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Hydrogen peroxide-induced oxidative stress and its impact on innate immune responses in lung carcinoma A549 cells

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Abstract

The immune responses, involved in recognition of cancer-specific antigens, are of particular interest as this may provide major leads towards developing new vaccines and antibody therapies against cancer. An effective treatment for cancer is still a challenge because there are many mechanisms through which the tumor cells can escape the host immune surveillance. Oxidative stress or respiratory burst which is host's mechanism to kill the foreign particles is used as defense mechanism by the tumor cells. The tumor cells uses this oxidative stress to form neo-antigens which in turn makes them undetectable and can escape the host immune surveillance. The human lung carcinoma (A549) cells were treated using 100 µM H_2O_2 to induce oxidative stress, and the extent oxidative modifications were detected at the level of membrane and proteins in form of lipid peroxidation and protein carbonyls respectively. Nitric oxide and iNOS levels were estimated by Griess assay and immunostaining, respectively. The oxidized tumor proteins were visualized on one-dimensional SDS–PAGE. The H_2O_2-treated (15 min and 24 h post-treatment) A549 cells were co-cultured with THP-1 cells to subsequently visualize the phagocytic activity by Giemsa and CFSE staining to understand the role of neo (oxidized) tumor antigens in eliciting alteration in immune responses. A significant decline in the percent engulfed cells and decrease in the levels of reactive oxygen species was observed. Immunohistostaining for $p47^{phox}$, which is an important indicator of the oxygen-dependent phagocytosis, showed a decrease in its levels when cells were treated for only 15 min with 100 µM H_2O_2, whereas at 24-h post-treatment there was no change in the $p47^{phox}$ levels. The study has established oxidative stress as a new pathogenic mechanism of carcinogenesis and will open new avenues for clinical intervention, adjunct therapies for cancer, and its control at the initial stage by targeting these neo-antigens.

Keywords Neo-antigens · Protein oxidation · Respiratory burst · Immune surveillance

Abbreviations

CFSE	5,6-Carboxyfluorescein diester
DAB	3,3′-Diaminobenzidine
DMSO	Dimethyl sulphoxide
DNPH	Dinitrophenylhydrazine
MDA	Malondialdehyde,
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBT	Nitroblue tetrazolium salt
NOX	NADPH oxidases
NO	Nitric oxide
PMA	Phorbol 12-myristate 13-acetate
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive species

Introduction

Production of reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), hypochlorite ion (HOCl), hydroxyl radical ($OH^{\cdot}$), superoxide anion ($O_2^{\cdot-}$), etc., is an inevitable consequence of metabolism (in situ). Besides certain exogenous sources (pollution, cigarette, smoke, radiation, and medication), some endogenous sources are also responsible for the generation of ROS. ROS elevations under normal physiological conditions, due to the default activity of biological scavengers, are required to drive regulatory pathways [1].

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However, the higher levels of intracellular ROS (beyond a threshold) can lead to apoptosis or necrosis [2]. Chronic and degenerative ailments including, autoimmune disorders, aging, cataract, rheumatoid arthritis, cardiovascular diseases, neurodegenerative disorders, and cancer-associated complications arise due to perturbed ROS equilibrium [3].

ROS is a double-edged sword which can either suppress or promote the cancerous growth [1]. The chemical entities can prolong the cell survival and contribute to malignant transformation [4, 5] in multiple ways, right from the oral cavity [6] to lung [7], breast [8], prostate [9], gut [2], and ovarian tissues [7], and also play a role in hepatitis B/C virus-induced hepatocellular carcinoma [10]. These by-products of oxidative stress induce conformational changes in DNA, proteins, and lipids which if not degraded by the ubiquitin–proteasome system can further result in glycosylation, glycation, phosphorylation, or oxidation which affect the function and stability of biomolecules [11]. ROS can react with cysteine, histidine, lysine, arginine, proline, or threonine residues of proteins to produce carbonyls [12]. Similarly, lipid peroxidation due to ROS production during various pathophysiological conditions generates malondialdehyde (MDA) and 4-hydroxynonenal. These resulting products are known to promote cell proliferation and block apoptotic pathway [13]. Identifying these oxidized products of the macromolecules can, therefore, serve to determine the extent of oxidative stress induced during an abnormal physiological condition.

The immune defense mechanisms and their interaction with the tumor microenvironment are another important aspects where the tumor immune escaping can be considered [14]. The macrophage-mediated innate immune responses are important to detect and kill the tumor. Macrophages constitute a major portion of the infiltrate surrounding the tumor; they interact with modified extracellular matrix (ECM) proteins, and instead of killing, at times can favor the tumor development. Due to high inflammatory molecules such as iNOS, COX2, and proinflammatory cytokines in the tumor microenvironment, the tumor supporting cells such as tumor-associated macrophages (TAM) are formed which stimulate the migration and metastasis of cancer cells by secreting Epidermal Growth Factor [15] and by expressing the receptor for Colony Stimulating Factor 1 [16]. ROS can also account for the phenotypic plasticity of macrophage and can downregulate the immune responses via neo-antigen formation [17, 18]. The altered antigens, generated while apoptosis and/or oxidation of proteins and lipids, can indulge in tumor resistance to radiotherapies and host immune system [19–21]. The chemical changes in amino acid residues induced by ROS can alter the coding sequences as well as tertiary and quaternary organization of a protein. These mutated peptides might create new epitopes, called as neo-antigenic determinants. Neo-antigens display a modified version of the usual antigenic determinant, and that is why, they require a different, specific antibody for recognition [22]. The neo-antigens are the characteristics of autoimmune pathogenesis as well as cancer, and can be detected by Serological expression cloning (SEREX) and ELISPOT. Somatic mutations and neo-antigen density has recently been shown to cooperate in the progression of non-small cell lung cancer. Further, the patients with higher mutation burden or neo-antigen load were suggested to be correlated with an enhanced clinical response. As a high tumor mutation is associated with increased neo-antigens and tumor-infiltrating lymphocytes, these mutations can act as potential biomarkers for deciding the efficacy of cancer immunotherapy. In this context, there are success reports available for colorectal and other cancers, but a fewer for lung cancer [23–25]. In brief, oxidative stress triggers cellular damage as well as boosts the inflammatory response, both of which can decrease the phagocytic activity of the activated macrophages towards apoptosis of cancer cells [26]. In the present study, we aimed to investigate that whether oxidative environment can aid the escape of cancer cells from phagocytic defenses. Our central hypothesis is that the oxidative stress may alter tumor proteins resulting in the formation of novel epitopes, which if not recognized by the host immune system, due to being neo-antigens, may lead to accumulation of tumor cells resulting in carcinogenesis. The in vitro system of the human lung carcinoma cell line (A549) is utilized as a model for inducing oxidative modification using H_2O_2 as an oxidant. The subsequent response towards oxidative stress is realized based on the macromolecular damage, and respiratory burst, and phagocytic activity. Our study will pave the unique role of ROS which by forming neo-antigens on tumor cells if identified may open new avenues for clinical intervention and adjunct therapies for cancer.

Materials and methods

Cell culture

Human lung carcinoma A549 cells were procured from National Center for Cell Sciences (NCCS), Pune, India, and were grown in complete media DMEM supplemented with 10% FBS in 5% CO_2 humidified incubator. Human monocytic leukemia THP-1 cells were generously gifted by Prof. Kanuri Rao, ICGEB, New Delhi, India. Morphologically, THP-1 cells are large, round single cells grown in suspension and differentiated into macrophages on stimulation with PMA. 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% FBS. THP-1 cells were treated with 10 ng/ml PMA for 48 h to induce terminal differentiation of these cells into macrophages which then show adherence.

Cell treatments and preparation of cell lysates

A549 cells (1×10^6) were seeded and treated with H_2O_2 (100 µm) for 24 h in serum-starved conditions. For the co-culturing experiments, the A549 cells were treated with 100 µm H_2O_2 for 15 min to see its effect at initial stages as soon as it enters the cells and for 24 h to see the effect of H_2O_2 after the oxidative stress is induced to bring any change in the macromolecules. After the treatment, the culture supernatant was stored and cell lysate was prepared using 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. Amount of protein in the samples was estimated using Bradford's assay and the aliquots of cell lysates were stored at − 80 °C till further use.

Cytotoxicity assay

A549 cells (1×10^5) plated in 24-well plates were treated with different concentrations of H_2O_2 (0, 20, 50, 100, 150, 200 µM) in FBS-free media. After 24 h, 20 µl of MTT solution (5 mg MTT dissolved in 1 ml sterile PBS) was added followed by incubation for 4 h at 37 °C. The purple product was solubilized using 200 µl of 10% SDS in acidified DMSO and absorbance was measured at 570 nm. The absorbance read is directly proportional to number of living cells [27]. The percent cell viability of each group was calculated with respective controls.

Detection of neo-antigens

A549 cell lysates from all the treated and untreated groups (20 µg of protein) were resolved on 10% SDS–PAGE on a Mini-Protean 3 system (Bio-Rad) 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% Coomassie blue G250, and images were acquired using advanced gel documentation system (Bio-Rad).

Detection of protein carbonyls

A549 cell lysates (50 µg) were derivatized with DNPH (100 mM in 2 M HCl) at 37 °C for 90 min. Protein precipitation was done with TCA (28%) followed by washing with ethanol and ethyl-acetate solution (1:1). After final washing, the samples were centrifuged at 6000 rpm for 6 min and the pellet resuspended in 6 M guanidine HCl. 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 21,000 $M^{-1}cm^{-1}$ and expressed as nanomoles (nmols) of protein carbonyls per mg of protein. For Western blotting, protein sample (35 µg) from untreated control and treated group were derivatized with 20 mM DNPH at 37 °C for 30 min followed by neutralization with 4M Tris–HCl and 30% Glycerol (pH 6.8 and). The derivatized samples were separated on 10% SDS–PAGE and proteins transferred to nitrocellulose membrane at 4 °C. Membranes were blocked for 2 h with 1% BSA washed and incubated overnight at 4 °C with rabbit Anti-DNP (1:1000; Santa Cruz) followed by incubation with HRP-conjugated goat anti-rabbit secondary antibody (1:5000; Santa Cruz) for 2 h. Enhanced chemiluminescence (ECL) reagent was used as a chemiluminescent substrate to detect the signal followed by imaging and data analysis using Bio-Rad Chemi-Doc imaging system.

Thiobarbituric acid reactive species (TBARS) estimation

A549 cell lysates (50 µg) were mixed thoroughly with 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/v) TBA, and 0.25 mol/L HCl. The reaction mixture was heated for 30 min in boiling water bath and 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 1.56×10^5 $M^{-1}cm^{-1}$ as molar absorption coefficient, and results were expressed as nanomoles (nmoles) per mg of protein.

Nitric oxide assay

The extracellular NO levels were assayed by using 100 µl of fresh cell culture supernatants of treated (100 µM H_2O_2) and untreated controls incubated with equal volume of Griess reagent (1% sulphanilamide, 5% phosphoric acid, and 0.1% N-[1-naphthyl] ethylenediamine dihydrochloride). The optical density was measured at 545 nm. The nitrate content was evaluated from the standard curve of $NaNO_3$ [28]. The intracellular iNOS/NOS2 localization and expressions were detected by using immunostaining. 2×10^5 A549 cells were seeded on glass coverslips in six-well plate; after 24 h the medium was removed from the wells and cells were washed three times with 1× PBS. The cells were fixed with 2% paraformaldehyde for 10 min in dark and permeabilized with 0.1% Triton X-100 for 5 min, then cells were blocked with 10% FBS for 1 h at 37 °C followed by incubation with Anti-NOS2 (iNOS) primary antibody (1:100, Santa Cruz) for overnight at 4 °C in a humidified chamber. The antibody binding was detected with FITC-labeled Anti-IgG Alexa Fluor-647 secondary antibody (1:200, Invitrogen). The nuclei of cells were stained with DAPI for 30 min. The coverslips were then mounted on microscopic glass slides and the immunofluorescence images were obtained using Olympus FV-1200 Laser Scanning Confocal Microscope

(Olympus) at Central Instrumentation Laboratory (CIL), Central University of Punjab, Bathinda.

Phagocytosis assays

Giemsa staining

1×10^5 A549 cells were treated with H_2O_2 for 15 min and 24 h. A total 0.5×10^5 THP-1 cells were allowed to differentiate into macrophages with PMA (10 ng/ml) treatment at 37 °C/5% CO_2 in a Lab-Tek II chamber slides. 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 Giemsa stain (1:20, v/v) for 20 min to visualize the macrophages and tumor cell uptake. Images were visualized under light microscope (CX25 OLYMPUS) at 100× [20]. The percentage of engulfed cells was calculated by number of cells engulfed per total number of cells.

CFSE assay

1×10^5 A549 cells were treated with H_2O_2 for 15 min and 24 h. Cells were trypsinized and stained with CFSE (16 µM/PBS) at 37 °C/5% CO_2 for 30 min. The A549 cells were then washed with PBS to remove the unbound dye and CFSE-stained cells were co-cultured overnight with PMA-differentiated THP-1 cells as described above in the Lab-Tek II chamber slides. The fluorescent cells which get phagocytosed by macrophages were visualized under CX25 OLYMPUS microscope. The percentage of engulfed cells was calculated by number of cells engulfed per total number of cells.

Respiratory burst assays

NBT assay

PMA-treated THP-1 cells (1×10^5) were co-cultured with control, untreated, and H_2O_2 (100 µ M)-treated A549 (1×10^5) cells for 15 min and 24 h in a six-well plate. After 24 h, 0.1% NBT (500 µl) was added and plates were incubated at 37 °C/5% CO_2 in the dark. After 45 min, the NBT was removed washed and DMSO and 2M KOH was used to dissolve the crystals. Absorbance was measured at 570 and 630 nm [20].

Immunohistochemistry for $p47^{phox}$

Control and H_2O_2-treated A549 cells were co-cultured with PMA-differentiated THP-1 in the Lab-Tek II-chambered slides for overnight. After washing with PBS, the cells were fixed with chilled acetone for 5 min and air dried. All the slides were then treated with 0.3% H_2O_2/PBS for 15 min. Then the blocking reagent (1% BSA/PBST) was added for 30 min. All slides were then washed twice, added $p47^{phox}$ mouse antibody (1:500, Santa Cruz) for 1 h at room temperature, and washed again with PBST. Followed by incubation for 30 min with anti-mouse-HRP-conjugated secondary antibody (1:1000, Santa Cruz), DAB (5 mg/10 ml) and 10 µl of 3% H_2O_2 were added for color development and counterstained with Mayer's hematoxylin (Invitrogen) and again washed with water and air dried. Finally, all the slides were mounted in DPX and images were taken in the CX25 OLYMPUS microscope. The percent positive cells were calculated by number of cells showing positive staining per total number of cells seeded [29].

Statistical analyses

All assays were repeated at least thrice. Data were presented as mean ± SD for control as well as experimental samples. For all statistical comparison, Student's *t* test was used with p value < 0.05 considered as statistically significant.

Results

Effect of H_2O_2 on cell viability

The response of H_2O_2 was determined by treating A549 cells with different concentrations of H_2O_2 (0–200 µM) for 24 h (Fig. 1). The A549 cells when treated with 50 µM of H_2O_2 showed no significant difference in percent viability as compared to the untreated control cells (83.1% ± 32.1 and 87.0% ± 35.4 vs. 100, respectively). However, 200 µM concentration of H_2O_2 showed significant decline in cell viability when compared to untreated control cells (63.1% ± 8.6 vs. 100, respectively) while 100 and 150 µM H_2O_2 showed a moderate to minimal toxicity to cells (78.9% ± 19.7 vs. 100, respectively). In further experiments, 100 µM H_2O_2 concentration was used as an oxidative stress inducer in A549 lung cancer cells.

H_2O_2 induced neo-antigen formation

Protein modifications which occur in the cells following oxidative stress might be responsible for the neo-antigen formation. In order to detect the neo-antigens, the H_2O_2-treated and control cell lysates (20 µg of protein) were resolved on 10% polyacrylamide gel. Figure 2 shows the gel stained with Coomassie brilliant blue (CBB) depicting bands that were observed only in H_2O_2-treated samples (marked with arrows) while, the corresponding bands were not visible in the control cell lysate suggesting the formation of oxidized neo-antigens or modified proteins. In vitro oxidized BSA was used for positive control.

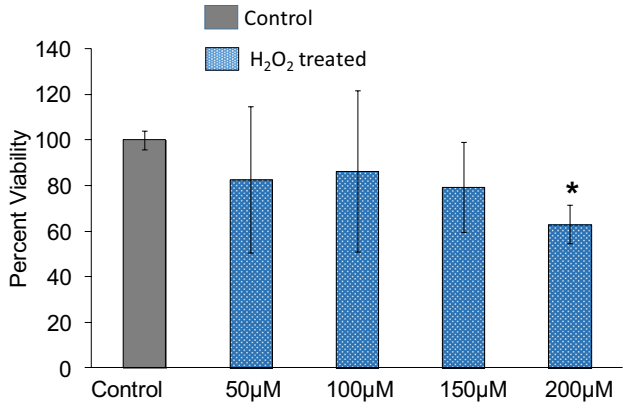

Fig. 1 Effect of H_2O_2 treatment on A549 cells. The A549 cells were treated with various concentrations of H_2O_2 (0–200 μM) and the cell viability was estimated by MTT assay. The data presented as percent viability of A549 cells when treated with increasing concentrations of H_2O_2. The results are expressed percent change ± standard deviation ($n = 3$). Student *t* test was performed to evaluate the significance of the results, the data were considered as statistically significant at $^*p \leq 0.05$ when H_2O_2 treated cells were compared with untreated controls

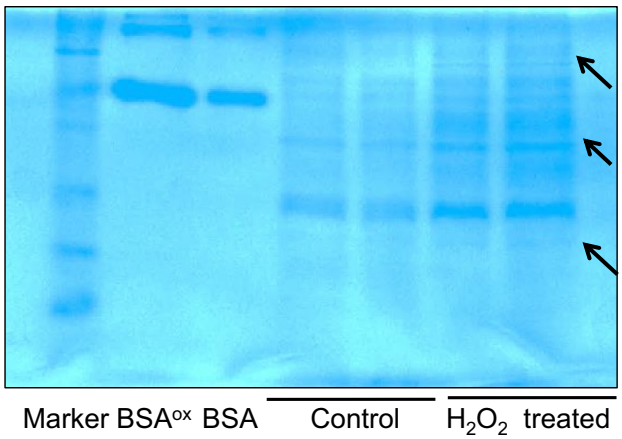

Fig. 2 Neo-antigen detection. The A549 cells treated with 100 μM of H_2O_2. The cell lysates were prepared and resolved on a SDS–PAGE then stained with Coomassie Brilliant Blue. *Lane 1*: marker (Protein ladder as size standard); *Lane 2*: BSA^{ox} (Positive control); *Lane 3*: BSA; *Lane 4 and 5*: control (without treatment); *Lane 6 and 7*: H_2O_2-treated samples (100 μM)

H_2O_2 induced protein carbonyls formation

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 method. A significantly high protein carbonyl control (20.6 ± 7.4) was observed for H_2O_2-treated cell lysates (24 h) when compared with untreated controls (7.7 ± 1.2) (Fig. 3a) which indicates that the oxidative stress induced protein modification due to the H_2O_2 treatment. Western blot showed a significant increase in protein carbonyl content in 100 μM H_2O_2-treated cell lysates for 24 h as compared to 100 μM H_2O_2-treated cell lysates for 15 min. Both derivatized oxidant-treated samples showed high content of protein carbonyls as compared to their respective derivatized untreated control for 15 min and 24 h (Fig. 3b-i). Densitometric analysis of Western blot showed twofold increase in carbonyl content in 100 μM H_2O_2-treated cell lysates after 24 h, indicating increased protein oxidation as compared to the untreated control cells (Fig. 3b-ii).

H_2O_2 induced lipid peroxidation

Oxidative stress might lead to oxidation of lipids present in cell membrane resulting in the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) resulting in the formation of thiobarbituric acid reactive species (TBARS). Control and H_2O_2 (100 μM)-treated A549 cell lysates were examined for lipid peroxidation, Fig. 3c shows a significant increase of 64.8% in lipid peroxidation level in H_2O_2-treated cell lysates.

H_2O_2 induced NO generation

NOS2 is often associated with NO generation which is further responsible for inflammation and may cause modification of certain other proteins in tumor cells via different mechanisms and thereby forming additional neo-antigens. To see the extracellular and intracellular generation of NO, Griess assay and confocal microscopy were performed. It was observed that 100 μM H_2O_2-treated A549 cells showed higher extracellular NO levels as compared to untreated A549 cell culture supernatants (Fig. 3d-i, d-ii, Table 1). The intracellular expression of NOS2/iNOS in 100 μM H_2O_2-treated A549 cells was elevated as compared to their respective controls (Fig. 3d-ii).

Immune escape by tumor cells

The PMA-differentiated THP1 macrophages were co-cultured with control and 100 μM H_2O_2-treated A549 cells, and the extent of innate immune response in the form of phagocytic activity and/or respiratory burst was determined.

Effect of neo-antigen formation on phagocytosis

Phagocytosis is an innate immune response exhibited by macrophages thereby engulfing the foreign particles. Neo-antigens formed due to oxidative stress may help tumor cells evade the host's immune response.

A549 cells were treated with 100 μM H_2O_2 for 15 min and 24 h followed by co-culturing with the THP-1 cells and were

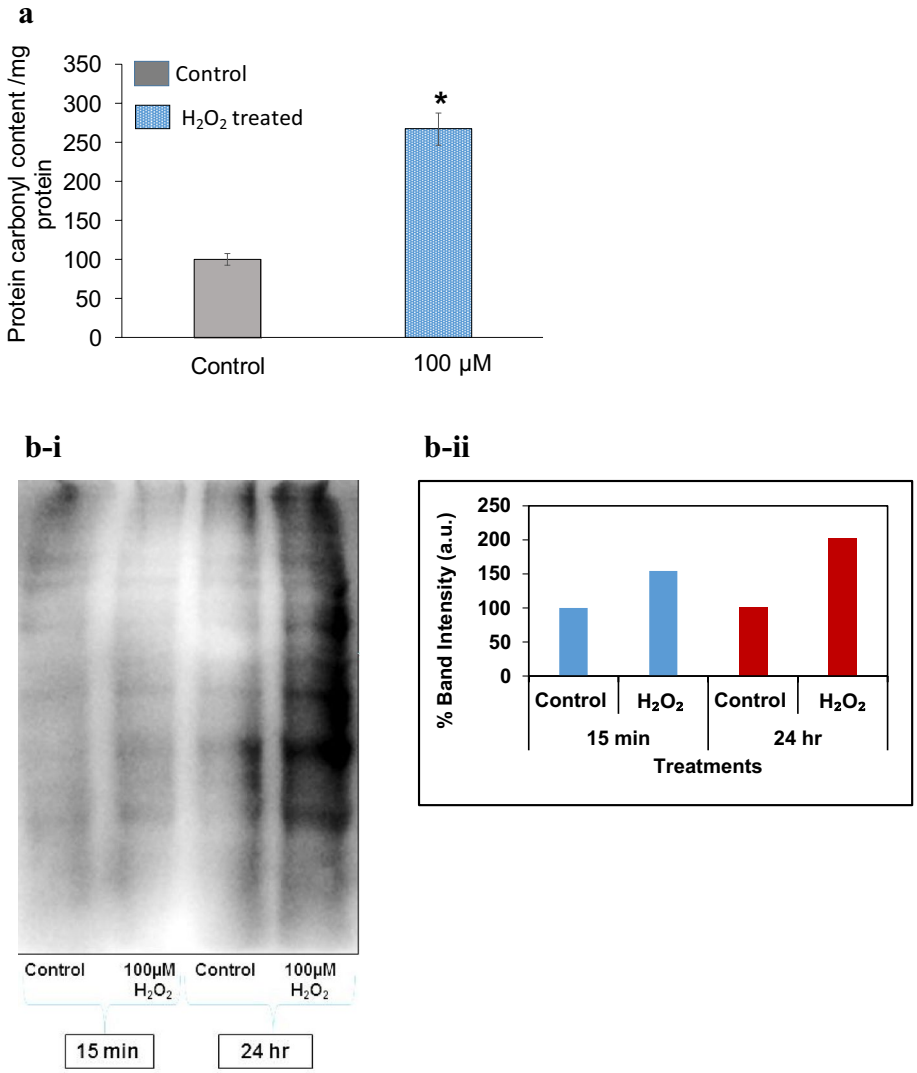

Fig. 3 Detection of oxidative stress and nitrosative stress. **a** Protein Carbonyls. The protein carbonyl content of control and H_2O_2-treated (100 µM) A549 cells was assessed with DNPH assay as described in "Materials and methods." The amount of protein carbonyls was detected in terms of nanomol of protein carbonyls per mg of protein when compared with untreated control A549 cell lysates. The results are expressed as the mean ± standard deviation ($n = 3$). Student *t* test was performed to evaluate the significance of the results, the data were considered as statistically significant at $^*p \leq 0.05$ when H_2O_2-treated cells were compared with untreated controls. **b-i** Western blot analysis of protein carbonyls in A549 cells: blot showing protein oxidation in A549 cells treated with 100 µM H_2O_2, where figure represents the derivatized samples for two time points 15 min and 24 h. **b-ii** Densitometric analysis of Western blot of derivatized protein carbonyls in A549 cells. **c** Thiobarbituric Acid Reactive Species (TBARS) Estimation. The lipid peroxidation content of control and H_2O_2-treated (100 µM) A549 cells was assessed with TBARS assay. The amount of lipid peroxidation was estimated in nanomol of per mg of protein when compared with untreated control A549 cell lysates. The results are expressed as the mean ± standard deviation ($n = 3$). Student *t* test was performed to evaluate the significance of the results, the data were considered as statistically significant at $^*p \leq 0.05$ when H_2O_2-treated cells were compared with untreated controls **d-i** Detection of extracellular NO levels in A549 cell culture supernatants. Extracellular NO was detected using Griess assay in 100 µM H_2O_2-treated A549 cell culture supernatants for 24 h. Student *t* test was performed. The results are expressed as the mean ± standard deviation ($n = 3$). Student *t* test was performed to evaluate the significance of the results, the data were considered as statistically significant at $^*p \leq 0.05$ when H_2O_2-treated cells were compared with untreated controls. **d-ii** Detection of H_2O_2-induced intracellular NOS2/iNOS in A549 cells. The cells were treated with 100 µM H_2O_2 for 24 h time points and the NOS2 (iNOS) was localized by using immunofluorescence staining. Images were taken at a magnification of 60× using Olympus FV-1200 Laser Scanning Confocal Microscope (Olympus) [Control (A–C); 100 µM H_2O_2 Treated 15 min (D–F)]

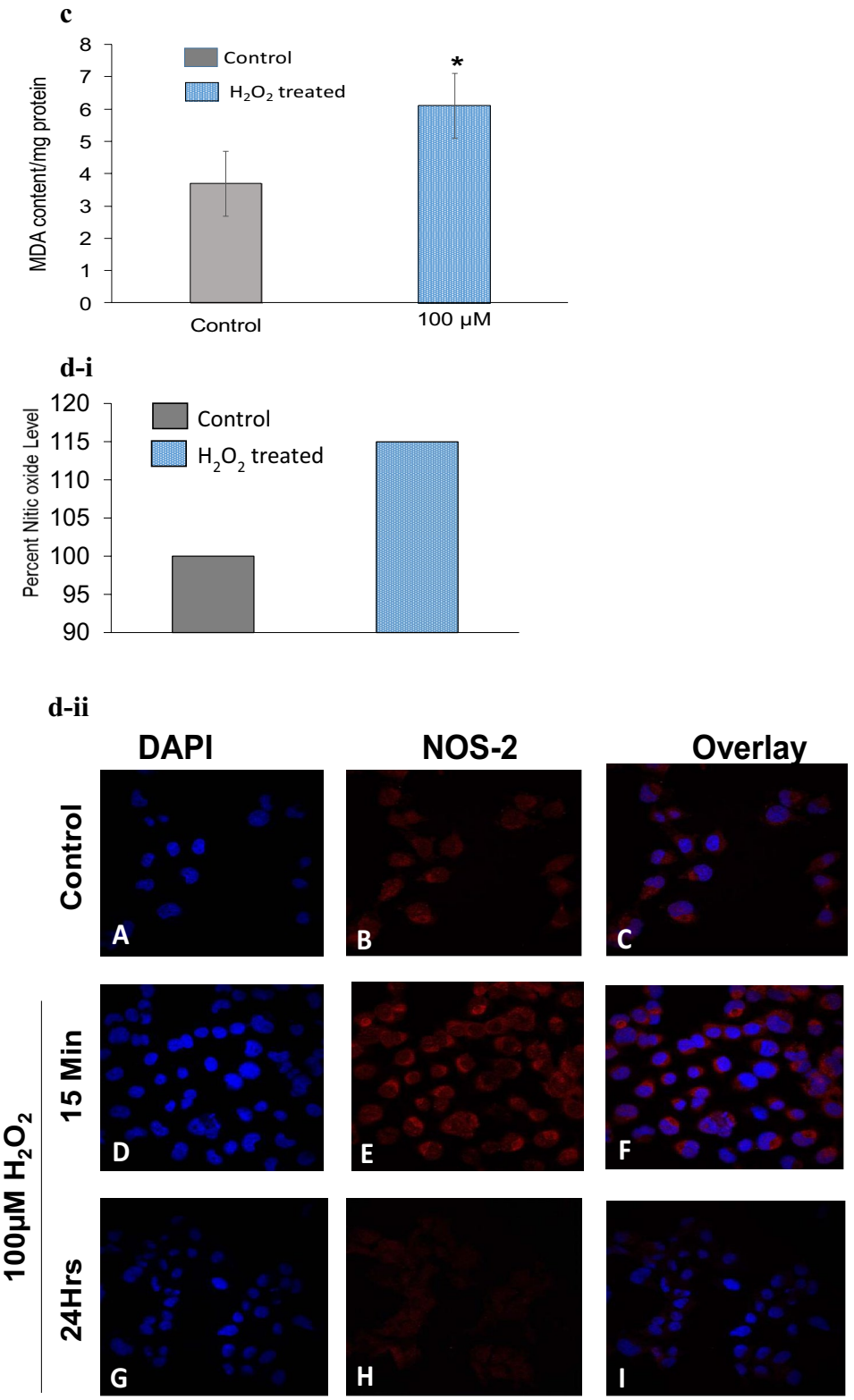

Fig. 3 (continued)

Table 1 Extracellular NO levels in the 100 μM H_2O_2-treated A549 cell culture supernatants

A549 cell culture supernatants	Percent NO generation
Control	100 ± 0
100 μM H_2O_2	114.97 ± 13.72

Table shows the percent NO levels (arbitrary units)

subsequently visualized for the presence of phagocytic activity by Giemsa staining under light microscope (Fig. 4a). Figure 4b depicts a significant 85% decline in percent engulfed cells in 24-h-treated cells, whereas A549 cells when treated for 15 min the proportion of engulfed cells was similar to the untreated control cells (Table 2).

As a second measure, CFSE-stained A549 cells were co-cultured with THP-1 cells to measure the phagocytosed cells using fluorescent microscope (Fig. 4c). The bright green-stained cells correspond to the free A549 cells which are not engulfed by THP-1 cells, whereas the engulfed A549 cells can be seen inside the THP-1 cells. The A549 cells treated for 24 h with H_2O_2 showed 46% decline in number of engulfed cells when compared to the control, whereas the A549 cells when treated for 15 min with H_2O_2 did not show any change as compared to untreated control cells (Table 2; Fig. 4d).

Effect of neo-antigen formation on respiratory burst

Respiratory burst is another defense mechanism of innate immune cells in response to foreign particles during phagocytosis. The phagocytosis-generated ROS is responsible for killing the cancerous cells and foreign particles. Keeping this in view, we measured ROS levels by NBT assay in macrophages and immunostaining for NADPH oxidase subunit $p47^{phox}$. A significant decrease of 25 and 36% in ROS levels was observed in THP1 cells co-cultured with A549 cells treated when treated with H_2O_2 for 15 min and 24 h, respectively, when compared with the untreated control (Fig. 5; Table 3).

$P47^{phox}$ is the cytosolic unit of NADPH oxidase which is one of the most important enzymes of the oxygen-dependent phagocytosis. Immunohistostaining was performed to detect the $p47^{phox}$ levels. The A549 cells treated with H_2O_2 for different time intervals were co-cultured with THP-1 cells and subsequently immunostained for $p47^{phox}$ (Fig. 6a). The THP-1 cells co-cultured with H_2O_2-treated A549 cells for 15 min showed a significant decrease of 55% in immunostaining, whereas there was no change when the A549 cells were treated for 24 h. These results suggest that during phagocytosis, the NADPH oxidase enzyme does not get compromised and its function is not impaired in the 24 h H_2O_2-treated A549 cells (Fig. 6a, b; Table 4).

Discussion

Malignant tumors of the lungs are the most prevalent oncological diseases making lung cancer one of the leading causes of deaths due to cancer [30]. ROS resulting from inflammation and other stresses are among the major factors responsible for the lung cancer incidences [31]. ROS accumulate in the form of oxidative stress and consequently triggers adaptive changes at molecular level. Cellular macromolecules suffer innumerable damages through ROS overproduction, which in turn can stimulate various signal transduction pathways and/or transform the resultant gene expression patterns [32]. 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 [33, 34]. On the other hand, increased endogenous ROS are also responsible for tumorigenesis, metastasis, and resistance to radiation and chemotherapy [35, 36].

In this study, we examined the role of oxidative stress on A549 lung carcinoma cell line and their escape from immune responses. Here, the oxidative stress was achieved by implicating H_2O_2 treatment. However, H_2O_2, 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 [34]. From cytotoxicity assay, the 100 μm concentration of H_2O_2 was observed to be inducing oxidative stress without killing cells [37]. Differences in the protein profile on SDS- PAGE was observed in 24-h-oxidized cells when compared with the untreated control. These additional bands could be associated to the structural changes in protein subunits that might be possibly resulting into the formation of some neo-antigens. Other reports on neo-antigen density in non-small cell lung cancer and pulmonary premalignant conditions also support our claim that oxidative stress promoted neo-antigen formation [25, 38].

ROS and their secondary products disrupt the structure and conformation of biomolecules via different mechanisms. Addition of carbonyl derivatives to protein causes fragmentation of the polypeptide chain, which may lead to altered cellular functions such as energy production, interference with the membrane potentials, and partial to total loss of enzymatic activity [39]. Similarly, the peroxidation of polyunsaturated fatty acids of the cell membranes alters their permeability and fluidity and may also affect membrane-bound proteins [40–42]. Protein carbonyl and lipid peroxidation have emerged as potent biomarkers of oxidative stress and tissue damage resulting from chronic inflammation, in various physiological disorders including, cardiovascular diseases and cancer [43–45]. A higher protein carbonyl and TBARS (a by-product of lipid peroxidation) content in H_2O_2-treated A549 cells in our study signified protein and membrane-lipid oxidation consistent to other reports

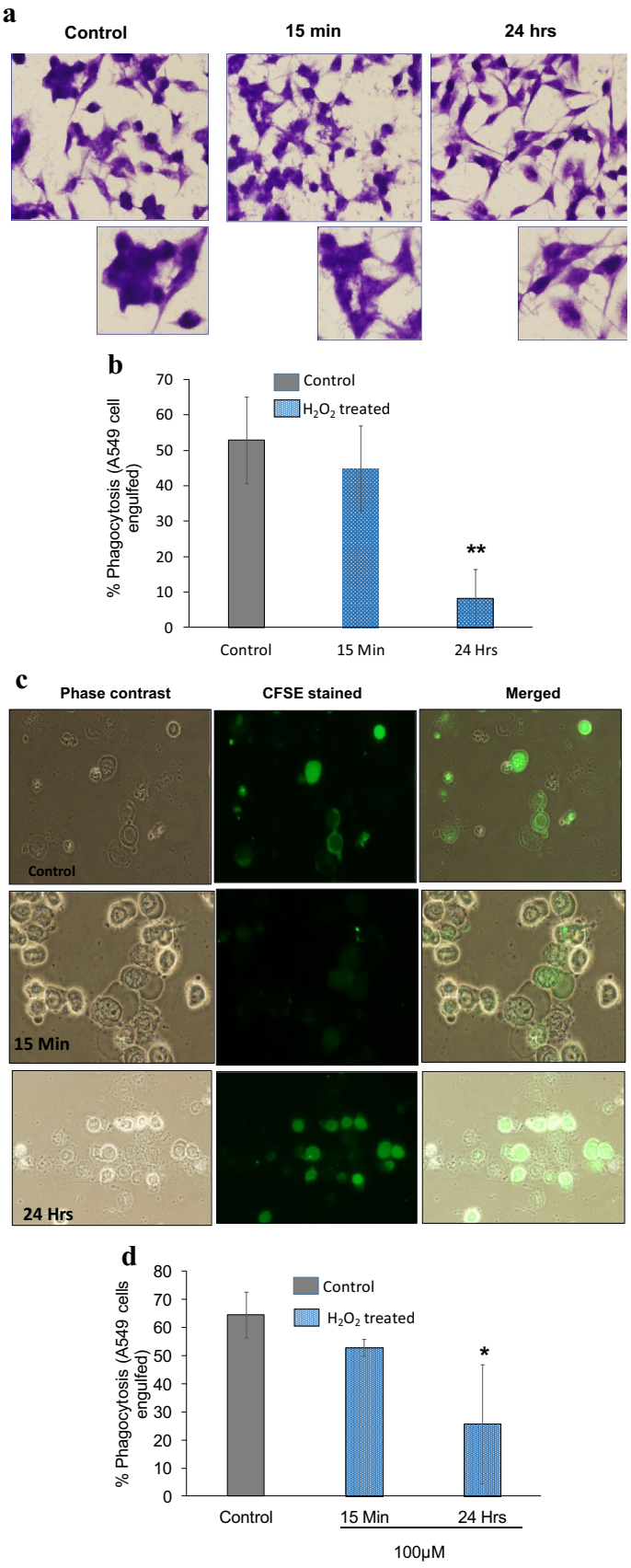

Fig. 4 Effect of Neo-antigen formation on phagocytosis. **a** Cells stained with Giemsa-exhibiting phagocytosis. The A549 cells treated with 100 µM H_2O_2 for 15 min and for 24 h were co-cultured with THP-1 cells; In Control and 15 min H_2O_2 treatment, most of the A549 cells (elongated cells) are engulfed by THP-1 macrophages; whereas in 24 h H_2O_2 treatment, A549 cells escape from THP-1 macrophages. **b** The percent A549 cells engulfed by THP1 macrophages following H_2O_2 (100 µM) treatments calculated as per the formula described in "Materials and methods." **c** The CFSE pre-stained A549 cells treated with 100 µM H_2O_2 for 15 min and for 24 h were co-cultured with THP-1cells. Representative images of Phase contrast (A–C), CFSE stained (D–F), and merged pictures (G–I) exhibiting phagocytosis of A549 cells. **d** Figure showing the proportion of A549 cells engulfed by THP1 macrophages following H_2O_2 (100 µM) treatments (significant at $*p < 0.05$)

Table 2 The A549 cells treated with 100 µM H_2O_2 for 15 min and for 24 h were co-cultured with the THP-1 cells

Treatment	% Engulfed cells (Giemsa stain) Mean ± SD	% Engulfed cells (CFSE-stained A549 cells) Mean ± SD
Control	53 ± 12.2	58 ± 8.18
15 min H_2O_2 treatment	45 ± 12.06	53 ± 3.01
24 h H_2O_2 treatment	8 ± 8.3**	25 ± 14.52*

The number of engulfed A549 cells observed after the Giemsa and CFSE staining (significance at $^{**}p < 0.01$, $^{*}p < 0.05$) are presented below

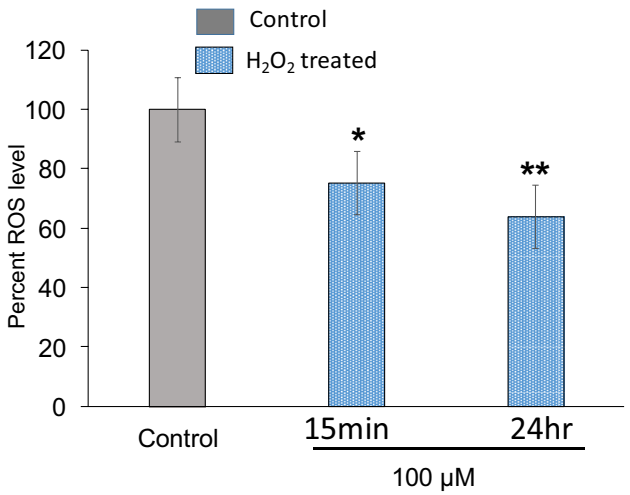

Fig. 5 Effect of neo-antigen formation on respiratory burst. The A549 cells treated with 100 µM H_2O_2 for 15 min and for 24 h were co-cultured with THP-1 cells and the levels of ROS was detected using NBT assay. The results are expressed as the mean ± standard deviation ($n = 3$). Student t test was performed to evaluate the significance of the results, the data were considered as statistically significant at $^{*}p \leq 0.05$ and $^{**}p \leq 0.005$ when H_2O_2-treated cells were compared with untreated controls

Table 3 The A549 cells treated with 100 µM H_2O_2 for 15 min and for 24 h were co-cultured with the THP-1 cells and NBT assay was performed to detect the ROS levels

Treatment	Percent ROS levels
Control	100 ± 11
15 min H_2O_2 treatment	75 ± 18*
24 h H_2O_2 treatment	63 ± 10**

Table shows the percent ROS levels (arbitrary units) and the percent decrease in ROS levels (significant at $^{*}p < 0.05$ and $^{**}p < 0.01$)

documenting the role of oxidative stress in promotion of tumorigenesis [46, 47]. Nitric oxide is produced enzymatically from L-arginine in the presence of NOS. The upregulation of inducible nitric oxide synthase (NOS2/iNOS) and prolonged generation of NO during inflammatory responses are undoubtedly involved in tumorigenesis. Under the influence of a long duration oxidative stress, decrease in NOS2 expression was observed. It may be due to the fact that oxidative stress-induced lipid peroxidation results in the formation of 4HNE which further subtracts inducible nitric oxide synthase by inhibiting the NF-kB activity. However, initially (15 min), the level of HNE might have been low which is reported by some previous groups [48, 49].

The human THP-1 (macrophage) cell line is a widely used in vitro model system for studying macrophages differentiation and function [50]. Macrophages participate in phagocytosis, respiratory burst, innate immune responses, initiate adaptive immunity, and also act as antigen presenting cell. However, ROS produced during phagocytosis can also change the morphological and marker expression and consequently contribute to tumor metastasis and invasion [51]. To demonstrate whether cancer cells can evade the macrophage attack after oxidation treatment in vitro, we employed Giemsa and CFSE staining. The 24 h-treated A549 cells co-cultured with THP1 cells showed interesting immune-escaping characteristics. Our experiments indicated that the induced amount of ROS favored the survival of A549 cells. From the protein profiles, it was further evident that ROS might have promoted the changes in antigen structure or conformation and hence, escaping from phagocytosis.

Phagocytes produce superoxide anion ($O_2^{\bullet -}$) and H_2O_2 via the intracellular enzymatic activity of NOX and superoxide dismutase during oxidative respiratory burst [52, 53]. These reactive molecules serve as inflammatory mediators constituting a part of host immune response activity by killing the invading pathogens [39]. Percent decrease in ROS levels detected by nitroblue tetrazolium (NBT) assay is an indirect measure of superoxide-dependent activity of the phagocytes [54]. Respiratory burst in THP-1 cells co-cultured with 24 h-treated A549 cells decreased significantly, whereas the 15 min treatment had minimal effect on ROS level. Furthermore, the THP-1 cells co-cultured with H_2O_2-treated A549 cells for 24 h showed no change in $p47^{phox}$ positive cells. From the normal flavocytochrome $p47^{phox}$, cytosolic component of NOX, it was inferred that the NOX function is unaffected during phagocytosis in the H_2O_2-treated cells. It can be explained by considering the fact that other sources like, mitochondrial respiration, or xanthine oxidase system might cause the release of ROS inside the cancerous cells or macrophages [55, 56] and hence, activate the NOX. In past few years, different combination of approaches is in demand to understand the early diagnosis and better therapeutics for cancer. Present study is focused on the role of oxidative stress in formation of epitopes (neo-antigens) as well as their respective effect on innate immune responses. Although, additional prerequisite for the preclinical and clinical evidence are required

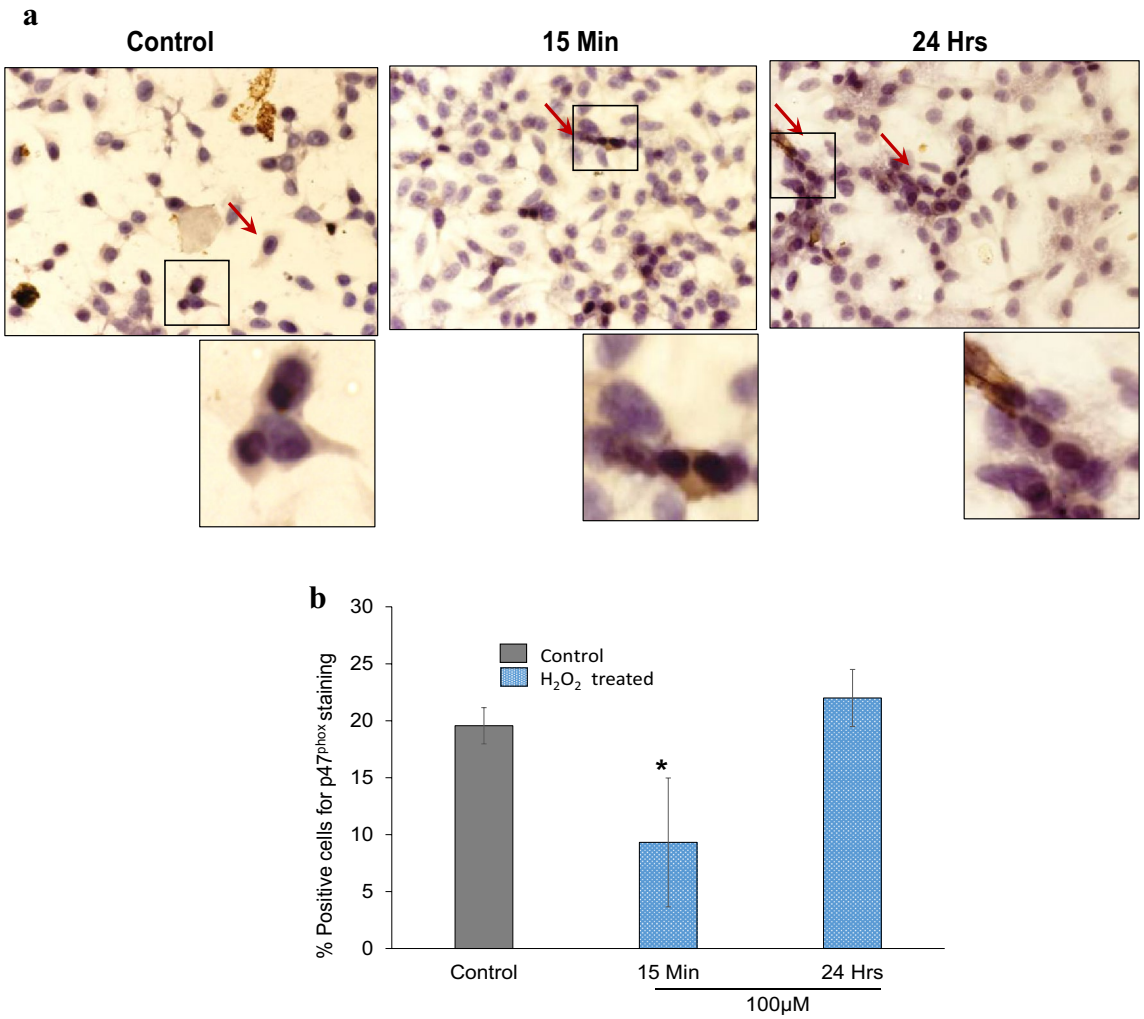

Fig. 6 Immunohistostaining for $p47^{phox}$ (cytosolic unit of NADPH oxidase). The A549 cells treated with 100 μM H_2O_2 for 15 min and for 24 h were co-cultured with THP-1cells, and immunohistostaining for $P47^{phox}$ was performed as described in "Materials and methods." **a** Representative images of the cells with positive staining (brown coloration) for $P47^{phox}$. **b** Percent cells with $P47^{phox}$ positive staining. significant at $^*p < 0.05$ and $^{**}p < 0.01$

Table 4 The A549 cells treated with 100 μM H_2O_2 for 15 min and for 24 h were co-cultured with THP-1 cells and immunohistostaining for $P47^{phox}$ was performed

Treatment	Percent positive cells Mean ± SD	% Change in positive cells
Control	20 ± 1.59	100
15 min H_2O_2 treatment	9 ± 5.66*	53*
24 h H_2O_2 treatment	22 ± 5.66	10

Table represents the percent positive for $p47^{phox}$ staining and percent change for $P47^{phox}$ staining (brown coloration) (significant at $^*p < 0.05$ and $^{**}p < 0.01$)

but identifying these tumor-specific neo-antigens that are formed due to oxidative modifications and correlating them with clinical patient's data will increase our understanding of cancer pathogenesis, and will provide opportunities for the development of adjunct therapies to control progression of the disease and detect cancer at very initial stages.

Conclusion

Present work has characterized the pathological significance of neo-antigens and has established oxidative stress as a new pathogenic mechanism of carcinogenesis. This study will open new avenues for clinical intervention and adjunct therapies for cancer, and it also advocates for new ventures to increase the efficacy of the chemotherapeutic drugs through neo-antigen formation in the cancer cells. In future studies, we propose to identify these neo-antigens using proteomics-based studies and will validate their effect

on a number of more cancer cell lines to see their effect on immune responses.

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Compliance with ethical standards

Conflict of interest The authors declared that they have no conflict of interest.

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