OXIDATIVE STRESS AND INNATE IMMUNE RESPONSES IN A549 LUNG CARCINOMA CELLS

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December, 2014

CERTIFICATE

I declare that the dissertation entitled "**Oxidative Stress and Innate Immune Responses in A549 Lung Carcinoma Cells**" has been prepared by me under the guidance of Dr. Monisha Dhiman, Assistant Professor,Centre for Genetic Disease and Molecular Medicine, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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I certify that Shishir Upadhyay has prepared his dissertation entitled "**Oxidative Stress and Innate Immune Responses in A549 Lung Carcinoma Cells**" for the award of M.Phil. Degree of the Central University of Punjab, under my guidance. He has carried out this work at the Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda.

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ABSTRACT

Oxidative stress and innate immune responses in A549 lung carcinoma cells

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Cancer immunology is the study of cross-talk between the immune response and cancer cells. The immune response, including the recognition of cancer-specific antigens is of particular interest as knowledge gained drives the development of new vaccines and antibody therapies. The molecular mechanisms which are disturbed in the susceptible patients who proceed to develop cancer are very complicated and still largely unknown. It proposed that apart from the reported genetic modifications on tumor cells there are modifications due to oxidative stress (resulting in the formation of chemical adducts, e.g. 3-hydroxynonenal, 3-nitrotyrosine, carbonyl etc.) at the vicinity of tumor where the immune cells infiltrate. The central hypothesis of the present work is that respiratory burst which is host's mechanism to kill the foreign particles (tumor cells) is used as defence mechanism by the tumor cells by forming neoantigen which in turn make them undetectable and can further help them to escape the host immune surveillance. The lung carcinoma A549 cells were treated with 100µM H_2O_2 and using 1-D gel electrophoresis the oxidized tumor proteins in normal and treated cells were visualized. To confirm the oxidized modifications at membrane levels and at proteins levels the lipid peroxidation and protein carbonyls were detected respectively. It was observed that the oxidative stress induces the lipid peroxidation and protein carbonyls in tumor cells. To determine if neo (oxidized) tumor antigens elicit any alteration in immune responses where they show compromised phagocytosis, thus resulting in a failure to elicit effector immune functions were analyzed via phagocytosis and respiratory burst using spectrophotometry and microscopic techniques.

The present study has identified a novel mechanism(s) of carcinogenesis initiation and which can further provide directions for the development of adjunct therapies to control cancer in its initial stages and at the same time it advocates for new ventures to increase the efficacy of the chemotherapeutic drugs.

Shishir Upadhyay — Dr. Monisha Dhiman

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LIST OF ABBREVIATIONS

S. No.	Full Form	Abbreviation
1.	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide	MTT
2.	3,3'-Diaminobenzidine	DAB
3.	5,6-carboxyfluoresceindiester	CFSE
4.	Degree Celsius	°C
5.	Dimethyl sulphoxide	DMSO
6.	Dinitrophenylhydrazine	DNPH
7.	Dulbecco's modified essential medium	DMEM
8.	Ethylenediaminetetraacetic acid	EDTA
9.	Fetal bovine serum	FBS
10.	Gram	G
11.	Hour(s)	hr(s)
12.	Hydrochloric acid	HCl
13.	Hydrogen peroxide	H_2O_2
14.	Liter	l
15.	Magnesium Chloride	$MgCl_2$
16.	Malondialdehyde	MDA
17.	Microgram	µg
18.	Microliter	µl
19.	Micromolar	µM
20.	Milligram	Mg
21.	Milliliter	ml
22.	Millimolar	mM
23.	Minute	min
24.	Molar	M
25.	Moles	mol

26.	NADPH Oxidases	NOX
27.	Nanogram	ng
28.	Nanometer	nm
29.	Nitrobluetetrazolium salt	NBT
30.	Nitric Oxide	NO
31.	Phenylmethylsulfonyl fluoride	PMSF
32.	Phorbol,12- myristate 13-acetate	PMA
33.	Phosphate buffer saline	PBS
34.	Polyacrylamide gel electrophoresis	PAGE
35.	Potassium chloride	KCl
36.	Roswell Park Memorial Institute	RPMI
37.	Sodium bicarbonate	$NaHCO_3$
38.	Sodium chloride	NaCl
39.	Sodium dodecyl sulphate	SDS
40.	Thiobarbituric acid	TBA
41.	Thiobarbituric acid reactive species	TBARS
42.	Trichloroacetic acid	TCA
43.	Water	H_2O

CHAPTER1

INTRODUCTION

The production of reactive oxygen species (ROS) is an inevitable consequence of metabolism *in situ* or produced from external sources (pollution, cigarette smoke, radiation and medication). However, high levels of ROS within a cell can be lethal and so the cell has a number of antioxidant mechanisms its cumulative effect called oxidative stress. The human body counteract towards oxidative stress by the antioxidants, which are either naturally produced *in situ*, or externally supplied through foods and/or supplements(Pham-Huy *et al.*, 2008). High levels of ROS within a cell have a number of direct and indirect consequences on cell signalling pathways and may result in apoptosis or necrosis. This process plays a major part in the development of chronic and degenerative illness such as cancer, autoimmune disorders, aging, cataract, rheumatoid arthritis, cardiovascular and neurodegenerative diseases, Chronic Lymphocytic Leukemia (CLL) (Pham-Huy *et al.*, 2008, England *et al.*, 2005). Under normal physiologic conditions, ROS (H_2O_2 and $O_2^{\bullet-}$) are constantly generated and eliminated in the biological scavenging system and are required to drive regulatory pathways (Gupta *et al.*, 2012). For tumor, ROS is a double edged sword by acting as cancer-suppressing as well as cancer-promoting factor (Gupta *et al.*, 2012).

ROS and secondary by-products of oxidative stress induce many different types of protein modifications that can affect their function and stability, including glycosylation, glycation, phosphorylation and oxidation chemistry (Murray *et al.*, 2008). For example, ROS react with cysteine-, histidine- and lysine-residues of proteins to form 4-hydroxynonenal (4-HNE). In addition, protein-derived aldehydes and ketones are produced by direct oxidation of arginine, lysine, proline, or threonine residues and collectively termed as protein carbonyls (Chevion *et al.*, 2000). In autoimmune diseases, epitope spreading is considered as neoantigenic determinants generates by reactive oxygen and nitrogen species (ROS and RNS). The oxidation of amino acids, and other macromolecules by peroxynitrite, hypochlorous acid and otherROSenhancing the antigeniccharacteristics of DNA, low density lipoproteins (LDL) and ImmunoglobulinG (IgG), generating ligands for which autoantibodies show higher avidity(Griffiths, 2008). There are evidences of ROS and RNS promoting the autoimmune responses as observed in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Plow *et al.*, 1972).

Cancer is caused by any defects at cellular level including cell metabolism, cell receptors and signaling and at its DNA. On the basis of WHO's GLOBOCAN-2012 data analysis, about 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012. Estimated 13% patients suffered from lung cancers worldwide (IARC, 2013).

Tumor microenvironment is one of the six hallmarks for evading immunesurveillance. In the tumor microenvironment, Tumor Associated Macrophages (TAM) promotes migration and metastasis through expression CSF-1 receptor and secreting EGF for cancerous cells (Goswami *et al.*, 2005). It is also documented that these growth factors increase ROS level within cells (Meng *et al.*, 2008). In the tumor microenvironment, ROS level is responsible for phenotypic plasticity of macrophage and in turn the macrophages generate enough ROS to downregulates immune responses through inhibiting the interaction of MHC and T cell peptide interaction (Schmid *et al.*, 2010; Zhang *et al.*, 2013). ROS also prompt to neoantigenic character as a result of alteration at molecular level. An alterationinprotein due to further modification within a biochemical pathway such as glycosylation, phosphorylation or proteolysis, responsible for generation of new epitopes which are called neoantigenic determinants and requireseparate, specific antibodies for recognition. Any refinement at these molecular levels such as genetically-coded amino-acid sequences, tertiary conformation and quaternary organization modify the antigenic expression (Ramsey *et al.*, 2006). Neoantigen generation are characteristics of autoimmune pathogenesis (Namazi, 2009) as well as cancer (Coulie *et al.*, 2001).

In present study, we investigated whether oxidative modification of tumor proteins results in the formation of new epitopes that, if not recognized by host immune system due to being neoantigens, would lead to accumulation of tumor cells and hence initiating carcinogenesis. We employed an *in vitro* system of the human carcinoma cell line as a model for oxidative modification using H_2O_2 as oxidizing agent. The oxidative stress induced neoantigen on the tumor cells, were detected on the SDS-PAGE gel. The innate immune responses of the phagocytes is important to detect and kill the tumor cell. Further, we used the co-culture technique to determine how the oxidative modifications on tumor cells can affect the innate immune response of macrophages due to neoantigen formation.

Phagocytosis was significantly reduced in the THP-1 cells co-cultured with H_2O_2 treated A549 cells for 24 hrs whereas the cells treated for a short period (15 min) were similar to untreated controls. The respiratory burst or ROS release was significantly declined in the THP-1 cells when co-cultured with H_2O_2 treated A549 cells for 24 hrs. The immunohistochemistry (IHC) for NADPH Oxidase enzyme which is one of the most important enzymes during the oxygen dependent phagocytosis showed very interesting results the THP-1 cells co-cultured with H_2O_2 treated A549 cells for 24 hrs showed a very high percent of cells positive for $P47^{phox}$ staining.

HYPOTHESIS

Respiratory burst which is host's mechanism to kill the foreign particles is used as defence mechanism by the tumor cells by forming neoantigen which in turn make them undetectable and can escape the host immune response.

OBJECTIVES OF THE STUDY

1. To detect oxidative stress and neoantigen formation in A549 lung cancer cells line.
2. To determine role of neoantigen in helping the tumor cells to escape the host immunesurveillance.

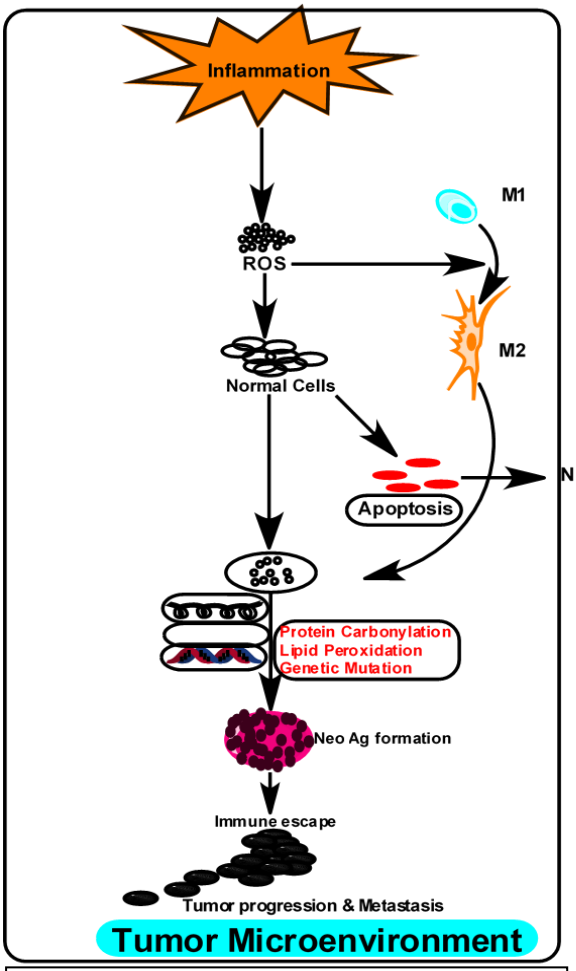


Figure 1.1: Inflammation generates ROS which leads to either apoptosis or neoantigen formation through protein carbonylation, lipid peroxidation which in turn helps tumor cells in immune escape and its progression and

SIGNIFICANCE OF THE STUDY

Although the indirect effects of ROS are well known, they can directly modify signalling proteins through different modifications such as nitrosylation, carbonylation, di-sulphide bond formation and glutathionylation but whether these modifications can modulate tumor cell protein into neoantigens which can further make them more resistant towards host immune responses is not known. This work will be critical to understand the possible factor responsible for the initiation of carcinogenesis. Apart from this these neoantigens if identified may provide highly specific targets for antitumor immunity which can be used as neoantigen vaccine further offering a promising new approach to eradicate cancer cells.

CHAPTER 2

REVIEW OF LITERATURE

2.1 REACTIVE OXYGEN SPECIES

A molecule with one or more unpaired electrons in its outer shell is called a free radical and is produced from molecules during breakage of a chemical bond such that each fragment keeps one electron; by cleavage of a radical to give another radical, and in various redox reactions. These free radicals are highly unstable molecules with freely available electrons enable them to react with various organic substrates such as lipids, proteins, DNA (Clerkin *etal.*, 2008; Pham-Huy *et al.*, 2008).

Figure 2.1: Endogenous and Exogenous source of ROS.

Reactive oxygenspecies (ROS) and oxidative stress have attracted a great deal of attention in recent years. ROS generated during normal physiological conditions and cellular metabolism in aerobic organisms (Pham-Huy *et al.*, 2008) include ions, free radicals [hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), singlet oxygen ($1O_2$), nitrogen dioxide ($NO_2^{\bullet}$), peroxyl ($ROO^{\bullet}$) and lipid peroxyl ($LOO^{\bullet}$)], and peroxides/ molecules [hydrogen peroxide (H_2O_2), ozone (O_3), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^{-}$), dinitrogen trioxide (N_2O_3), lipid peroxide (LOOH). Under normal physiological conditions, ROS begin to deplete due to their very short half- life (Mittal *et al.*, 2014).

ROS and RNS formation endogenously takes place via two mechanisms: enzymatic and non-enzymatic reactions. Mitochondrial respiratory chain and partial electrons coupling reactions add to the endogenous $O_2^{\bullet-}$ levels. Phagocytosis (oxidative burst), xenobiotic metabolism and NADPH oxidase, cyclooxygenases, or lipooxygenases and other inflammation reactions bring forth ROS. Phagocytes and neutrophils, highly active during immune cell response, are the chief source of these enzymes. In addition, mental stress, excessive exercise,

ischemia, infection, cancer and ageing are some other physiological sources(Pham-Huy *et al.*, 2008). Smoking, drugs namely, tacrolimus, cyclosporine and those affecting mitochondrial respiratory chains, ionizing and nonionizing radiation etc. (Pham-Huy *et al.*, 2008) are exogenous sources. On the other hand, antioxidants such as GSH (glutathione peroxidase), SOD (superoxide dismutase), catalase, peroxidredoxin, thioredoxin maintain ROS levels to normal (Mittal *et al.*, 2014; Pham-Huy *et al.*, 2008) (Figure 2.1).

2.2 CANCER

Cancer is a worldwide most pernicious disease and has no perfect one definition. Cancer is a disease characterized by genetic instability, defective signalling and unregulated transcription factors. The tremendous array of genetic alterations unique to each tumor could provide unique peptide sequences termed neoantigens, which when presented on a tumor's major histocompatibility complex (MHC) molecules would represent tumor-specific neoantigens capable of being recognized by T cells. The tumor specific antigen are distinct from the majority of tumor antigens recognized by antibodies, which tend to recognize ubiquitously expressed cell-surface antigens whose structure is modified in tumors by posttranslational events, such as glycosylation (Pardoll, 1999). Only about one third of the neoantigens or mutated epitopes are immunogenic and elicit immune response as compared to wild type sequence(Castle *et al.*, 2012).

2.3 ROS AND CANCER

ROS form a part and parcel in a variety of various physiological processes such as cell signaling, cell metabolic activities and maintaining homeostasis and also in apoptosis (Matés *et al.*, 2000). ROS are classically defined as partially reduced metabolites of oxygen that possess strong oxidizing capabilities. They are deleterious to cells at high concentrations but at low concentrations (exact concentrations still remaining to be defined), they serve in complex signaling functions (Mittal *et al.*, 2014).

ROS is an important hallmarks of cancer which promote cancer by prolonging cell survival (impeding apoptosis), promoting angiogenesis, extending the response of growth factor receptors and its signaling cascade and by

increasing the activity of various transcription factors thereby destabilizing genetic composition (Fiaschi *et al.*, 2012; Laurent *et al.*, 2005). These are also known to be involved in cell proliferation and malignant transformation (Wang *et al.*, 2008), ROS accumulation can alter the composition or configuration of macromolecules (DNA, lipids and proteins) and hence, changing the normal biological phenomena ultimately culminating into cancer of oral cavity (Gurudath *et al.*, 2012), lung (Luanpitpong *et al.*, 2010), breast (Knaus, 2002), prostate (Khandrika *et al.*, 2009), gut (Lotfy *et al.*, 2011) and ovary (Luanpitpong *et al.*, 2010). It is well documented that different factors are responsible for changes on tumor cells at molecular levels such as ROS generation during chronic inflammation and chronic ROS induces chronic inflammation which provide a mechanism during promotion, progression and metastasis (Reuter *et al*., 2010).

A cancerous cell shows high metabolism and NADPH oxidase (NOX) activity which by increasing the ROS level within cells may inhibit apoptosis by mutating PI-3 Kinase/ Akt, PTEN andp53 signaling pathways (Clerkin *et al.*, 2008). It is experimentally proved that higher endogenous or exogenous doses of $O_2^{\bullet}$and H_2O_2 caused cellular transformation and tumorigenicity in NIH 3T3 cells, through phosphorylating signaling responsible for survival of GSK-3b, Bad, FOXO (transcription factors), caspase-9, AKT, p42/p44 and p38/ MAPK (mitogen-activated protein kinase) (Clerkin *et al.*, 2008). But, till date it is unclear whether and how ROS control the self-renewal/differentiation process and the tumor-initiating capacity of cancer cells (Sato *et al.*, 2014).

It is elucidated that p53 maintains redox potential in normal cells by regulating the genes responsible for antioxidant activity and hence, keeping a check on the ROS levels. The transformed cells where the p53 is mutated showed a higher redox potential (Clerkin *et al.*, 2008). Antioxidant property of GSH also regulates the redox balance by controlling the ROS level within the cells. In case of chronic lymphocytic leukemia, GSH displayed excessive spontaneous apoptosis *in vitro* while in tumor microenvironment, bone marrow stromal cells promoted GSH metabolism and cell survival rate by disturbing the ROS level and redox potential (Zhang *et al.*, 2012).

Higher ROS are known to cause lipid peroxidation during various pathophysiological conditions. MDA(Malondialdehyde) and 4-HNE, products of

lipid peroxidation are linked to carcinogenesis by promoting cell proliferation and blocking apoptotic pathway (Marquez *et al.*, 2007). Moreover, ROS play a crucial role in hepatitis B (HBV) and hepatitis C virus (HCV) induced hepatocellular carcinoma. ROS levels rises during infections causing oxidation of intracellular macromolecules and also allowing replication of virus and cell survival by activation of transcription factors (Fiaschi *et al.*, 2012).

In fact, most of the chemotherapeutic and radiotherapeutic agents kill cancer cells by augmenting ROS stress. ROS regulates the expression of various tumor supressor genes such as p53, retinoblastoma gene (Rb), and phosphatase and tensin homolog (PTEN) (Gupta *et al.*, 2012). It is suggested that many therapeutic medicines for combating various diseases utilizes ROS dependent mechanism such as Cucurbitacin B (cucB) - acolon cancer drug (Hanafi *et al.*, 2012)andfor killing the multidrug resistant cancer cells (Maiti, 2012).

2.4 ROS, CANCER AND IMMUNE CELLS

Immune cells in the tumor microenvironment are particularly important, resulting in both positive and negative consequences for tumor growth.Natural killer (NK) cells, monocytes and macrophage cells, body's first line of defense, are capable of killing a broad spectrum of tumor cells without apparent specificity (Jäättelä *et al.*, 1993). Macrophage number exceeds in developing tumor than normal as host responds and constitutes more than half of the total cellular infiltrates surrounding the tumor. Human monocytes and tumor associated macrophages (TAM) show spontaneous cytotoxicity after coming in contact with tumor cells but not with normal cells (Davies *et al.*, 1992) by releasing tumor necrosis factor α (TNF-α), ROS and RNS. This is clearly evidenced by chemiluminiscence response associated with $O_2^{\bullet-}$ and $OH^{\bullet}$ production (Mytar *et al.*, 1999). Chemokines produced by these cells are also responsible for oxidative bursts and excessive ROS generation during phagocytosis and tumoricidal activity.

At low or moderate concentrations, ROS and RNS are necessary for normal cellular processes.Thesecan act asweapons for the host defense system.Innate immune cells releases ROS creating oxidative burst and destroying the invading pathogens (Elbim *et al.*, 2001) and even tumor cells (Davies *et al.*, 1992). ROS can

also suppress tumor growth through sustained activation of cell-cycle inhibitor and induction of cell death as well as senescence by damaging various macromolecules(Ramsey *et al.*, 2006).Human phagocytic cells such as polymorphonuclear leukocytes (PMN) are readily mobilized to sites of infection and ingest pathogen or foreign paricle by a process known as phagocytosis. Ingested bacteria are killed by ROS derived from superoxide produced by an activated, phagosome-bound NADPH-dependent oxidase. The granulomatous patients showed the significance of ROS generated by immune cells. Multiple and persistent infections has been noticed in patients with defective NADPH oxidase system or are unable to produce superoxide anion radical ($O_2^{\bullet-}$) (Henriet *et al.*, 2011).

Paradoxically, researchers found that tumor infiltrate constituting mainly of monocytes and macrophages also promotes tumor survival, progression and metastasis (Lee *et al.*, 2013). ROS is one of the major stimulator for M2 differentiation and this TAM is also noticed in promoting tumorigenesis (Almatroodi *et al.*, 2014). TAM also releases tumor protective chemokines (MFG-E8 & IL-6) (Jinushi *et al.*, 2011) and high ROS as seen in cancer microenvironment (Trulson *et al.*, 1994). It is suggested that exogenous ROS drives the aggressiveness of cancer in tumor microenvironment(Marquez *et al.*, 2007). In another finding it is reported that some markers CD18, CD29 and CD44 are pre-requisite for tumor-monocyte interactions which further result in ROS generation (Mytar *et al.*, 2001). Within cancer microenvironment, ROS produced due to inflammation, oxidative bursts and other sources dramatically decrease the phagocytic activity of macrophage (Kirkham *et al.*, 2003).

2.5 CONCEPT OF NEOANTIGEN IN CANCER

Neoantigen is not only the characteristics of autoimmune disease but tumor cells often express new antigens ("neoantigens") not present on normal ones. Tumor antigens or neoantigens are the antigens presented by MHC I or MHC II molecules on the surface of tumor cells (Sloan *et al.*, 2002). During Cancer immunoediting, cells of innate immune system and the cytotoxic T lymphocytes of the host recognize these antigens and destroy the tumor cells and prevents from

their outgrowth (Runthala *et al.*, 2009). The numbers of neoantigens within cancers is proportional to their mutation frequency (Shukla *et al.*, 2013).

Cancer cells frequently elude immunesurveillance and remain dormant. After developing tumor microenvironment, it undergoes a number of changes leading to metastasis. Tumor cell generates numerous fragile neoantigens and also shares self-proteins acting a positive selection during their evasion. Neoantigens formation in tumor cells can be result of multiple mutations and alterations in oncogenes, tumor suppressor genes and DNA repair genes due to various reasons, including the excessive oxidative stress *in vivo*(Prendergast, 2008). These tumor neoantigens could differ within the patients as well as in region *in vivo*(Brown *et al.*, 2014).

Tumor neoantigens are the consequences of gene aberration. Numerous neoantigens have been recognized through whole exom sequencing (WES) of HLA typing (Shukla *et al.*, 2013). Tumor neoantigens or tumor specific, shared antigens can originate from cancer germline genes MAGE (human), P1A (mouse), tumor specific transcripts (TRP2), oncogenic virus (HPV-16, EBV), Mucins, by differentiations of antigens (MART-1/Melan-A) from CEA (carcinoembryonic antigen), overexpression of antigens (for example, HER-2/neu expressed in more than 30% in breast and ovarian cancer) (Coulie *et al.*, 2001). With the help of molecular and cellular techniques, more than 500 tumor antigens have been recognized. These neoantigen can be detected with the help of SEREX (Krackhardt *et al.*, 2002) and ELISPOT (Castle *et al.*, 2012).

Figure 2.2: ROS modify lipid, protein and genetic material prompting neoantigen.

2.6 ROS AND NEOANTIGEN FORMATION

Presence of ROS in the microenvironment of tumor activate several pathways of protein modifications. ROS induces certain posttranslational modification and genetic aberration modifying the epitopes forming neoantigens in respective tissues and organs (Eggleton *et al.*, 2008Kalluri *et al.*, 2000) that prompt autoimmune diseases through epitope spreading-antibodies showing cross reactivity with native antigen (Griffiths, 2008). But in the case of tumor, the 'self/non-self' theory of immune recognition teaches that new antigens produced by the tumor should lead to its immune rejection (Hao *et al.*, 2012). If tumors express antigens which can be recognized by the immune system, then they must be eliminated normally, but it is still not understood that if they are known to possess so specific antigenic targets then how they escape the host immunesurveillance and develop into cancer. In present work, we propose that apart from genetic modifications in tumors, there are some additional modifications caused due to oxidative burst (reactive oxygen species) induced as a result of host immune cells infiltrating in the vicinity due to which it can escapes the host immunity and escape phagocytosis and lead to the formation of tumors (Figure 2.2).

2.7 ROS AND IMMUNOEDITING

Macrophage is a link of innate to adaptive immune response. These originate from myeloid progenitor cells from monocytic cells except Langerhans and microglial cells (Hao *et al.*, 2012). Based on phenotypes, macrophages have been categorized into two subtypes: M1 (classically activated) and M2 (alternatively activated). In the tumor microenvironment, interactions between tumor cells and stromal cells are responsible for tumor growth and metastasis. Macrophages tend to destroy tumor cells by releasing lysozymes, generating IFN-γ and by activating T cells; and also by producing reactive oxygen intermediates and nitric oxide (NO) (Runthala *et al.*, 2009). ROS on the other hand, also play a critical role in the differentiation of alternatively activated macrophages, TAM via MAPK-ERK signaling pathway (Zhang *et al.*, 2013). Within the tumor microenvironment, TAM provides an inflammatory and favorable conditions such as chemokines for angiogenesis, lymphogenesis, metalloproteinases and

immunosupression that might result into metastasis (Solinas *et al.*, 2009). As seen in M2-polarized macrophages promote metastatic behavior of Lewis lung carcinoma cells by inducing vascular endothelial growth factor-C (VEGF-C) expression. Potent immunosuppressive functions of macrophages, reveal that macrophages can suppress T cell responses via ROS production and suggest that ROS inhibitors may be useful in promoting antitumor immune responses (Hamilton *et al.*, 2014). The role of TAM / M2 in tumor microenvironment supported with Metastatic tumor growth is inhibited if these TAM / M2 macrophages are killed (Qian *et al.*, 2009). Rac2, small GTPase protein controls macrophage M1 to M2 differentiation and the metastatic phenotype *in vivo*. In the absence of Rac2, macrophages and/or neutrophils display suppressed reactive oxygen species (ROS) production, defective chemotaxis, impaired phagocytosis, and decreased microbial killing (Joshi *et al.*, 2014).

2.8 NEOANTIGEN AND THERAPEUTICS

Some immune-based therapies targeting neoantigens are in phase III studies assessing whether immunizing against these antigens affects overall survival (Klebanoff *et al.*, 2011).In a revolutionized cancer immunotherapy, neoantigens generated during autoimmune disease that mimics some unique tumor specific antigens are taken as a promising targets for immunotherapy (Kirkham *et al.*, 2003). Now a days, ionizing radiation are supplemented with chemotherapy that induce neoantigens on tumor cells marking them for destruction and reducing the overall systemic toxicity of drugs (Corso *et al.*, 2011). Recently, animal model systems have provided the first indications how cancer exome data may be used for immunotherapy, by demonstrating not only that vaccination against neoantigens within a mouse melanoma model can be used to increase tumor control, but also that immune-based selection against such neoantigens can lead to epitope loss *in vivo*(van Rooij *et al.*, 2013).

Another study in murine melanoma tumor B16F10 showed that immunization withneoantigens (potential immunogenic epitopes generated by mutations) control the disease both prophylactically and therapeutically. In a clinical study therapeutic vaccine from peptides derived from human papillomavirus (HPV) derived by novel neoORF generated via genetic processes was successfully tested (Hacohen *et al.*, 2013).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

Dimethyl sulphoxide (DMSO), phosphate buffer saline (PBS), tris base, ethylenediaminetetraacetic acid (EDTA), phenol, fetal bovine serum (FBS), hydrogen peroxide (H_2O_2), dinitrophenylhydrazine (DNPH), sodium dodecyl sulphate (SDS), β-mercaptaethanol, acrylamide-bisacrylamide solution, sodium bicarbonate ($NaHCO_3$), acetic acid, trichloroacetic acid, 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Essential Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI), 0.25% Trypsin-EDTA, methanol, ethanol, ethyl acetate, sodium chloride (NaCl), formaldehyde, tris HCl (pH 7.4), phenylmethylsulfonyl fluoride (PMSF), potassium chloride (KCl), hydrochloric acid (HCl), thiobarbituric acid (TBA), glycerol, bromophenol blue, sulphnilamide, guanidium HCl, chloromethyl 2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), 5,6-carboxyfluoresceindiester (CFSE), Penicillin/ Streptomycin, orthophosphoric acid , glacial acetic acid , Geimsa stain , nitrobluetetrazolium salt (NBT), phorbol 12 myristate 13-acetate (PMA), 3,3'-Diaminobenzidine (DAB), $p47^{phox}$ antibody, Malondialdehyde(MDA), NADPH Oxidase (NOX), Mayer's hematoxylin (Invitrogen).

3.2 Cell Culture

3.2.1 Culturing A549 cells.

The human alveolar epithelial cell line (A549) is derived from adenocarcinomic human alveolar epithelial cancer cells. Squamous in shape, they grow as monolayer adherent cells on surface of petriplates.

A549 cells were grown in complete medium DMEM supplemented with 10% FBS in 5% CO_2 humidified incubator. During treatment, the FBS media was removed and cells were washed twice with 5mL sterile PBS and with 5 ml of serum-free (SF) medium.

3.2.2 Culturing THP-1 cells

THP-1 cells, a human monocytic leukemia cell line, are derived from acute monocytic leukemia patient. These are large, round and grow as single cell in

suspension and differentiate into macrophage on stimulation with PMA. THP-1 cells were kindly gifted by Prof. Kanuri Rao, ICGEB, New Delhi.

For experiments in objective 2, THP-1 cells were cultured. The suspension THP-1 cells were maintained in RPMI 1640 medium with 2 mM L-glutamine, 1.5 g/L $NaHCO_3$, 4.5 g/l glucose, 10 mM HEPES and 1.0 mM sodium pyruvate and supplemented with 0.05 mM of 2-mercaptoethanol and 10% fetal bovine serum. Cells were treated with 10 ng/ml PMA which induced terminal differentiation of THP-1 cells into macrophages which then show adherence.

3.2.3 Co-Culturing of A549 cells with THP-1 cells

For some experiments, terminally differentiated THP-1 cells were used for the co-culturing experiments. The H_2O_2 (100 µm) for 15 min (short time) and 24 hrs (long time) treated A549 cells were co-cultured with THP-1 cells for overnight.

3.3 Cell Treatments

A549 cells ($1X10^6$) were seeded onto petriplates containing culture media supplemented with 10% FBS. During the treatment the media was changed to serum free media and cells were treated with H_2O_2 (100 µm) for 24 hrs (long time). After treatments of 24 hrs, cell supernatants were collected for lysis of cells using lysis buffer.

Table 3.1: Treatment Plan for Various Experiments

Treatment	**Duration**
Control	-
H_2O_2 (100 µm)	24 hrs (Objective 1& 2)
H_2O_2 (100 µm)	15 min (Objective 2)

3.4 Bradford Assay

Estimation of protein concentration is essential for quantifying proteins. Bradford assay is preferred over the Lowry method because it is simpler, faster and more sensitive.

Diluted the Bradford's reagents fivefold in distilled H_2O (1 part Bradford: 4 parts H_2O), filtered using Whatman-540 paper and stored at 4°C. Added 10-20 µl of the protein extract to 1ml of diluted reagent mixed and measured the blue color formed at 595 nm wavelength. Prepared the standard curve using a serial dilution series (0.1-1.0 mg/ml) of BSA as a known protein sample concentration (Krüger *et al.*, 2002).

3.5 Cytotoxicity Assay

MTT assay is a colorimetric assay that measures the reduction of MTT by NAD(P)H-dependent cellular oxidoreductase enzymes to purple formazon product for assessing cell viability. A solubilization solution, an acidified DMSO (0.6% acetic acid in DMSO), is added to dissolve insoluble formazan forming a colored solution, the absorbance of which can be quantified by spectrophotometer at the wavelength of 570 nm.

Cells ($1x10^5$) plated in 24 well plates were treated with different concentration of H_2O_2 (0, 20, 50, 100, 150, 200 µM) (SRL) and incubated for 24 hrs in FBS free media. Then, added 20 µl of MTT solution (5 mg MTT dissolved in 1ml sterile PBS) and incubated for 4 hrs in 37°C. After 4 hrs, the purple product was solubilized using 200 µl of 10% SDS in acidified DMSO (0.6 ml acetic acid per 100 ml of solvent) and absorbance was measured at 570 nm. The absorbance read is directly proportional to number of living cells (Dhiman *et al.*, 2012). The cell viability of each group was calculated as percentage (%) by the following equation:

$$\text{Percent viabilty} = \frac{\text{Number of living cells X 100}}{\text{Total number of cells (both living and dead)}}$$

3.6 Preparation of Cell Lysate

A549 cells treated with H_2O_2 (100 µM) for long-time (24 hrs) and non-treated cells (taken as controls) were washed with PBS [25 mM Tris-HCl (pH 7.4), 5 mM KCl, 137 mM NaCl]. Cells were trypsinized and centrifuged at 1500 rpm for 5 min at 4ºC. The pellet was homogenized in lysis buffer [20 mM Tris HCl (pH 7.2), 250 mM sucrose, 0.6% nonidet P-40, 2 mM EGTA, 40 mM KCl, 0.5 mM PMSF, 10 mM leupeptin and 10 mg/ml aprotinin)]. After incubation for 15 min in ice,

homogenates were centrifuged at 3000 g at 4 ºC for 10 min and the resulting supernatant (total cell lysates) aliquots were stored at -80 ºC (Wen *et al*., 2004).

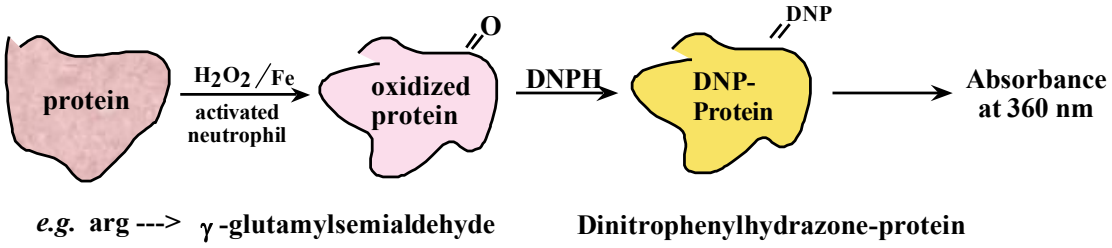
3.7 Neoantigen Detection

Gel electrophoresis is a useful method to separate and/ or identify proteins and nucleic acids. In SDS- polyacrylamide gel electrophoresis (SDS-PAGE), proteins are separated on the basis of their molecular weight using two sequential gel system. The top or stacking gel is slightly acidic (pH 6.8) and the lower separating or resolving gel is more basic (pH 8.8).

Cell lysates from all the treated groups and controls (20 µg of protein) were boiled and resolved on 10% acrylamide gels on a Mini-Protean3 system (BioRad) using 0.2 mol/L tris- HCl anode buffer (pH 8.8) and 0.1 mol/L Tris-Tricine cathode buffer containing 0.1% SDS. Gels were stained with 0.05% Commassie blue G250 and images were acquired using advanced gel documentation system (BioRad).

3.8 Protein Carbonyls Detection

Protein carbonyl formation indicates oxidative stress and free radical-induced protein modification. Carbonyl (CO) groups (aldehydes and ketones) are produced on protein side chains (especially on proline, arginine, lysine and threonine) upon oxidation (Dalle-Donne *et al.*, 2003). DNPH reacts with protein carbonyls to form hydrazones, which can be detected spectrophotometrically at 360 nm.



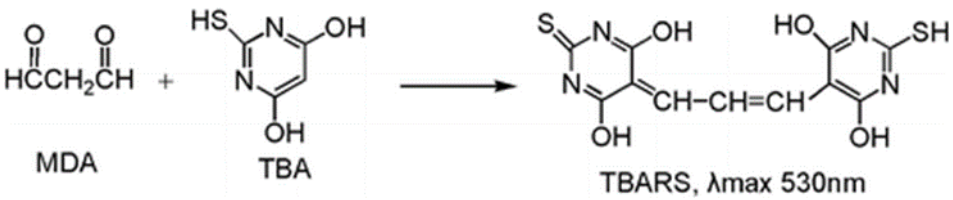
Protein sample (50 µg) was taken in 1.5 ml tubes and 500 µl of DNPH (100 mM in 2 M HCl) was added to the tubes. Incubated the tubes at 37^0C for 90 min. Samples were cooled and 1ml of TCA (28%) was added to precipitate the protein followed by vortexing for 5 min. Centrifuged the samples at 6000 rpm for 3 min and suspended the pellet in 1 ml of ethanol and ethyl-acetate solution (1:1). The

mixture was vortexed for 2 min and again centrifuged at 6000 rpm for 6 min. Pellet was repeatedly washed with ethanol-ethyl acetate (1:1) solution, resuspended in 6M guanidium HCl and vortexed for 1min. Centrifuged at 6000 rpm for 3 min to remove any insoluble particles. The absorbance was read at 360 nm using spectrophotometer (Shimadzu UV- 2450). The amount of protein carbonyls were determined by using an extinction coefficient of 21000 $M^{-1}cm^{-1}$ and expressed as nanomoles (nmols) of protein carbonyls per mg of protein.

$$\text{Protein carbonyls} = \frac{\text{O. D X Total sample X Dilution Factor}}{\text{21000 X Protein (mg) X Sample volume}}$$

3.9 Thiobarbituric Acid Reactive Species (TBARS) Estimation

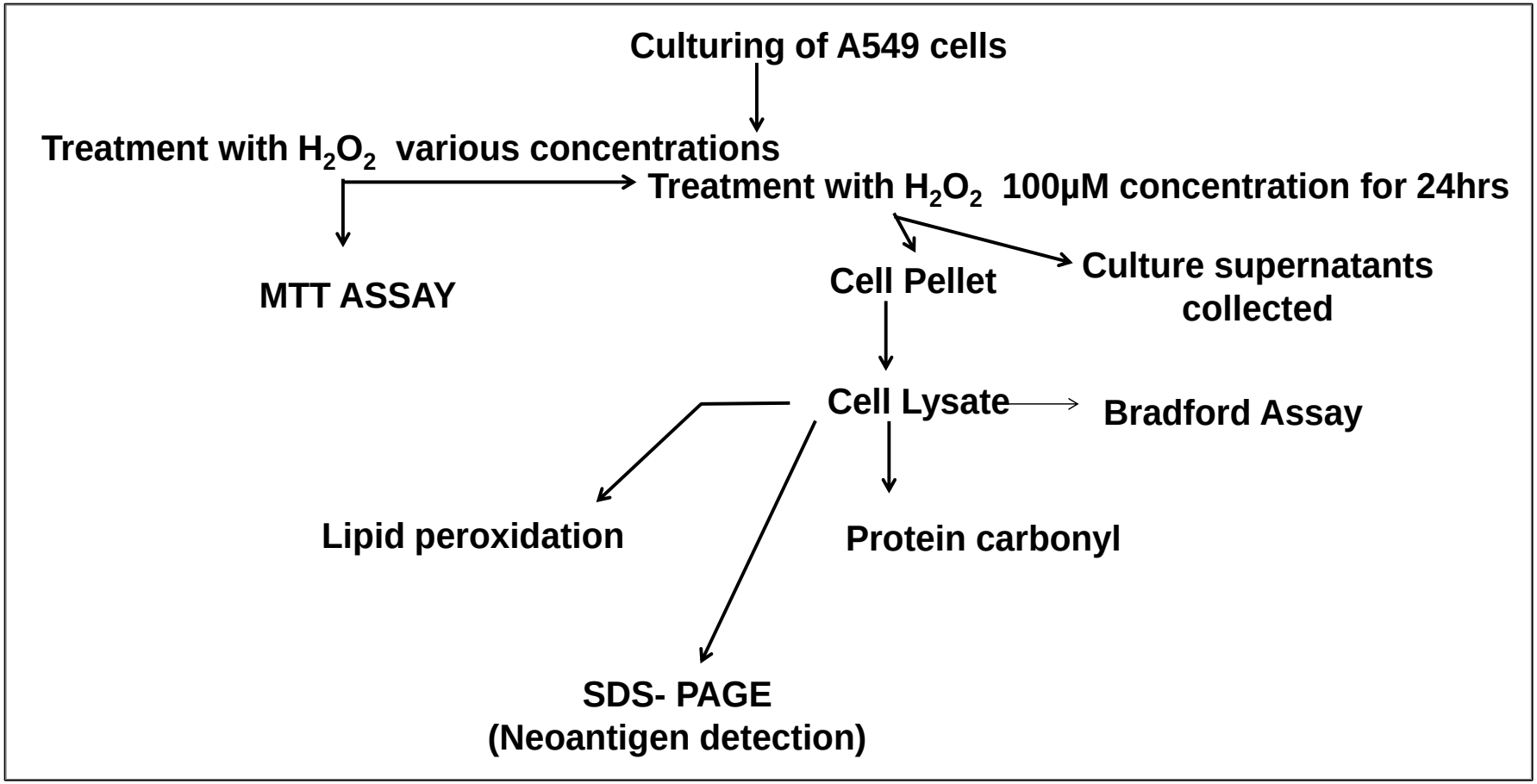
Polyunsaturated fatty acids (PUFA) are prone to oxidative stress and can react to form lipid peroxides, which are unstable and form complexes including reactive carbonyls upon decomposition. PUFA peroxides further react to form malondialdehyde (MDA) which reacts with TBA forming colored complexes, called TBARS (Gaweł *et al.*, 2003). It is the most widely reported analyte for estimating oxidative stress effects in lipids or as a convenient biomarker for lipid peroxidation (Lykkesfeldt, 2007).



100 µl of control and H_2O_2 (100 µM) treated A549 cell lysates were mixed thoroughly with 15%(w/v) trichloroacetic acid, 0.375% (w/v) TBA and 0.25 mol/l HCl. The mixture was heated for 30 min in boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 min. The absorbance of the supernatant was determined at 530 nm and the TBARS concentration was calculated using 1.56 X 10^5 $M^{-1}cm^{-1}$ as molar absorption coefficient and results were expressed as nanomoles (nmoles) per mg of protein.

$$\text{TBARS concentration} = \frac{\text{total volumeXO. D.}}{\text{0.156XvolumeXprotein(mg)}}$$

Work Outline for Objective 1



3.10 Phagocytosis Assay

3.10.1 Giemsa Staining

Giemsa stain is used in cell biology to differentiate nuclear and/or cytoplasmic morphology of various cell types such as platelets, RBCs, WBCs etc. and for histopathological studies. It is specific for the phosphate groups of DNA and attaches itself to regions of DNA where there are high amounts of adenine-thyminebonding.

1x10^5 A549 cells were treated with H_2O_2 for 15 min and 24- 48 hrs. The 0.5x10^5 THP-1 cells were allowed to differentiate into macrophages with PMA (10 ng/ml) treatment at 37º C/ 5%CO_2 in a Lab-Tek II chamber slide. THP-1 cells were then co-cultured with A549 cells (2:1 ratio) for overnight. Cells were fixed in absolute methanol (200 µl) for 5 min, and stained with diluted Giemsa stain (1:20, v/v) for 20 min to visualize the macrophages and tumor cell uptake. Images were visualized under light microscope (Cx25 iOLYMPUS) at 100x (Sim Choi *et al.*, 2006).

$$Percent\ engulfment = \frac{Number\ of\ engulfed\ A549cells}{Total\ number\ of\ cells}\ X\ 100$$

$$Percent\ difference\ in\ engulfed\ cells = \frac{Mcontrol - Mtreated}{Mcontrol}\ X100$$

Where Mc and Mt represents Mean of total no cells counts of control and treatment respectively.

3.10.2 CFSE Assay

CFSE is a fluorescent cell staining dye. Itretains within the cells for longer periods due to covalent coupling reaction of its succinimidyl group to intracellular molecules (intracellular lysine residues and other amine sources). Also, due to this stable linkage, once incorporated within cells the dye is not transferred to adjacent cells.

1X10^5 A549 cells were treated with H_2O_2 for 15 min and 24 hrs. Cells were trypsinized and stained with CFSE (16 µM/ PBS) at 37º C/ 5% CO_2 for 30 min

(Vander Top *et al.*, 2006). The cells were then washed with PBS to remove the unbound dye and stained cells were co-cultured overnight with PMA differentiated THP-1 cells as above in a Lab-Tek II chamber slide. The fluorescent cells which get phagocytosed by macrophages were visualized under Cx25 iOLYMPUS microscope.

$$Percent\ engulfment = \frac{Number\ of\ engulfed\ A549cells}{Total\ number\ of\ cells}\ X\ 100$$

$$Percent\ difference\ in\ engulfed\ cells = \frac{Mcontrol - Mtreated}{Mcontrol}\ X100$$

Where Mc and Mt represents Mean of total number cells counts of control and treatment respectively.

3.11 Assay to Detect Respiratory Burst

3.11.1 NBT Assay

NBT assay is spectroscopic method to determine the production of superoxide anion $O_2^{\cdot-}$ by various phagocytic cells. NBT is water soluble, yellow colored compound. Upon reduction by ROS, it forms blue formazen crystals.

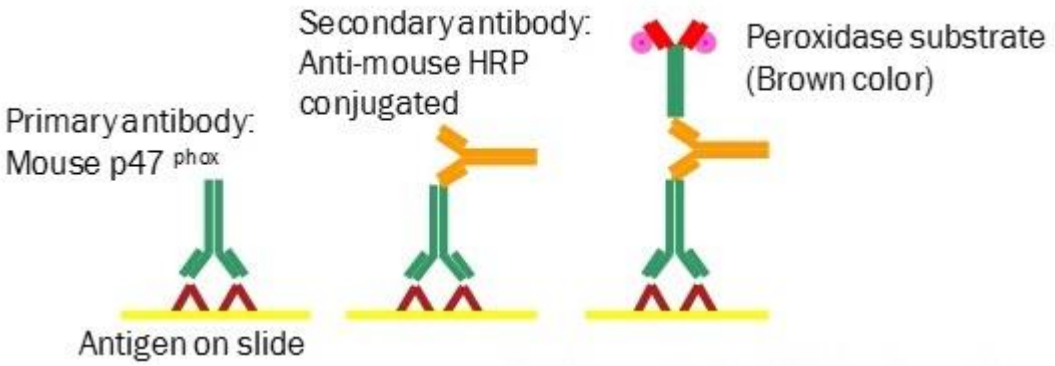
PMA treated THP-1 cells ($1x10^5$) were co-cultured with control, short term (15 min) and long term (24 hrs) H_2O_2 treated A549 ($1x10^5$) cells in 6 well plates for 24 hrs. After 24 hrs, cells were treated with 0.1% NBT (500 µl) and plates were incubated at 37ºC/ 5%CO_2 for 45 min in dark. NBT was removed; cells were washed with 1 ml PBS and snap cooled with 1ml chilled methanol. 600 µl of 2 M KOH and 700 µl of DMSO were used to dissolve crystals. Absorbance was measured at 570 nm and 630 nm(Sim Choi *et al.*, 2006).

$$\text{Percent ROS level} = \frac{\text{Mean treated (OD)}}{\text{Mean control (OD)}}\text{X100}$$

3.11.2 Immunohistochemistry

Immunohistochemistryrefers to the process of detecting antigens byusing the principle of antibody-antigen interaction, which can be visualized by numerous methods such as, antibody conjugated to an enzyme, that can catalyze a color-

producing reaction. It is commonly used for estimating the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue.



Adopted from R&D Systems Tools for Cell Biology Research™

The p-47^{phox} Antibody (A-7) (Santa Cruz) is a mouse monoclonal IgG_1 antibody. It is human in origin, also referred as NCF (neutrophil cytosolic factor 1), is required for NADPH oxidase activity. NADPH oxidase activity during ROS production causes p-47^{phox} to migrate to the membrane(Dhiman *et al.*, 2011).

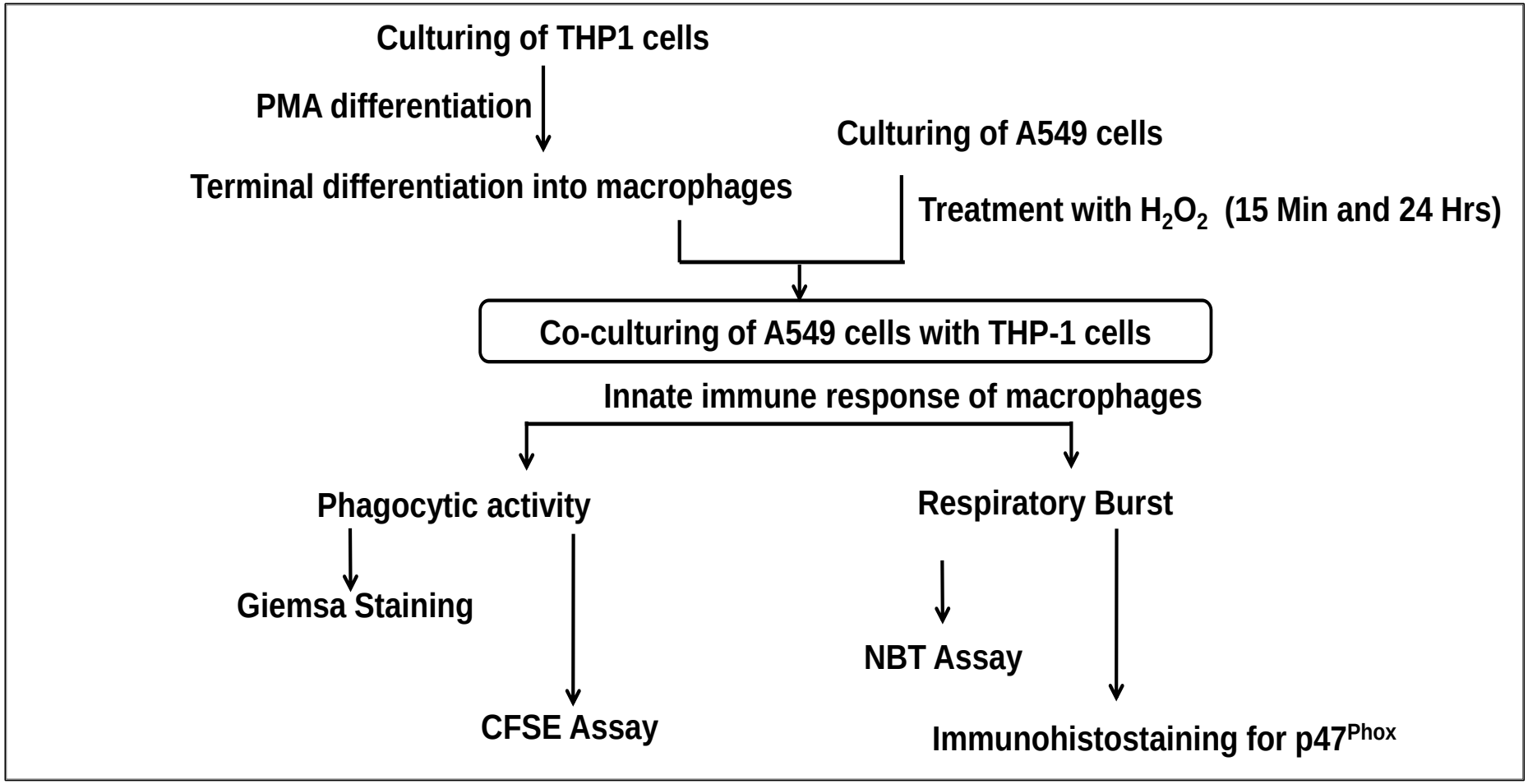
Control and H_2O_2 treated A549 cells were co-cultured with differentiated THP-1 (PMA, 10 ng/ul) in 8 Lab-Tek II chamber slide for overnight incubation at 37ºC/ 5%CO_2 in sterile conditions. After washing with PBS fixed the cells with chilled acetone for 5 min and dried. The slide was then treated with 0.3% H_2O_2/ PBS for 15 min. The blocking reagent (1% BSA/ PBST) was added for 30 min. The slide was then washed twice, added p-47^{phox} mouse antibody (1:500 dilution) for 1 hr at room temperature and washed again with PBST. It was incubated for 30 min with anti-mouse-HRP conjugated secondary antibody (1:1000 dilution). DAB (5 mg/10ml) and 10µl of 3% H_2O_2 was added for color development. After washing with distilled water, counterstained with Mayer's hematoxylin (Invitrogen) and again washed with water and dried. Finally the slide was mounted in DPX and images were taken in the Cx25 iOLYMPUS microscope.

$$Percent\ positive\ cells = \frac{Positive\ staining\ cells}{Total\ cells} X100$$

3.12. Statistical analysis

The sample analysis was done in triplicates and assays repeated at least thrice. Data was presented as mean ± S.D for control as well as experimental samples. For all statistical comparison, Student's t-test was used with p value < 0.05 was considered statistically significant.

Work Outline for Objective 2



CHAPTER 4
RESULTS

Assessment of Oxidative Stress and Neoantigen Formation

The cytotoxic and oxidative effect of H_2O_2 was estimated. The neoantigen once formed due to oxidative stress was using various techniques.

Effect of different concentrations of H_2O_2 on cell viability

Cytotoxic response of H_2O_2 was determined by treating A549 cells with different concentrations of H_2O_2 (0-200 µM) for 24 hrs (Figure 4.1). The cells when treated with 50 and 100 µM of H_2O_2 showed no significant difference in percent cell viability as compared to untreated control (83.1% ± 32.1 and 87.0% ± 35.4 vs. 100, respectively). However, 20 and 200 µM conc. of H_2O_2 showed significant decline in viability when compared to untreated control (70.1% ± 4.5 and 63.1% ± 8.6 vs. 100, respectively). Thus, these two concentrations were significantly toxic to cells while, 150 µM too had minimal toxicity (78.9% ± 19.7 vs. 100).

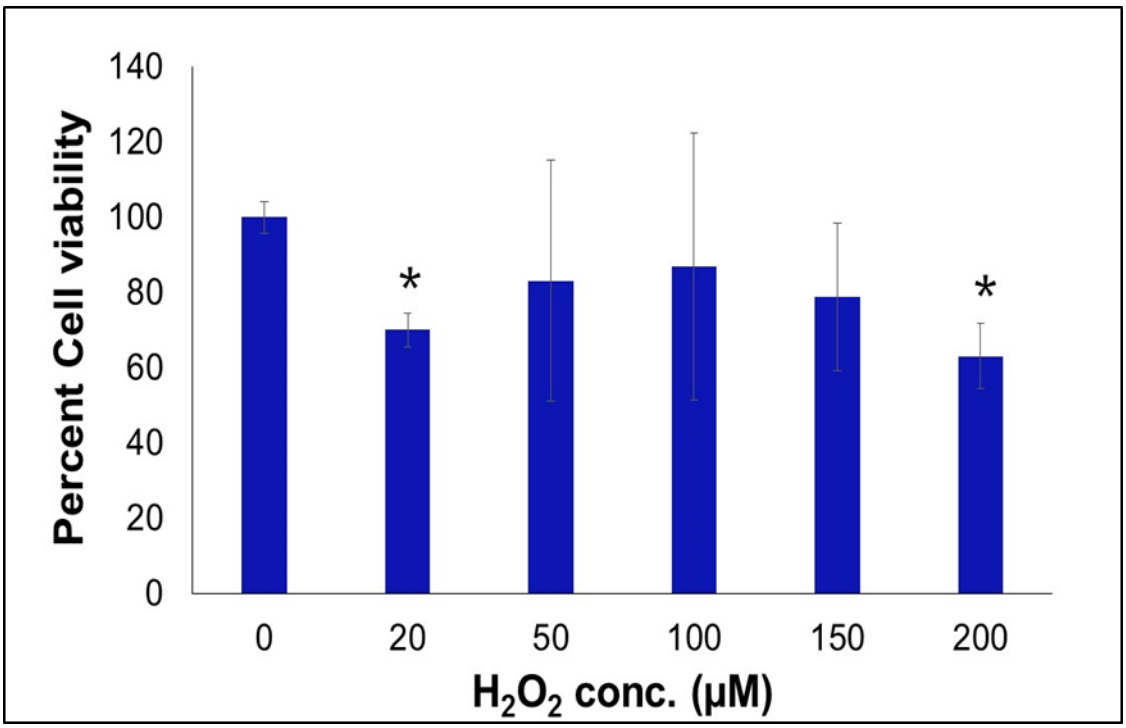


Figure 4.1: The percent viability of A549 cells when treated with increasing concentrations of H_2O_2 (significant at *p<0.05)

100 µm did not show significant effect on cell viability with respect to untreated control. For the further experiments this concentration was used as an oxidative stress inducer in A549 lung cancer cell line.

4.1.2. Neoantigen detection

Some modification might occur in the cells following oxidative stress, in form of H_2O_2 treatment that might be responsible for the neoantigen formation. In order to detect the neoantigens, the H_2O_2 Treated and control cell lysates (20 µg of protein) were resolved on 10% polyacrylamide gel. Figure 4.2 shows the gel stained with coommasie brilliants blue (CBB) depicting a few bands that were observed only in H_2O_2 treated samples while, the corresponding bands were not visible in case of lysate from control cells suggesting the formation of oxidised neoantigens or modified proteins. We used *in vitro* oxidised BSA as our positive control.

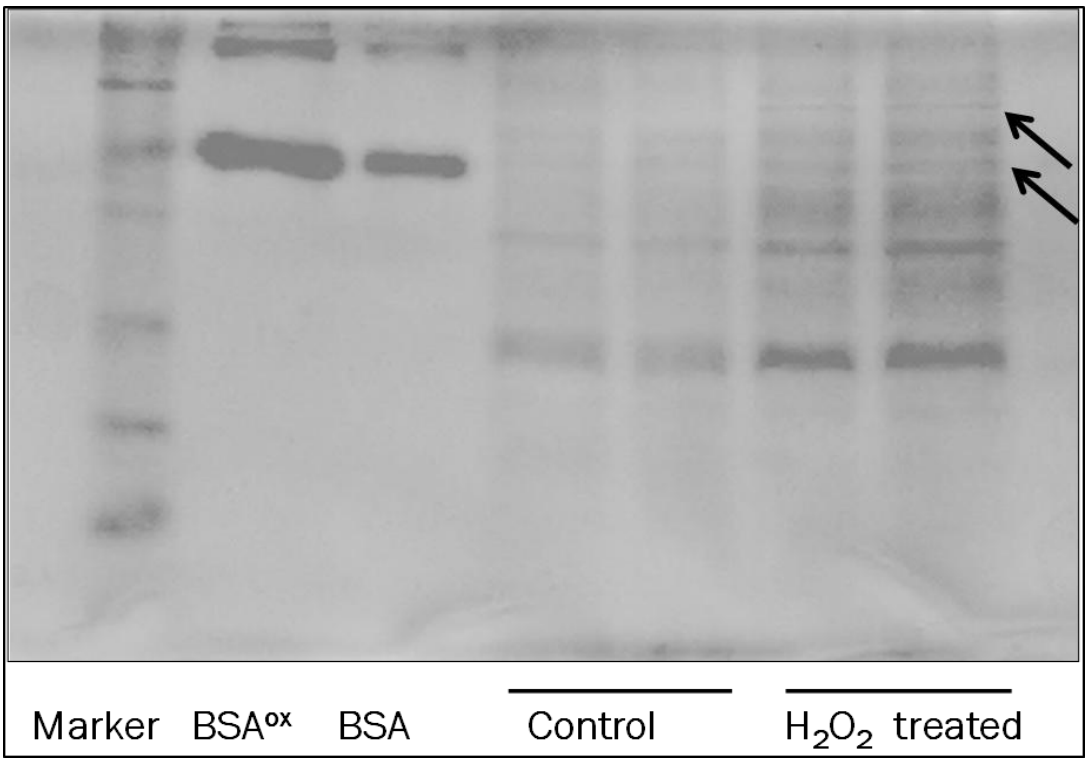


Figure 4.2: Polyacrylamide Gel stained with Commassie Brilliant Blue. Lane 1: Marker (Protein ladder as size standard), Lane 2: BSA^{ox} (Positive control), Lane 3: BSA, Lane 4, 5: Control (without treatment), Lane 6, 7: H_2O_2 treated samples (100 µM).

4.1.3 Protein Carbonyls Detection

Protein carbonyl content is a measure of oxidative damage in proteins produced due to ROS. Hydrazones formed after reaction of DNPH and carbonyls

(by product of protein oxidation) were estimated by spectrophotometric analysis at 370 nm.

A significantly high protein carbonyl control (20.6 ± 7.4) was observed for H_2O_2 treated cell lysate (24 hrs) when compared with untreated controls (7.7 ± 1.2) (Figure 4.3) which indicates the oxidative stress induced protein modification due to H_2O_2 treatment.

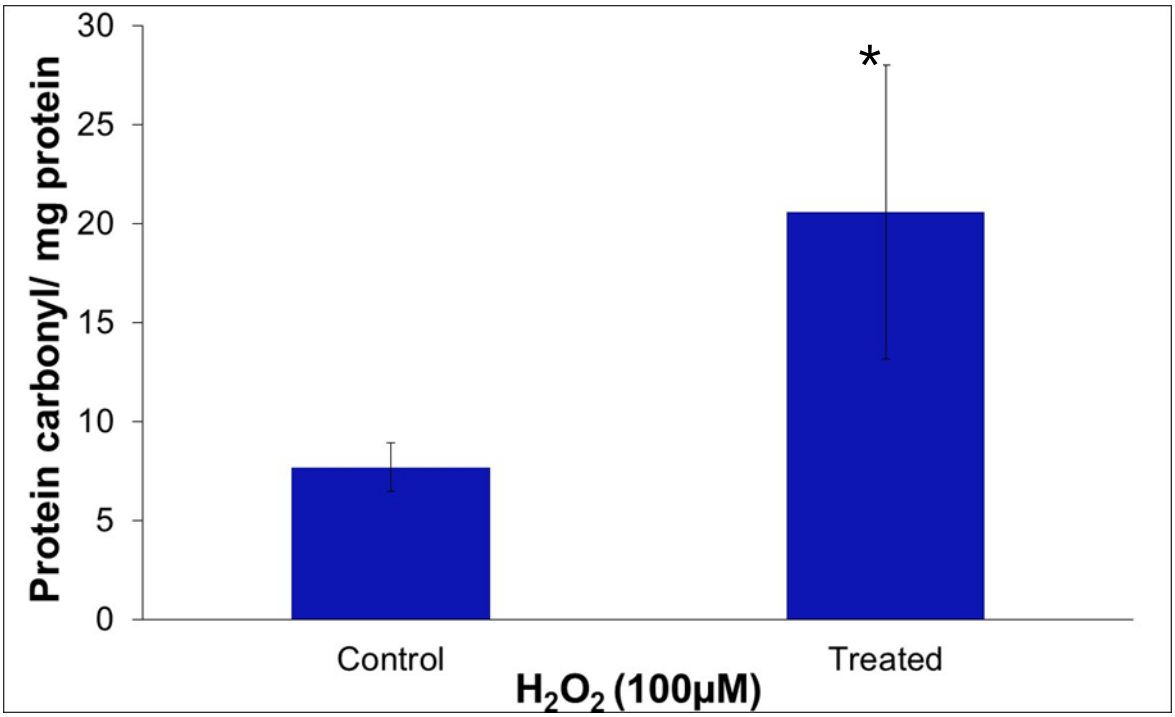


Figure 4.3: Protein carbonyl content of control and H_2O_2 treated (100 µM) A549 cells. Amount is expressed as nanomols of protein carbonyls per mg of protein (significant at *p<0.05).

4.1.4 TBARS Estimation

Oxidative stress might lead to oxidation of lipids present in cell membrane resulting reaction of malondialdehyde (MDA) (end product of unsaturated fatty acid peroxidation) with thiobarbituric acid (TBA) result in formation of thiobarbituric acid reactive species (TBARS). Control and H_2O_2 (100 µM) treated A549 cell lysates were examined for lipid peroxidation. Figure 4.4 shows the significant increase (64.8%) in level of lipid peroxidation in H_2O_2 treated cells. The mean TBARS content in treated A549 cell culture was 6.1 ± 1.5 when compared with untreated control (3.7 ± 0.9).

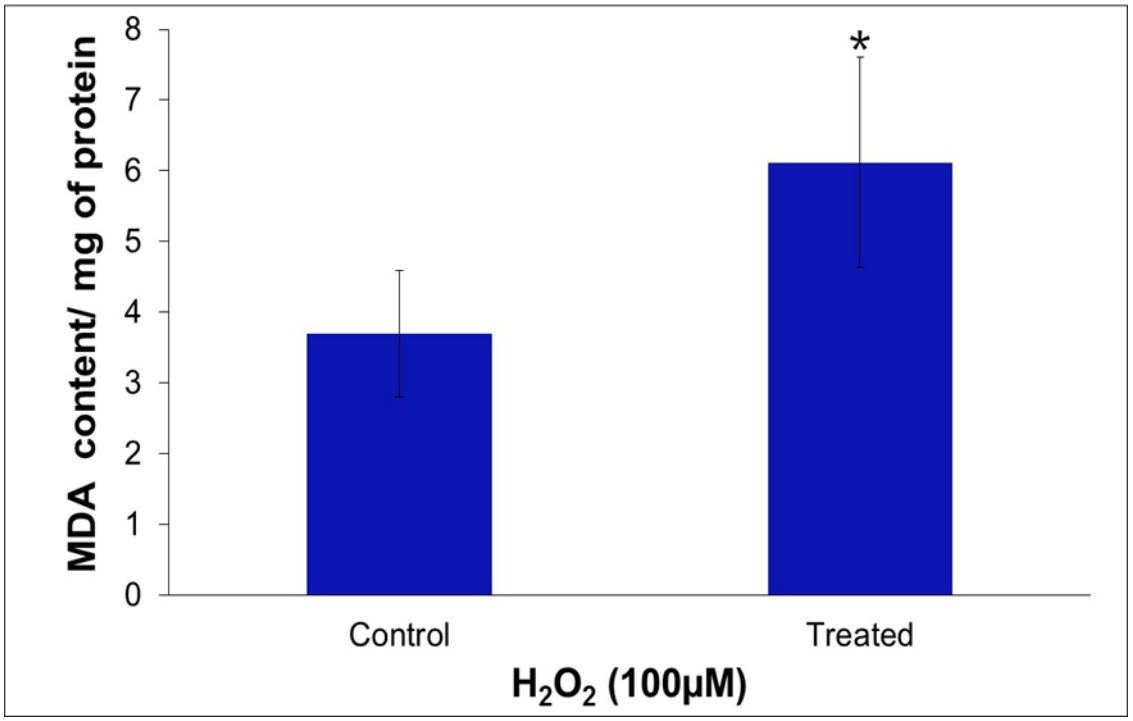


Figure 4.4: TBARS content is expressed as nanomoles (nmoles) per mg of protein of control and H_2O_2 treated (100 µM) A549 cells (significant at *p<0.05).

4.2 Neoantigens and Immune Escape by Tumor cells

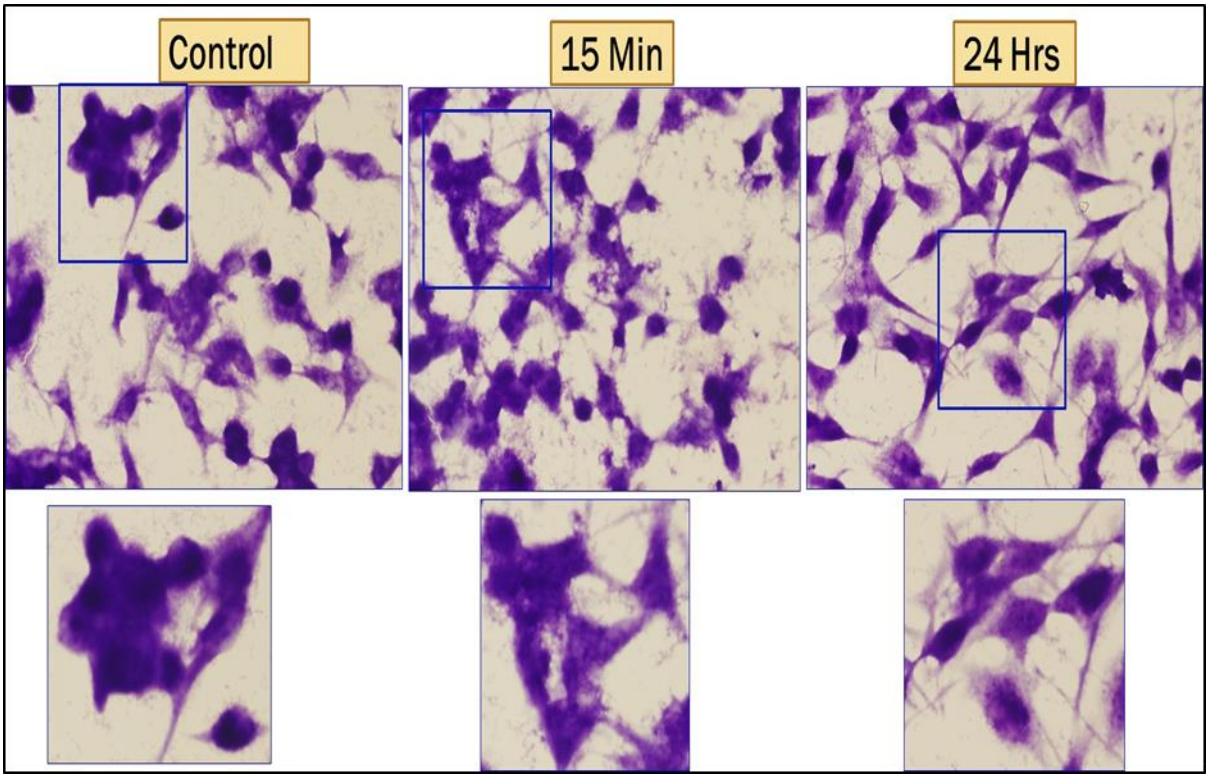
The innate immune responses are important to detect and kill the tumor cells. Oxidative stress is responsible for causing damages at molecular level as verified from the above results. These changes leads to generation of neoantigen and whether or not neoantigens are responsible for immunoescaping of cancer cells from innate immune response, phagocytosis based on staining methods and respiratory burst was assessed. For this, the differentiated THP1 macrophages were co-cultured with control as well as short and long-term treated A549 cells and extent of innate immune response in form of phagocytic activity and/ or respiratory burst was determined.

4.2.1 Phagocytosis Assay

Phagocytosis is an innate immune response exhibited by macrophages thereby engulfing the foreign particles. Neoantigens formed under the influence of oxidative stress may help tumor cells evade the host's immune response.

4.2.1.1 Giemsa Staining

A549 cells were treated with H_2O_2 for 15 min and 24 hrs followed by co-culturing with THP-1 cells and were subsequently visualized for the presence of phagocytic activity by Giemsa staining under light microscope (Panel 1).



Panel 1: Cells stained with Giemsa exhibiting phagocytosis of A549 cells by THP-1 cells.

Figure 4.5 depicts a significant reduction in percent engulfed cells (85%) for long term (24 hrs) treated cell culture whereas, in case of short term treated A549 cells the difference in the proportion of engulfed cells (15%) was not significant in comparison to control (Table 4.1).

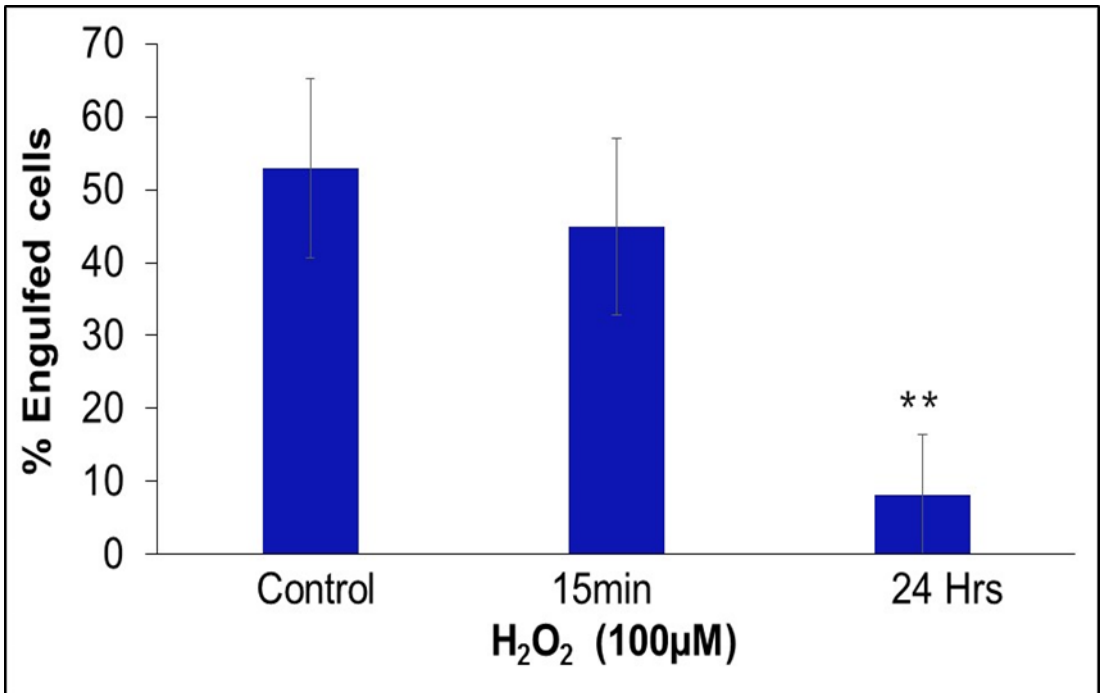


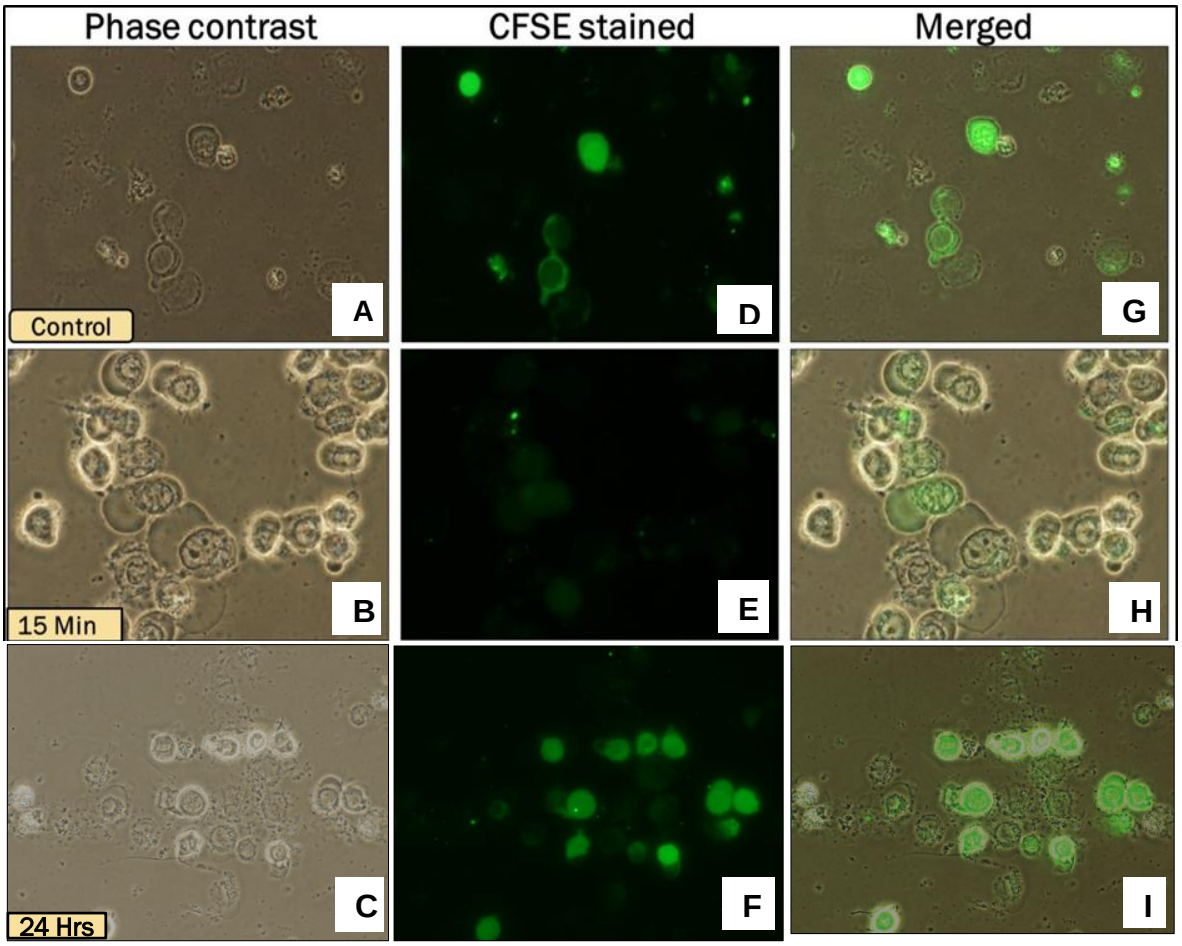
Figure 4.5: Percent A549 cells engulfed by THP1 macrophages following H_2O_2 (100 µM) treatments (significance at **p<0.01)

Table 4.1: Percent engulfed A549 cells following various treatments of H_2O_2 after co-culturing with THP1 cells (significance at **p< 0.01) as observed during Giemsa staining.

Treatment	No. of THP-1 cells	No. of Free A549 cells	No. of Engulfed A549 cells	% Engulfed cells	Mean ± S.D.
Control	153	5	64	41.8	53 ± 16.9
15 min H_2O_2 treatment	147	3	72	49.0	45 ± 17.8
24 hrs H_2O_2 treatment	293	5	69	23.5	8 ± 8.3**

4.2.1.2 CFSE Assay

The co-cultured cells were stained with CFSE to measure the phagocytosed fluorescent A549 cells by THP-1 cells using fluorescent microscope (Panel 2). In Panel 2, the stained cells (bright green) correspond to the free A549 cells that are not engulfed by THP-1 cells.



Panel 2: Phase contrast (A-C), CFSE stained (D-F) and merged pictures (G-I) exhibiting phagocytosis of A549 cells.

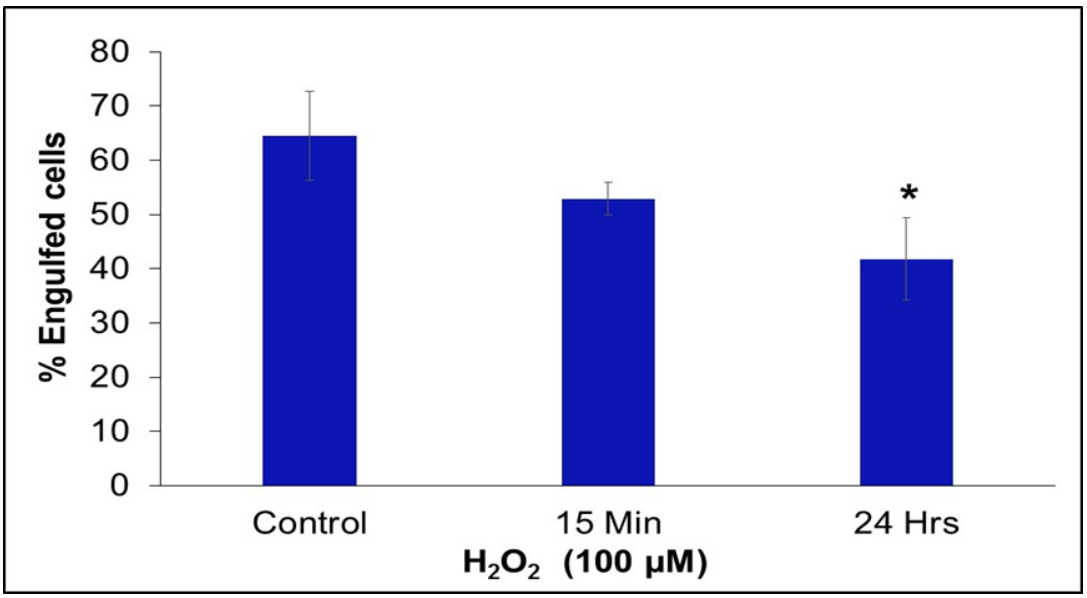


Figure 4.6: Proportion of A549 cells engulfed by THP1 macrophages following H_2O_2 (100 µM) treatments (significant at *p<0.05)

The long term (24 hrs) treated A549 cells showed 35% decline in number of engulfed cells when compared to control whereas, in case of short term treated A549 cells the difference in the proportion of engulfed cells (23%) was not statistically significant (Table 4.2 and Figure 4.6).

Table 4.2: CFSE stained cells depicting percent engulfed A549 cells following various treatments of H_2O_2 after co-culturing with THP1 cells (significance at *p< 0.05)

Treatment	No. of THP-1 cells	No. of Free A549 cells	No. of Engulfed A549 cells	% Engulfed cells	Mean ± S.D.
Control	67	5	45	67	65 ± 8.18
15 min H_2O_2 treatment	241	2	164	68	53 ± 3.01
24 hrs H_2O_2 treatment	67	9	31	44	42 ± 7.5*

4.2.2 Respiratory Burst Estimation

Respiratory burst is another defence mechanism of innate immune cells in response to foreign particles during phagocytosis. The phagocytosis generated ROS responsible for killing the cancerous cells and foreign particles. The ROS levels were measured by NBT assay and immunostaining for NADPH Oxidase subunit p-47^{phox}.

4.2.2.1 NBT Assay

Free radical production by macrophages was determined from NBT assay. Intracellular ROS are responsible for reduction of NBT inside the cells. A significant decrease of 25% and 36% in ROS activity was observed for THP1 co-culture with short term (15 min) and long term (24 hrs) H_2O_2 treated respectively when compared to the untreated control (Figure 4.7, Table 4.3).

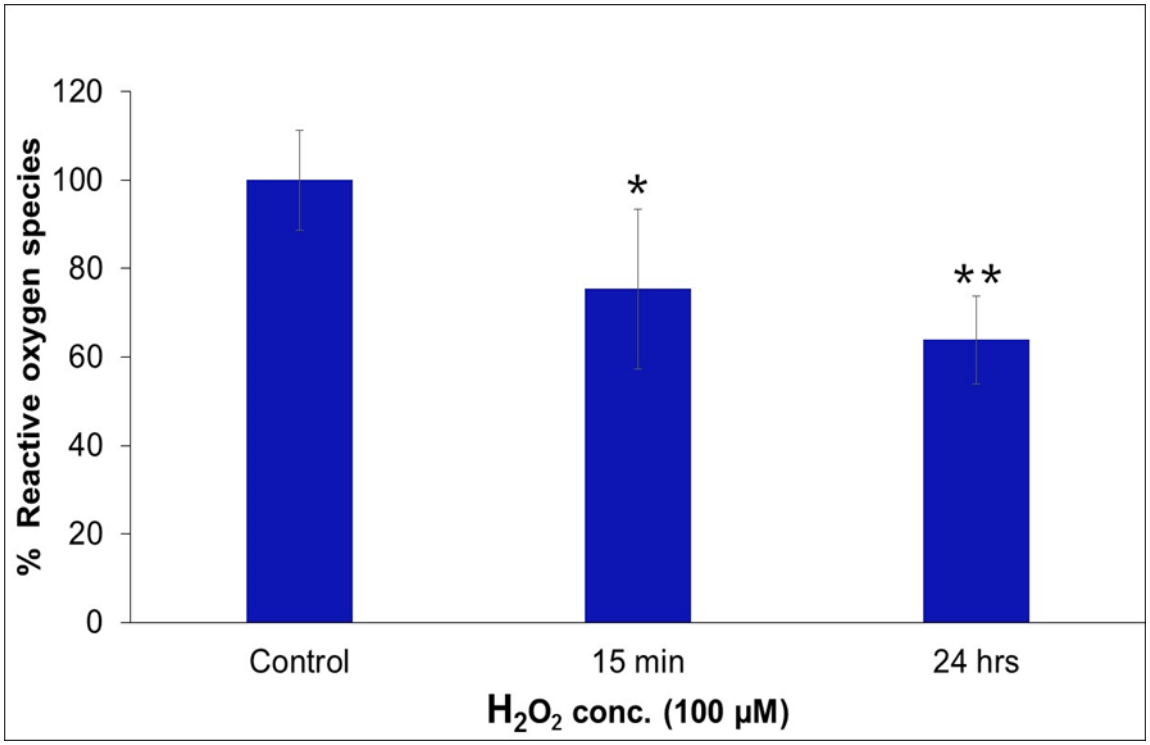


Figure 4.7: Percent ROS generated in co-cultured cells following various treatments (significant at *p<0.05, **p<0.01)

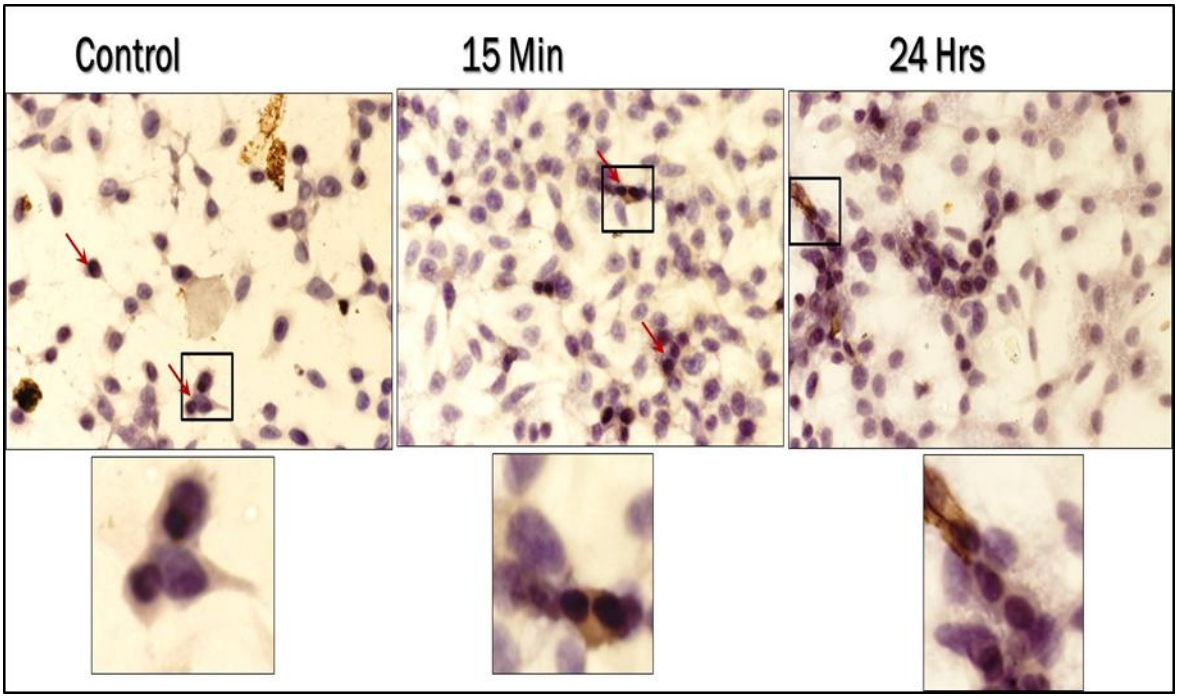
Table 4.3: ROS levels detected by NBT assay (significant at *p<0.05, **p<0.01).

Treatment	ROS conc. (Mean ± SD)	%ROS level
Control	0.27 ± 0.03	100
15 min	0.20 ± 0.04*	75.4
24 hrs	0.17 ± 0.02**	63.9

4.2.2.3 Immunohistochemistry (IHC)

To analyse the source of ROS generation in phagocytic cells during phagocytosis, immunohistostaining experiment was performed. The cytosolic unit of NADPH oxidase which is one of the most important enzymes in the oxygen dependent phagocytosis was used for the IHC.

A549 cells were treated with H_2O_2 for different time intervals followed by co-culturing with THP-1 cells and subsequently stained with anti $p47^{phox}$ immunostaining under microscope (panel 3).



Panel 3: Immunohistaining pictures to detect the respiratory burst in co-cultured A549 cells after various treatments.

The THP-1 cells co-cultured with H_2O_2 treated A549 cells for 24 hrs showed two fold increase in $p47^{phox}$ positive cell staining whereas in the cells treated for short time showed a significant change of 55%. These result suggest that during the phagocytosis the NADPH-oxidase enzyme is not getting compromised and its function is not impaired as it is getting activated even more in the H_2O_2 treated A549 cells (24 hrs) (Figure 4.8, Panel 3, Table 4.4).

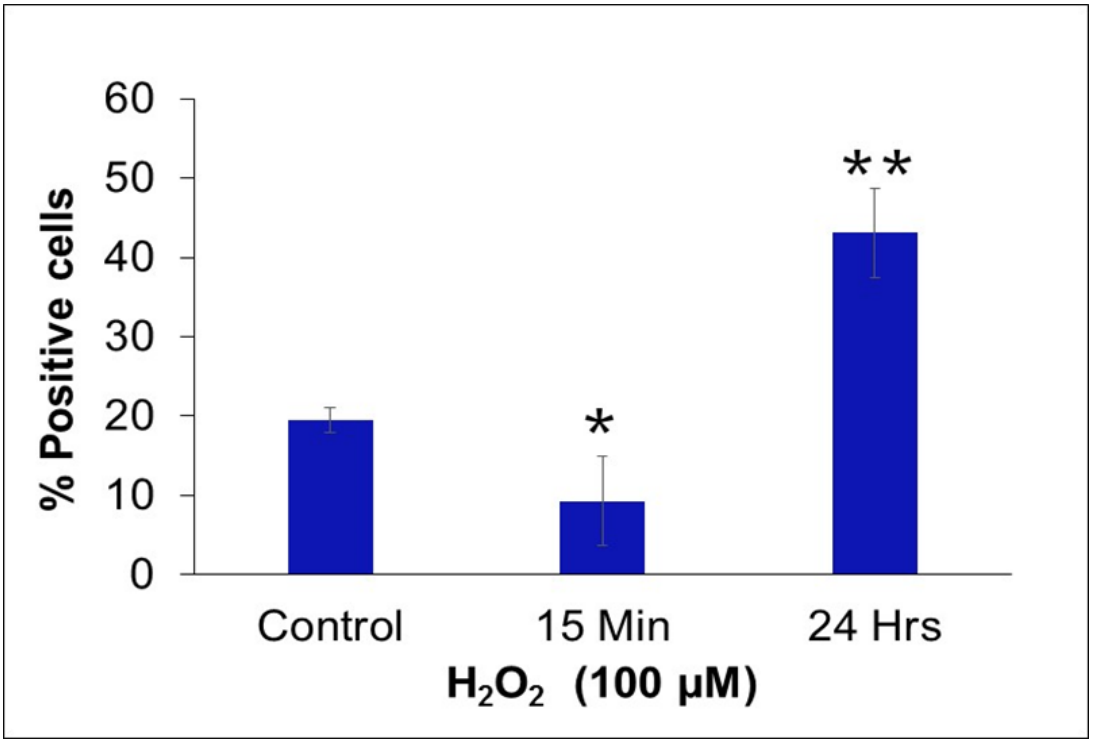


Figure 4.8: Percent positive cells showing respiratory burst for immunohistostaining (significant at *p<0.05, **p<0.01)

Table 4.4: Percent p-47^{phox}positive cells estimated by immunohistochemistry (significant at *p<0.05, **p<0.01)

Treatment	Total no. of cells	Positive for p47^{phox}	% Positive cells	Mean ± SD
Control	344	78	22.7	20 ± 1.59
15 Min	552	69	12.5	9 ± 5.66*
24 Hrs	614	208	33.9	43 ± 5.66**

CHAPTER 5

DISCUSSION

Malignant tumors of the lungs are the most prevalent oncological diseases making lung cancer one of the leading cause of deaths due to cancer (Ferlay *et al.*, 2010). Reactive oxygen species (ROS) resulting from inflammation and other stresses are among the major factors responsible for the disease (Azad *et al.*, 2008). Several mechanisms have been proposed by which oxidative stress can result into carcinogenesis through ROS production. Commonly produced intracellular ROS include superoxide ($O_2^{\bullet-}$),hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$), which can react with proteins, lipids and DNA. Cellular macromolecules can suffer innumerable damages through ROS overproduction, which in turn can stimulate various signal transduction pathways and/ or transform the resultant gene expression patterns (Klaunig *et al.*, 2010). ROS when accumulate in form of oxidative stress consequently trigger adaptive changes at molecular level. Since the endogenous ROS levels are much higher in cancer cells therefore, an above threshold value to induce toxicity can be achieved faster than in normal cells (Khan *et al.*, 2012; Laurent *et al.*, 2005). On the other hand, increased endogenous ROS are also responsible for tumorgenesis, metastasis and resistance to radiation and chemotherapy (Ishikawa *et al.*, 2008; Shi *et al.*, 2012).

In this study, we examined the role of oxidative stress on A549 lung carcinoma cell line and their escape from immune response where the oxidative stress was achieved by implicating H_2O_2 treatment. From cytotoxicity assay, we found that 100 µm concentration of H_2O_2 can induce oxidative stress without killing cells (Kim *et al.*, 2012). Treatment duration of 24 hrs was demonstrated to bring molecular changes (protein and lipid) to a significant level as estimated by biochemical assays. The protein profile on SDS- PAGE displayed some additional bands in 24 hrs oxidized cells as compared to untreated control. This could be associated to structural changes in protein subunits that might be possibly resulting into the formation of some neoantigen. It is known that hydrogen peroxide, a precursor to hydroxyl radical, is less reactive and more readily diffusible and thus more likely to be involved in the formation of oxidized bases through Fenton and Haber-Weiss reactions (Klaunig *et al.*, 2010).

Protein carbonyl and lipid peroxidation have emerged as potent biomarkers of oxidative stress and tissue damage resulting from chronic inflammation, in

various physiological disorders *viz*., sepsis, cardiovascular diseases and diabetes mellitus and cancer (Kirkham *et al.*, 2003).

Proteins are among highly structurally and functionally diverse biological macromolecules and act as the chief ROS scavenger inside the cells. These reactive chemical entities or their secondary products disrupt the protein structure and conformation via different mechanisms; peptide backbone cleavage, cross-linking, amino acid side chain modification.Addition of carbonyl derivatives may change the protein conformation, and/or even cause fragmentation of the polypeptide chain, thus responsible for partial or total inactivation of proteins. In addition, it can even result in the loss of enzymatic activity, increased proteolytic degradation, altered cellular functions such as energy production, interference with the creation of membrane potentials and changes in the type and level of cellular proteins(Silva *et al.*, 2010).Determination of protein carbonyl, by spectrophotometric assay like DNPH (a highly sensitive approach), is an indicative of protein modifications caused by oxidation (Dalle-Donne *et al.*, 2003; Popadiuk *et al.*, 2005). A higher protein carbonyl content in H_2O_2 treated A549 cells indicates a significant protein oxidation. An elevated protein carbonyl level has also been documented previously patients suffering from colorectal cancer (Yeh *et al.*, 2010), lung cancer (Bartling *et al.*, 2011)and breast cancer (Rossner *et al.*, 2007), suggesting the role of oxidative stress in tumorigenesis.

Lipids are the primary constituents of cell membranes. Polyunsaturated fatty acids (PUFAs), because of their multiple double bonds, are also extremely sensitive to oxidation by free radical attack. In addition, PUFAs can be formed enzymatically by the action of lipoxygenases. Arachidonic and linoleic acids are the main PUFAs in the mammalian membranes and are able to undergo both enzymatic and non-enzymatic lipid peroxidation(Niki *et al.*, 2005).Any modifications in lipids can lead to the variation in properties of membranes; hampering the fluidity and affecting membrane-bound proteins, which in turn may influence normal cellular function and permeability. ROS contribute to cellular damage by causing peroxidation of lipid (Dalle-Donne *et al.*, 2006; Rahman *et al.*, 2002) the decomposition products, malondialdehyde, can be detected by TBARS assay. A significant alteration in membrane lipids was observed as exhibited by TBARS (a by-product of lipid peroxidation) in the H_2O_2 treated A549 cells as

compared to the untreated cells. These results were well supported by previously conducted studies on other cell lines that displayed an increased lipid peroxidation products (Bowie *et al.*, 1997; Estrada-García *et al.*, 2002; Zhang *et al.*, 2010).

The human THP-1 (macrophage) cell line is a widely used *in vitro* model system for studying macrophages differentiation and function (Grodzki *et al.*, 2013). Macrophages are produced by monocyte differentiation and participate in non-specific defense (innate immunity) and initiate specific defense system (adaptive immunity) as well by acting as antigen presenting cell. The primary function of macrophages is phagocytosis and production of ROS which are the major effectors of the process to kill or to engulf the altered or non- self cells. To test this, we performed phagocytic staining. As a measure we performed Giemsa and CFSE staining to have a light microscope and fluorescent based assay respectively. The long term treated A549 cells co-cultured with THP1 macrophages in both assays (Giemsa and CFSE) showed some interesting immune escaping characteristics. Our experiments indicated that this amount of ROS favored the survival of A549 cells by promoting changes in antigen structure or conformation and hence, escaping from phagocytosis. ROS produced during phagocytosis by tumor associated macrophages (TAMs) are responsible for bringing changes in morphological and marker expression *in vitro* andconsequently contribute to metastasis and cells invasion (Liu *et al.*, 2013).

ROS levels were detected by nitroblue tetrazolium (NBT) test, which is an indirect measure of superoxide- dependent bactericidal activity of the phagocytes (James *et al.*, 1998). Phagocytes produce ROS, such as superoxide anion ($O_2^{\bullet-}$) via NADPH oxidase and hydrogen peroxide (H_2O_2) via superoxide dismutase, during oxidative respiratory burst (Cathcart, 2004; Sarna *et al.*, 2010). These reactive molecules serve as inflammatory mediators constituting a part of host immune response activity by killing the invading pathogens (Choi *et al.*, 2006). Respiratory burst in THP-1 cells co-cultured with 24 hrs treated A549 cells showed significant decrease whereas, the 15 min treated A549 cells showed slight change in ROS level.

NADPH oxidase activation is a key downstream event of phagocytosis and a central player for pathogen killing in phagocytic leukocytes. The NOX2 NADPH

oxidase is composed of a membrane-bound flavocytochrome b_{558} (composed of NOX2/gp91^{phox}/cytb and p22^{phox}) and cytosolic components p67^{phox}, p47^{phox}, and p40^{phox}(Huang *et al.*, 2009).

We observed very surprising results with immunohistostaining for NADPH oxidase (NOX) activity when used p47^{phox}when used as antibody.The THP-1 cells co-cultured with H_2O_2 treated A549 cells for 24 hrs showed very high p47phox positive cellsinferring that although the NOX levels are high, but this level of ROS produced by macrophages during respiratory burst is not enough to phagocytose the cell as noticed in previous experiments. Several other sources including mitochondrial respiration, xanthine oxidase, superoxide dismutase, myeloperoxidase, glutathione peroxidase system might be responsible for the release reactive species inside the cancerous cells or macrophages (Bur *et al.*, 2014; Kirkham, 2007) which may be activating the NOX activity further.Oxidative stress boosts the inflammatory response and decrease the phagocytic activity of the activated macrophages towards apoptotic or cancer cells (Neyen *et al.*, 2013). This is due to the interaction of macrophages with modified extracellular matrix (ECM) proteins Since, macrophages constitute a major portion of the infiltrate surrounding the tumor during earlier phases and instead of being recognized usually as cytotoxic, may promote growth of tumor(Quail *et al.*, 2013).

In the previous studies it is reported that macromolecular changes linked to oxidation of lipids and proteins can also provide protection to cancerous cells from phagocytosis (Reuter *et al.*, 2010).The macrophage mediated innate immune responses are important to detect and kill the tumor cells. However, the interactions with this inflammatory microenvironment somehow favour tumor development, approach to inhibit these interactions without disturbing the normal physiology can work for the associated cancers (Sethi *et al.*, 2012).The altered antigens, generated while apoptosis and oxidation of proteins and lipids, can also indulge in tumor resistance to radiotherapies and host immune system (Brown *et al.*, 2014; Catera *et al.*, 2008).Targeting these neoantigens can be adopted as a strategy in the anti-cancer therapy. The present study has identified novel mechanism(s) of carcinogenesis initiation which can further provide directions for the development of adjunct therapies to control cancer in its initial stage.

SUMMARY

Immune system helps in protection against invading pathogen and cancer. Cross talk between the immune response and cancer cells decides the fate of tumor development. The altered molecular mechanisms underlying cancer development needs to be delineated. In addition to reported genetic modifications on tumor cells, oxidative stress-mediated modifications (such as formation of chemical adducts, e.g. 3-hydroxynonenal, 3-nitrotyrosine, carbonyl etc.) takes place at the vicinity of tumor where immune cells starts infiltrating. The central idea of the present work is that respiratory burst which is host's mechanism to kill the foreign particles (tumor cells) is used as defence mechanism by the tumor cells by forming neoantigen which in turn makes them undetectable and further helps them to escape the host immunesurveillance. The non-small cell lung carcinoma A549 cells were treated with oxidant (100µM H_2O_2); and using 1-D gel electrophoresis technique, the oxidized tumor proteins in normal and treated cells were visualized. Oxidatively-modified lipids (lipid peroxidation products) and proteins (protein carbonyl products) were detected respectively. It was determined if neo (oxidized) tumor antigens elicit any alteration in immune responses where they show compromised phagocytosis, thus resulting in a failure to elicit effector immune functions. This was analyzed via phagocytosis and respiratory burst using spectrophotometry and microscopic techniques.

Present study has identified a novel mechanism(s) of carcinogenesis initiation which can further direct the development of adjunct therapies to control cancer in its initial stages, and at the same time it also advocates for new ventures to increase the efficacy of the chemotherapeutic interventions. The interconnections between signaling pathways that control immune escape and those that control proliferation, senescence, apoptosis, metabolic alterations, angiogenesis, invasion and metastasis remain virtually unexplored, offering rich new areas for investigation. Learning how immune escape evolves during the integrated processes of oncogenesis and immuneediting may therefore yield more powerful insights into cancer pathophysiology and therapy than achieved to date.

These advances of neoantigen concept may lead us to design of new therapeutic agents and their modulation will be exploited as a major mechanism to selectively kill cancer cells.

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