMA. After 48 h, whole cell lysates were prepared as described in materials and methods. Lysates were subjected to western blotting with an antibody LC-3 (upper panel). The blot was stripped and reprobed with an antibody against β- actin (lower panel).

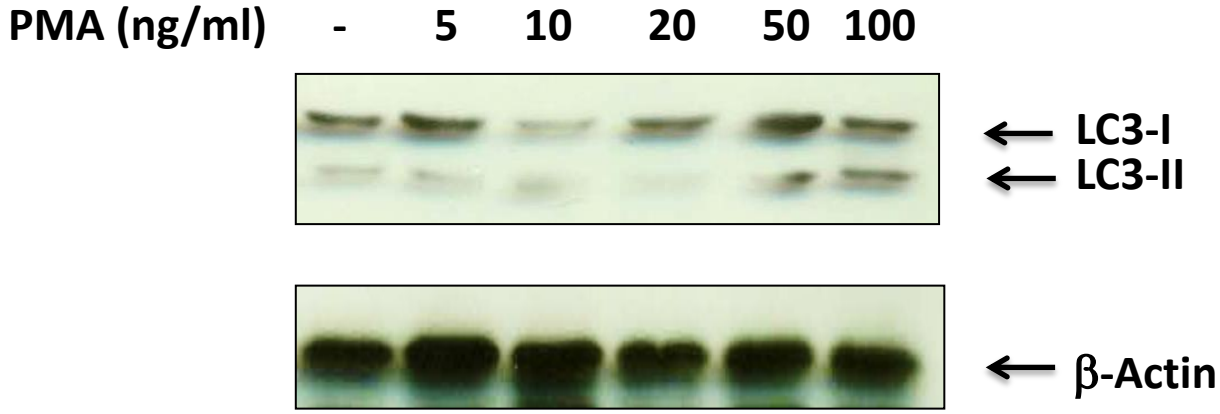

Figure 9: Effect of PMA (0, 5, 10, 20, 50, and 100 ng/ml) on LC-3 degradation in differentiated THP-1 cells after 48 h of treatment. Differentiated THP-1 cells were treated with 10 to 100 ng/ml of PMA. After 48 h, whole cell lysates were prepared, as described in materials and methods. Lysates were subjected to western blotting with an antibody against LC-3 (upper panel). The blot was stripped and reprobed with an antibody against β- actin (lower panel).

6.3. Effect of sub optimal dose of PMA on the activity of Rapamycin

At lower level of PMA, autophagy was not induced and to confirm this, we performed another experiment with PMA and Rapamycin (autophagy inducer). We found that PMA at 50 ng/ml did not induce autophagy but when rapamycin is added with PMA at the same concentration, autophagy is induced. Autophagy is caused due to rapamycin and not PMA as rapamycin is an autophagy inducer.

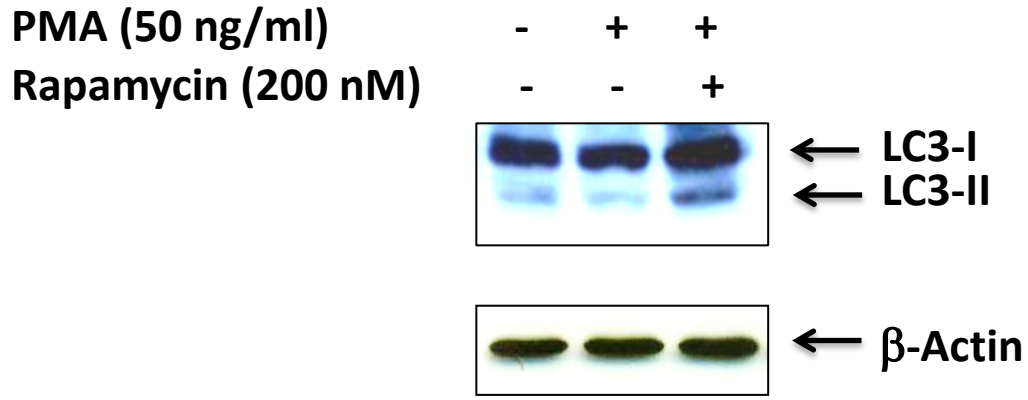

Figure 10: Effect of PMA and Rapamycin on LC-3 degradation in differentiated THP-1 cells after 48 h of treatment. Differentiated THP-1 cells were treated with 50 ng/ml of PMA and 200 nM of Rapamycin (autophagy inducer). After 48 h whole cell lysates were prepared as described in materials and methods. Lysates were subjected to western blotting analysis with an antibody against LC-3 (upper panel). The blot was equipped and reprobed with an antibody against β- actin (lower panel).

7. DISCUSSION

The factors leading to monocyte macrophage differentiation and the mode of autophagy remains partially characterized. In this study we tried to study the effect of PMA on autophagy of THP-1 cells.

First we tried to see the time kinetics cell viability of THP-1 cells under varying concentrations of PMA (from 1 ng/ml to 100 ng/ml) and we found that PMA at a concentration of 100 ng/ml were not at all toxic to THP-1 cells at 24,48 and 72 h after treatment.

Then we tried to see the effect of two variable concentration of PMA, lowest (1 ng/ml) and highest (100 ng/ml) on autophagy of THP-1 cells. We found that in comparison to the lowest dose of PMA highest dose was potentially enough to convert LC-3 I to LC-3 II form. Further to pinpoint the exact dose of PMA having ability to induce autophagy, we perform concentration kinetics experiment and proved that no other dose lower than 100 ng/ml is inducing autophagy in THP-1 cells. Since for further studies in our lab and as reported in literature 50 ng/ml PMA will be used to differentiate THP-1 cells, we were interested to cross check further the autophagy with that dose of PMA in the presence and absence of an autophagy inducer, Rapamycin (200 nM). We found that 50 ng/ml of PMA is not inducing any autophagy in comparison to Rapamycin. This result will give us a leverage of using PMA at a dose of 50 ng/ml for differentiation of THP-1 cells for further experiments in our lab.

8. CONCLUSION

From the experiments that were conducted we concluded that

- PMA does not have any cytotoxic effect on THP-1 cells, as all the cells were viable.
- Higher dose of PMA i.e. 100 ng/ml induces autophagy in THP-1 cells.
- Lower dose of PMA i.e. 1 ng/ml does not induce autophagy.
- Out of the variable doses of PMA (0, 5, 10, 20, 50, 100 ng/ml) that were checked, autophagy was induced only at a concentration of 100 ng/ml. Concentrations below 100 ng/ml could not induce autophagy.
- Sub optimal dose of PMA used for the differentiation of the monocytes did not have any interference with the effect of Rapamycin

9. REFERENCES

Auwerx, J., Chait, A., and Deeb, S. S., Transcriptional regulation of the LDL-receptor and HMG-CoA reductase genes by protein kinase C and a putative negative regolatory protein. Proc. natl Acad. Sci. USA 86 (1989) 1133-1137.

Auwerx, J., Chait, A., Wolfbauer, G., and Deeb, S., Involvement of second messengers in regulation of the low-density lipoprotein recep- tor gene.Molec.cell. Biol. 9 (1989) 2298-2302.

Auwerx, J., Deeb, S., Brunzell, J. D., Peng, R., and Chait, A., Tran- scriptional activation of the lipoprotein lipase and apolipoprotein E genes accompanies differentiation in some human macrophage-like cell lines. Biochemistry 27 (1988) 2651 2655.

Auwerx, J., Deeb, S., Brunzell, J. D., Wolfbauer, G., and Chait, A., Lipoprotein lipase gene expression in THP-1 cells. Biochemistry 28 (:1989) 4563-4567.

Auwerx, J., Staels, B., Van Vaeck, E, Verhoeven, G., and Ceuppens, J., IgG Fc receptor expression during macrophage differentiation of the monocyticleukemia cell line, THP-1. (1990)

Auwerx, J., Staels, B., and Sassone-Corsi, P., Coupled and uncou- pled induction of fos and jun transcription by different second mes- sengers in ceils of hematopoietic origin. Nucl. Acids Res. 18 (1990) 221 - 228.

Auwerx, J., Staels, B., Van Vaeck, E, Verhoeven, G., and Ceuppens, J., IgG Fc receptor expression during macrophage differentiation of the monocyticleukemia cell line, THP-1. (1990) submitted.

Auwerx, J., The human leukemia cell line, THP-1 : A multifacetted model for the study of monocyte-macrophage differentiation. (1991)

Basu, S. K., Brown, M. S., Ho, Y. K., Havel, R. J., and Goldstein, J. L., Mouse macrophages synthesize and secrete a protein resem- bling apolipoprotein E. Proc. natl Acad. Sci. USA 78 (1981) 7545 7549.

Biddison, W. E., Rao, P. E., Talle, M. A., Goldstein, G., and Shaw, S., Possible involvement of the OKT4 molecule in T cell recognition of class II HLA antigens. J. exp. Med.156 (1982) 1065 1076.

Bishop, J. M., Viral oncogenes. Cell 42 (1985) .

Brown, M. S., and Goldstein, J. L., Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclero- sis. A. Rev. Biochem. 52 (1983) 223 261.

Brown, M. S., Goldstein, J. L., Krieger, M., Ho, Y. K., and Ander- son, R. G. W, Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. J. Cell Biol. 82 (1979) 597 613.

Collins, S. J., The HL-60 promyelocyticleukemia cell line: prolifera- tion, differentiation, and cellular oncogene expression. Blood 70 (1987) 1233 1244.

Drage, M.G., Pecora, N.D., Hise, A.G., Febbraio, M., Silverstein, R.L., Golenbock, D.T., Boom, W.H., and Harding, C.V. 2009. TLR2 and its co-receptors determine responses of macrophages and dendritic cells to lipoproteins of Mycobacterium tuberculosis. Cell Immunol 258:29-37.

Fenton, M.J., Vermeulen, M. W., Clark, B. D., Webb, A. C., and Auron, P. E., Human pro-IL-I beta gene expression in monocytic cells is regulated by two distinct pathways. J. Immunol. 140 (1988) 2267-2273.

Global Tuberculosis Report (2013), W.H.O.

Gutierrez, M.G., Master, S.S., Singh, S.B., Taylor, G.A., Colombo, M.I., and Deretic, V. 2004. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 119:753-766.

Hara, H., Tanishita, H., Yokayana, S., Tajima, S., and Yamamoto, A., Induction of acetylated low density lipoprotein receptor and suppression of low density Epoprotein receptor on the cells of a human monocyticleukemia cell line.Biochem.biophys. Res. Com- mun.146 (1987) 802 808.

Jagannath, C., Lindsey, D.R., Dhandayuthapani, S., Xu, Y., Hunter, R.L., Jr., and Eissa, N.T. 2009. Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. Nat Med 15:267-276.

Johnston, R. B., Monocytes and macrophages. N. Engl. J. Med. 318 (1988) 747-751.

Kodama, T, Reddy, P., Kishimoto, C., and Krieger, M., Purification and characterization of a bovine acetyl low density lipoprotein re- ceptor. Proc. natl Acad. Sci. USA 85 (1988) 9238-9242.

Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P., and Krieger, M., Type I macrophage scavenger receptor contains e-helical and collagen-like coiled coils. Nature 343 (1990) 531-535.

Kostura, M.J., Tocci, M. J., Limjucco, G., Chin, Ji, Cameron, P., Hillman, A. G., Chartrain, N. A., and Schmidt, J. A., Identification of a monocyte specific pre-interleukin 1 beta convertase activity. Proc. natl Acad. Sci. USA 86 (1989) 5227-5231.

Kradin RL, McCarthy KM, Preffer FI, Schneeberger EE (1986) Flow- cytometric and ultrastructural analysis of alveolar macrophage maturation. J LeukocBiol 40: 407–417.

Leemans, J.C., Florquin, S., Heikens, M., Pals, S.T., van der Neut, R., and Van Der Poll, T. 2003. CD44 is a macrophage binding site for Mycobacterium tuberculosis that 102 mediates macrophage recruitment and protective immunity against tuberculosis. J Clin Invest 111:681-689.

Lubbert, M., and Koeffler, H. P., Myeloid cell lines: tools for studying differentiation of normal and abnormal hematopoietic cells. Blood. Rev 2 (1988) Lubbert, M., and Koeffler, H. P., Myeloid cell lines: tools for study- ing differentiation of normal and abnormal hematopoietic cells. Blood. Rev 2 (1988) 121 133.

Mitchinson, M. J., and Ball, R. Y., Macrophages and atherogenesis.Lance 2 (1987) 146 149.

Molina, J.M., Scadden, D.T., Byrn, R., Dinarello, C.A., and Groopman, J. E., Production of tumor necrosis factor alpha and interleukin 1 beta by monocytic cells infected with human immuno- deficiency virus. J. clin. Invest. 84 (1989) 733-737.

Nathan, C., Secretory products of macrophages. J. clin. Invest. 79 (1987) 319-326.

Olivecrona, T., and Bengtsson-Olivecrona, G., Lipoprotein lipase from milk-the model enzyme in lipoprotein lipase research, in: Lipo- protein Lipase, pp. 15-58. Ed. J. Borensztajn. Evener press. Chicago 1987.

Ponta, H., Sherman, L., and Herrlich, P.A. 2003. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 4:33-45.

Remijsen, Q., Berghe, T. V., Wirawan, E., Asselbergh, B., Parthoens, E., De Rycke, R., ... & Vandenabeele, P. (2011). Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell research*, *21*(2), 290-304.

Ross, R., Atherosclerosis: a problem of the biology of the arterial wall cells and their interactions with blood components. Arterioscle- rosis 1 (1981) 293-311.

Schlesinger, L.S. 1993. Macrophage phagocytosis of virulent but not attenuated strains of Mycobacterium tuberculosis is mediated by mannose receptors in addition to complement receptors. J Immunol 150:2920-2930.

Schwartz, S. M., and Reidy, M., Common mechanisms of prolifera- tion of smooth muscle in atherosclerosis and hypertension. Hum. Path. 18 (1987) 240-247.

Schwende H., Fitzke E., Ambs P., and Dieter P., Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1 ,25-dihydroxyvitamin D3 (1996)

Semba, R.D. 1999. Vitamin A as "anti-infective" therapy, 1920-1940.J Nutr 129:783- 791.

Sokol RJ, Hudson G, James NT, Frost IJ, Wales J (1987) Human macrophage development: a morphometric study. J Anat 151: 27–35.

Stromhaug, P.E., and Klionsky, D.J. 2001. Approaching the molecular mechanism of autophagy. Traffic 2:524-531.

Sudhof, T. C., Russell, D. W., Brown, M. S., and Goldstein, J. L., 42 bp element from LDL receptor gene confers end-pr0duct repression by sterols when inserted into viral TK promotor. Cell 48 (1987) 1061-1069.

Tavera-Mendoza, L., Wang, T.T., Lallemant, B., Zhang, R., Nagai, Y., Bourdeau, V., Ramirez-Calderon, M., Desbarats, J., Mader, S., and White, J.H. 2006. Convergence of vitamin D and retinoic acid signalling at a common hormone response element. EMBO Rep 7:180-185.

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., and Tada, K., Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int. J. Cancer 26 (1980) 171 176.

Turner, M., Chantry, D., and Feldmann, M., Post-transcriptional control of IL-1 gene expression in the acute monocyticleukemia line THP-1. Biochem.biophys. Res.Commun.156 (1988) 830-839.