Exposures to 2,4-Dichlorophenoxyacetic acid with or without endotoxin upregulate small cell lung cancer pathway

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Abstract

Background: Pesticide residues in food and environment along with airborne contaminants such as endotoxins pose health risk. Although herbicide 2,4-Dichlorophenoxyacetic acid (2,4-D) has been associated with increased risk of lung cancers such as small cell lung cancer (SCLC) among agricultural workers, there are no data on the SCLC signaling pathway upon 2,4-D exposure alone or in combination with endotoxin.

Methods: We exposed Swiss albino mice (N=48) orally to high (9.58 mg kg^{-1}) and low (5.12 mg kg^{-1}) dosages of 2,4-D dissolved in corn oil for 90 days followed by *E. coli* lipopolysaccharide (LPS) or normal saline solution (80µl/animal. Lung samples and broncho-alveolar fluid (BALF) were subjected to Total histological score (THS) and TLC and DLC analyses, respectively. We used microarray and bioinformatics tools for transcriptomic analyses and differentially expressed genes were analyzed to predict the top canonical pathways followed by validation of selected genes qPCR and immunohistochemistry.

Results: Total histological score (THS) along with broncho-alveolar fluid (BALF) analyses showed lung inflammation following long term dietary exposure to high or low doses of 2,4-D individually or in combination with LPS. Microarray analysis revealed exposure to high dose of 2,4-D alone or with endotoxin upregulated 2178 and 2142 and downregulated 1965 and 1719 genes, respectively ($p<0.05$; minimum cut off 1.5 log fold change). The low dose alone or with LPS upregulated 2133 and 2054 and downregulated 1838 and 1625 genes, respectively. Bioinformatics analysis showed SCLC as topmost dysregulated pathway along with differential expression of Itgb1, NF-κB1, p53, Cdk6 and Apaf1. Immunohistological and qPCR analyses also supported the transcriptomic data.

Conclusions: Taken together, the data show exposures to high and low dose of 2,4-D with/without LPS induced lung inflammation and altered pulmonary transcriptome profile with the involvement of the SCLC pathway. The data from the study provides the insights of the potential damage on lungs caused by 2,4-D and endotoxin interaction and helps to better understand the mechanism of this complex relation.

Keywords: 2,4-D; SCLC; p53; LPS; lungs; Apaf1

1. Introduction

Agricultural workers are commonly exposed to pesticides during production, transportation, planning and utilization of pesticides [1]. Among pesticides, herbicides are used to kill weeds and are frequently used (36%) followed by insecticides (25%), fungicides (10%) and other pesticides (29%) [2]. Herbicide, 2, 4-Dichlorophenoxyacetic acid (2,4-D) is the most widely used herbicide due to low cost, high effectiveness and good water solubility. It mimics the growth hormone auxin (Indole acetic acid) resulting in uncontrolled and unorganized growth and eventually death of weeds. However, the frequent use of 2,4-D leads to environmental contamination and damage to non-target species [3] including increased risk of non-Hodgkin's lymphoma, soft tissue sarcoma or as recently reported lung cancer in humans especially agricultural workers [4]. Agricultural workers especially those in animal confinement or grain handling buildings may potentially get exposed to both pesticides and environmental bacterial lipopolysaccharides (LPS) [5]. Previous studies from our laboratory indicate that endotoxin interaction with various classes of pesticides increases the pesticide induced lung damage [6-10]. LPS ligate TLR-4 to activate various signaling pathways including NF-κB leading to the production of inflammatory mediators and lung inflammation [11]. LPS may also promote tumor growth *in vivo* by amplifying the release of proinflammatory cytokines [12]. Currently, there is scarcity of data on the activation of lung cancer pathways following chronic exposure to 2,4-D alone or in when co-exposed with endotoxin/LPS.

Lung cancer is the main cause of cancer-related death around the world and small cell lung cancer (SCLC) accounts between 13% and 15% of diagnosed lung cancers resulting in about 250,000 death worldwide yearly [13]. SCLC arises from neuroendocrine cells in the bronchial epithelium and is characterized by rapid tumour growth, high vascularity, genomic instability and early metastatic dissemination [14]. The development of SCLC disrupts normal DNA repair mechanisms by involving signature genes including p53, Itgb1, Cdk6, NF-κB and

Apaf1. Currently, there are no data on the expression of SCLC signature genes in animals exposed to 2,4-D alone or in combination with LPS.

The tumor suppressor gene, p53, plays critical role in preventing cancer development [15] and is the most frequently altered gene in around 50% of cancers in human such as breast, colon, lung, liver, prostate, bladder and skin cancer [16]. Overproduction of mutant p53 proteins promotes invasion, loss of migration directionality and metastatic behavior resulting in integrin signaling via integrin such as Itgb1 and epidermal growth factor receptor (EGFR) signaling [17]. Integrin β1 (Itgb1) mediates cellular adhesion to the extracellular matrix, and is correlated with highly invasive and metastatic behavior of tumor and its high expression is associated with poor prognosis of patients with lung cancer and non-SCLC [18, 19]. Integrin-mediated proliferation depends on the ERK pathway activation via phosphorylation of the cyclin D-Cdk4/6 complex, which are involved in regulation of cell cycle and apoptosis [20]. Cyclin dependent kinases are involved in regulation of cell cycle and apoptosis [21]. Cdk6 activates the nuclear factor κB (NF-κB) by phosphorylating NF-κB at serine 536 and are highly expressed in various cancers such as SCLC [22], lymphoma, and leukemia [23, 24]. Activated NF-κB itself plays an important role in lung cancer pathogenesis and is highly expressed in SCLC as compared to Non-SCLC [25]. Apoptotic protease activating factor 1 (Apaf1) and caspase-9 are essential downstream targets of p53-mediated apoptosis and their loss promotes oncogenic transformation [26]. Apaf1 expression is significantly reduced in primary human melanomas [27] and Non-SCLC [28]. It is obvious that a complex interplay of many dysregulated genes leads to formation of various cancers.

Currently, there is very little known of expression of genes involved in cancer and inflammation following exposure to 2,4-D alone or combined with LPS. Therefore, we tested the hypothesis that 2,4-D induces lung inflammation and up-regulates SCLC pathway by using a microarray approach along with bioinformatics tool followed by protein analyses of selected

genes. We report the first data that 2,4-D exposure dysregulates SCLC pathway with increased protein expression of Itgb1, NF-κB, p53 and Cdk6 and decreased expression of Apaf1.

2. Material and methods

2.1 In vivo experiments. Institutional Animal Ethics Committee (IAEC), Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana approved the experimental protocols with guidelines from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Swiss albino healthy male mice aging 6-8 weeks (N=48) were procured from Disease-Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India. Mice were maintained in polypropylene cages with 12 hr light and 12 hr dark cycle at small animal housing hall, GADVASU, Ludhiana. The animals were given synthetic pelleted diet and water *ad libitum*. These mice were acclimatized for one week prior to the start of the experiment.

2.2 Experimental design. Animals were randomly divided into two treatments and one control group (n=16/group). The treatment groups were administered high (9.58 mg kg^{-1}) and low (5.12 mg kg^{-1}) dose of 2,4-D orally dissolved in corn oil for 90 days. Corn oil was orally administered to control group. At the end of experiment eight animals from each group were anaesthetized with xylazine ketamine combination anaesthesia (1/10th of the body weight) and challenged with LPS@ 80 µl/animal intranasally. The remaining mice were administered 80µl of normal saline solution (NSS) intranasally. Animals were sacrificed with xylazine ketamine combination (0.1µl/10gm of body weight) after 9 hours of LPS/NSS challenge.

2.3 Collection of samples. The blood sample was collected by cardiac puncture and bronchioalveolar lavage (BAL) fluid was collected from left lung [7]. BAL fluid was centrifuged at 500g for 10 min at 4°C to collect pellet and supernatant. The supernatant was stored at -80°C for further analysis and the pellet resuspended in PBS for total leukocyte count (TLC) and differential leukocyte count (DLC) analysis on the same day. Right lung samples were stored in RNA later solution at -80°C for RNA isolation. Left lung was fixed *in situ* in paraformaldehyde solution and used for histopathology and immunohistochemistry.

2.4 Total leukocytes and differential leukocyte count analysis. Blood and BAL fluid samples were processed for TLC and DLC analysis on the same day as described earlier [6]. Briefly, 380µl of the white blood cell diluting fluid was mixed with 20µl of the blood/BAL fluid for TLC analysis. For DLC analysis clear blood smear was prepared and stained with Leishman stain. Neutrophils and lymphocytes were counted on each slide at 40X. About 100 cells per sample were identified and counted by an evaluator blinded to the identity of the samples and count was expressed as absolute number of neutrophils and lymphocytes per microlitre of blood.

2.5 Hematoxylin and eosin staining. The paraformaldehyde fixed lung (6 animals from each group) was processed to obtain 5 µm thick paraffin sections which were stained with hematoxylin and eosin for histopathological analysis. Pathological features (peribronchial infiltration, perivascular infiltration, blood vessel congestion, increase in perivascular space, thickening of alveolar lining and inter alveolar oedema) were graded to obtain cumulative total histology score (THS) in a blinded fashion as described previously [29] Further, each feature was scored from 0 to 3 based on its absence (0) or presence to a mild (1), moderate (2) and severe (3) degree.

2.6 Microarray gene expression and analysis. About 50mg of right lung from each animal was used to isolate RNA using the Trizol method (Ambion, Life Technologies, USA). Two RNA samples from each group were randomly used for microarray analysis. The purity and concentration of extracted RNA was checked by using the Nanodrop spectrophotometer (Thermo Fisher). The quality check of the total isolated RNA was also performed in Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano Kit. RNA samples with an RNA Integrity Number (RIN) > 7 were selected for microarray analysis. Microarray analysis was performed using the mouse microarray slide of format 8 x 60K (ID No: 0307760384; Agilent Technologies). Labelling of total RNA (100 ng) was done with Low Input Quick Amp WT Labelling Kit followed by hybridization and scanning. After generating the microarray scan images, the feature extraction was done by Feature Extraction software version 10.7.3. Data generated were further analyzed by Genespring version 14.9 to identify the differentially expressed genes (DEGs) with cut off of 1.5 log fold change and $p<0.05$.

2.7 Functional annotation and kyotoencyclopedia of genes and genomes (KEGG) pathway enrichment analysis. Gene Ontology (GO) enrichment analysis including molecular function, biological processes and cellular components was performed on DEGs as well as uniquely expressed genes by the Database for Annotation, Visualization and Integrated Discovery (DAVID), a web-based bioinformatics tool (https://david.ncifcrf.gov/) by using *Mus musculus* as background and default options and annotation settings. Further gene lists containing gene identifiers (probe set IDs) and corresponding expression values (fold change) were uploaded to DAVID Bioinformatics Resources (version 6.7) to identify top dysregulated pathways.

2.8 Quantitative real-time PCR. Microarray data for the mRNA expression of p53, Itgb1, Cdk6, NF-κB1 and Apaf1 was validated by qRT-PCR. Briefly, 400 ng/µl of total RNA for each sample was reversed transcribed into cDNA using a Revert transcriptase cDNA synthesis kit (Thermo Scientific). qRT-PCR was performed using SYBR green chemistry with published primer sequences for p53 gene [30], Itgb1 [31], NF-κB1 [32], Apaf1 [33] and β- actin as an endogenous control [33]. Primer sequences for Cdk6 was self-designed. The relative expression of each sample was calculated by using the ΔΔCT methods [34].

2.9 Immunohistochemistry. The paraffin lung sections were subjected to immunohistochemical staining as described earlier [35, 36]. The sections were stained with primary antibodies (rabbit polyclonal) against mouse p53 (E-AB-32468; dilution 1:20), Itgb1 (E-AB-10403; dilution1:50), Cdk6 (E-AB-10222; dilution 1:10), NF-κB1 (E-AB-35022; dilution 1:25) and Apaf1 (E-AB-15478; dilution 1:10) followed by appropriate horseradish peroxidase conjugated secondary antibody (Polyclonal goat anti-rabbit; Santa cruz; dilution 1:400). The colour development was carried out with commercial kit (SK4600, Vector Laboratories, USA) and methyl green was used as a counter stain. Controls included staining without primary antibody.

2.10 Grading for immunohistochemistry. Immuno-positive p53, Itgb1, Cdk6, NF-κB1 and Apaf1 cells were counted in the lung tissue sections of five animals from each group. The cells were counted manually in 10 fields/section in an area of 0.2mm^2 under the 40X objective lens of the microscope so as to maintain the uniformity [35, 36]. The evaluator was blinded to the identity of treatment groups.

2.11 ELISA. Flat-bottomed (Nunc, Maxisorp) plate was coated with BAL fluid diluted in coating buffer (Carbonate-bicarbonate buffer, pH 9.3) and incubated at 4^{0}C, overnight. BAL

fkuid was discarded followed by addition of 100 µl blocking buffer (2.5% Skimmed milk powder in PBS) and incubation at 37°C for 1 hour. Plates were washed thrice with PBS-T (pH 7.4). Primary antibody (50 µl) (p53, Itgb1, Cdk6, NF-κB1 and Apaf1; dilution 1:20 in blocking buffer) was added into each well and incubated for 1 hour at 37°C. After incubation the plate was washed thrice with PBS-T buffer (pH 7.4) and incubated with 50 µl horseradish peroxidase-conjugated secondary antibody (dilution 1:100 in blocking buffer) at 37^0C for 1 hour. Following thrice washing with PBS-T (pH 7.4), 200 µl of OPD substrate (dissolved in phosphate –citrate buffer, pH 5.0) was added into the wells and kept at room temperature till the color appeared (2-3 minutes). The reaction was stopped by adding 50 µl of 3M H_2SO_4 and the absorbances were recorded at 490nm in a Synergy Hi Hybrid Reader (Biolek).

2.12 Statistical analysis. Data from TLC, DLC, histopathology, immunohistochemistry, ΔCT values and ELISA were presented as mean ±SE. Data were subjected to analysis of variance (ANOVA) followed by Tukey's posthoc test, using GraphPad Prism software (evaluation version). We considered a *P*-value of < 0.05 to significant.

3. Results

3.1 Total leukocyte count and differential leukocyte count analysis.

Blood: Exposure to LPS or high (9.58 mg kg^{-1}) dose of 2,4-D increased TLC of blood along with increase in neutrophils count and decrease in lymphocytes count. Further, exposure to high dose of 2,4-D combined with LPS increased in TLC compared to LPS alone (Table 1). Although treatment with low (5.12 mg kg^{-1}) dose of 2,4-D alone did not alter the TLC of blood, but in combination with LPS increased TLC compared to control group. There was significant increase in neutrophils count and decrease in lymphocytes count following exposure to low dose of 2,4-D alone or in combination with LPS compared to control group.

3.2 Bronchoalveolar lavage fluid. LPS alone or in combination with both high and low dose of 2,4-D increased the TLC and neutrophils in BAL fluid compared to control group. Mice treated with either high or low dose of 2,4-D had increased TLC but not neutrophils in BAL fluid compared to control and LPS only mice (Table 1).

3.3 Histopathological examinations. Hematoxylin and eosin stained lung sections from the control mice showed normal histoarchitecture (Fig. 1 A). Exposure to LPS, high or low doses of 2,4-D individually or the combined with LPS treatments caused lung inflammation characterized by congestion in blood vessels, peribronchial and perivascular accumulation of mononuclear cells and increase in the total histological score (THS) in all the treatment groups compared to the control (Fig. 1 B-F; Suppl Table 1).

3.4 Differentially expressed genes (DEGs) and functional analysis. A total of 5351 genes were differentially expressed ($p < 0.05$; fold change $> \pm 1.5$) following exposure to LPS and high or low dose of 2,4-D alone or a combination of 2,4-D and LPS. LPS treatment alone upregulated 671 genes and down-regulated 655 genes. Treatment with high dose (9.58 mg kg^{1}) caused the upregulation of 2178 genes and downregulation of 1965 while the same dose in combination with LPS up regulated 2142 genes and down regulated 1719 genes. Low dose (5.12 mg kg^{-1}) upregulated 2133 genes and down-regulated 1838 genes while the same dose when combined with LPS upregulated 2054 genes and down-regulated 1625 genes. The gene overlap studies between differentially expressed genes (DEG) in all the groups showed 356 (216 upregulated and 140 down-regulated) commonly expressed genes in all the treatment groups as compared to control. The relative expression levels of these genes are illustrated as a Venn diagram (Fig. 2).

3.5 Biological classification and pathway enrichment analysis of DEGs. Gene ontology enrichment analysis revealed that DEGs were significantly enriched in genes involved in various biological processes including response to oxygen containing compound, tissue

development, regulation of protein modification process, regulation of cell population proliferation and nucleic acid metabolic process (Suppl Table 2). KEGG pathway enrichment analysis revealed that SCLC pathway was the topmost dysregulated pathway following exposure to high or low dose of 2,4-D with or without LPS. KEGG pathway enrichment analysis also suggested that p53, Itgb1, Cdk6, NF-κB1 and Apaf1 genes were hub genes primarily associated with SCLC pathway.

3.6 Validation of microarray data by real-time RT-PCR and immunohistochemistry.

3.6.1 p53: Lung transcriptomic analysis revealed the up regulation of *p53* mRNA following exposure to high or low doses of 2,4-D with or without LPS. There was 3.28, 3.29, 3.09, and 3.16 folds increase in the mRNA expression of p53 following exposure to high dose alone or in combination with LPS and low dose alone or in combination with LPS, respectively. The RTPCR data were found to be in concordance with the microarray data (Fig. 3a)

The lung sections incubated without primary antibody were used as a negative control during immunohistochemical staining and did not show any colour development (Fig. 4). Lung tissues from control and LPS-treated mice showed weak staining for p53 in the airways epithelial and alveolar septal cells (Suppl Fig. 1). However, the high or low doses of 2,4-D alone or in combination with LPS showed strong reaction for p53 (Suppl Fig. 1). There was a significant increase in the number of p53-positive cells in lungs of mice exposed to both doses of 2,4-D alone or in combination with LPS compared to control group (Fig. 4a).

3.6.2 Integrin β1: Lung tissues from mice exposed to LPS alone showed downregulation of *Itgb1* by 0.94 folds. Exposure to the high dose alone or in combination with LPS and the low dose alone or in combination with LPS increased *Itgb1* mRNA expression by 1.76, 1.69, 1.71, and 1.68 folds, respectively. The RTPCR data were found to be in concordance with microarray data (Fig 3b).

There was a mild staining for integrin β1 protein in the airway epithelial cells, alveolar septal cells and occasionally in large septal cells/macrophages in lungs from control and LPS group (Suppl Fig. 2). However, the high or low doses of 2,4-D alone or in combination with LPS caused strong staining for integrin β1 in alveolar epithelium, alveolar septa and macrophages (Suppl Fig. 2). Compared to the control group, lungs from mice exposed to both doses individually or in combination with LPS had increased number of integrin β1 positive cells (Fig. 4b).

3.6.3 Cdk6: LPS treatment downregulated Cdk6 mRNA by 1.85 folds. However, exposure to the high dose alone or in combination with LPS and to the low dose alone or in combination with LPS up regulated mRNA expression of Cdk6 gene by 4.61, 4.62, 4.64, 4.62 folds, respectively. The RTPCR data were found to be in concordance with the microarray data (Fig. 3c).

Lung sections from the mice in control and LPS groups showed weak to mild reactivity for Cdk6 in the airways epithelial and alveolar septal cells (Suppl Fig. 3). However, the high or the low dose alone or in combination with LPS increased the number of lung cells positive for Cdk6 compared to the control group (Suppl Fig. 3Fig. 4c).

3.6.4 NF-κB1*:* Microarray analysis revealed the upregulation of NF-κB1 gene following exposure to high and low doses of 2,4-D with or without LPS. There was 4.16, 4.24, 4.25, 4.23 folds increase in the expression NF-κB1 following exposure to the high dose alone, the high dose combined with LPS, the low dose alone or the low dose in combination with LPS, respectively, compared to control. LPS reduced NF-κB1 mRNA by 0.12 folds. The RTPCR data were found to be in concordance with the microarray data (Fig. 3d).

A mild NF-κB1 staining was localized in the airway epithelial cells and alveolar septal cells in lungs of mice from control and LPS group (Suppl Fig. 4). The high or the low dose alone or combined with LPS induced strong staining in alveolar epithelium cells, alveolar septal cells

and macrophages compared to the control and LPS group (Suppl Fig. 4). There was a significant increase in the number of NF-κB1-positive cells in the lungs from mice treated with either of the 2,4-D doses with or without LPS compared to control group (Fig. 4d).

3.6.5 Apaf1: The Apaf1 mRNA decreased in lungs of mice treated with high dose alone (-1.73 fold), high dose compbined with LPS (-1.15 fold), the low dose (-1.28 fold), low dose combined with LPS (-1.51 fold) . The Real Time PCR was found to be in concordance with the microarray data (Fig. 3e).

Lungs from control mice showed strong staining for Apaf1 in the alveolar cells (Suppl Fig 5). LPS exposure also showed moderate to strong Apaf1 reactivity in the alveolar cells (Suppl Fig. 5). Further lungs from the mice exposed to the high or the low dose alone or in combination with LPS showed moderate reactivity for Apaf1 protein in alveolar cells (Suppl Fig. 5). There was a significant decrease in the number of Apaf1 positive cells in lungs of mice exposed to either of the doses of 2,4-D alone or in combination with LPS compared to control group (Fig. 4e).

3.7 Expression of proteins in BAL Fluid. Indirect ELISA was carried out to compare the relative differences in absorbances as a readout of concentrations of p53, Itgb1, Cdk6, NF-κB1 and Apaf1 proteins in the BAL fluid (Fig. 5). LPS treatment did not alter the protein concentration of p53, Itgb1, Cdk6, NF-κB1 and Apaf1 in BAL fluid compared to control group. However, exposure to the high dose of 2,4-D alone or in combination with LPS increased the concentration of p53 (0.886 and 0.857 folds), Itgb1 (0.905 and 0.835 folds), Cdk6 (0.833 and 0.822) and NF-κB1 (0.833 and 0.860 folds) in BAL fluid. Similarly, exposure to the low dose alone or in combination with LPS increased the concentration of p53 (0.898 and 0.888 folds), Itgb1 (0.848 and 0.782 folds), Cdk6 (0.874 and 0.745 folds) and NF-κB1 (0.867 and 0.879 folds). There was no change in the expression of Apaf1 in BAL fluid following exposure to the low dose alone (0.150 folds) and LPS or/and high dose (0.176 and 0.137 folds) as compared to

control. However, the BALF from mice treated with low dose combined with LPS showed decrease in the concentration of Apaf1 (0.077 folds) as compared to control (Fig. 5).

4. DISCUSSION

We present new data indicating lung inflammation and SCLC pathway as the top most dysregulated pathway along with altered expression of Itgb1, NF-κB1, p53, Cdk6 and Apaf1 following exposure to 2,4-D with or without LPS. These data add to understanding of effect potential role of 2,4-D in the development of lung cancer in the agricultural workers.

First, we wanted to understand whether exposure to 2,4-D alone or in combination with LPS causes lung inflammation. We used LPS exposure alone as a control and its exposure caused lung inflammation as reported previously [7, 10]. We used a combination of BAL analyses and Total Histological Score (THS) to determine lung inflammation. Because activated blood cells migrate into lung alveoli through coordinated actions of adhesion molecules and chemokines, BAL analysis is a reliable marker of lung inflammation [12]. High and low dose of 2,4-D with or without LPS caused significant increase in the TLC of BAL fluid. There was increased peribronchial and perivascular infiltration of mononuclear cell in the lung and significant increase in the THS in all groups compared to the control. Herbicide 2,4-D had been previously reported to cause lung injury [37]. Our previous work also showed long term dietary exposure to pesticide such as fipronil [7, 38], ethion [10], lindane [9], imidacloprid [6] and indoxacarb [39] cause lung injury. In addition, similar to observations made with other pesticides [6, 40, 41], there was increase in blood TLC, neutrophils and lymphocytosis with the high dose alone or the low dose combined with LPS to indicate systemic immune response. The histopathological and BAL fluid data taken together show that both high and low doses of 2,4-D irrespective of co-exposure with LPS inflamed the lungs.

The major focus of the work however was explored the lung responses to 2,4-D exposure considering there is epidemiological linkage of lung cancer with exposure to 2,4-D [4]. There was differentiation in the effects of exposure of high dose of 2,4-D as it globally dysregulated higher number of genes compared to low dose. Among the dysregulated genes, SCLC pathway was the topmost dysregulated pathway along with upregulation of p53, Itgb1, Cdk6, NF-κB1 and downregulation of Apaf1 following exposure to the high or the low dose of 2,4-D alone or in combination with LPS. We examined the expression of p53, Itgb1, Cdk6, NF-κB1 and downregulation of Apaf1 at the mRNA and protein levels in the lung to further understand their potential roles in 2,4-D induced cellular changes in the lung.

The p53, Itgb1, Cdk6, NF-κB1 and Apaf1play important roles in cell signaling and functions including cell cycle regulation and repair. High or low doses of 2,4-D alone or in combination with LPS upregulated the p53 and decreased Apaf1 mRNA and protein expression in the lung. p53 trans-activates expression of genes involved in apoptosis, cell cycle regulation, and DNA damage repair [42]. While tumor suppressor *p53* gene plays an important role in the onset of SCLC development by enabling the invasion, metastasis, proliferation and cell survival of malignant cells [43], Apaf1 is involved in apoptosome formation and its low expression is observed in aggressive tumors [44]and occurs in lung tumors such as NSCLC [45]. There is a close linkage between p53 and Apaf1 in chronic myeloid leukemia patient as APAF-1 is a transcriptional target of p53 [46]. Taken together, dysregulation of p53 and Apaf 1 may underly in induction of lung cancer following exposure to 2,4-D.

Both high and the low doses of 2,4-D alone or in combination with LPS significantly increased Itgb1 expression in the lung tissues along with its increase in BAL fluid. Interestingly, integrin β1 promotes cell adhesion to the extracellular matrix and is associated with highly invasive and metastatic behaviour in SCLC [18]. The increased expression of Itgb1 is correlated with poor prognosis in lung cancers [47]. Further there was increase in the

expression of Itgb1 protein in macrophages, alveolar epithelium and alveolar septal cells. Other integrins such as αvβ3, a5β1 and αvβ6, are expressed at low or undetectable levels in adult epithelia, but their expression increases in some tumors [48]. The increased expression of Itgb1 may activate macrophages and facilitate their surveillance role in the lungs and [49] and have a role in neutrophil recruitment (Werr et al 1998). The data suggest that high or low dose of 2,4-D with or without LPS caused a significant increase in the Itgb1 expression in lungs and BAL fluid.

Integrins such as Itgb1 in addition to their roles in activation of inflammatory cells also phosphorylate and activate the focal adhesion kinase resulting phosphorylation of the cyclin D/Cdk4/6 complex via ERK pathway to promote the cell cycle progression [50]. Cdk6 is frequently amplified or overexpressed in a variety of human tumors [51]. However, little is known about its expression and role in SCLC. We observed upregulation of Cdk6 protein expression in lungs following exposure to high or low dose of 2,4-D with or without LPS. Aberrant expression of Cdk6 protein has been reported in many tumors suggesting that Cdk6 protein promotes tumor progression [52] and contribute to chronic inflammation and neoplasia through NF-κB [23]. The data indicate pulmonary upregulation of Cdk6 during 2,4-D induced lung damage.

Inflammation and angiogenesis proceed in a coordinated manner and sustain one another during wound healing and tissue repair in many chronic inflammatory diseases and in cancer [12]. NF-κB pathways are important in broad range of cellular processes including inflammation and cancer progression [53]. NF-κB expression is elevated in Kras induced lung adenocarcinomas and blocking NF-κB significantly reduces tumor growth in mice [54]. Blockade of NF-κB activity is associated with suppression of angiogenesis, invasion and metastasis [55]. NF-κB1 is a pleiotropic transcription factor that promote tumor cell invasion and angiogenesis by regulating expression of various factors that very important

in tumorigenesis including matrix metalloproteinases, cyclooxygenase-2 (COX-2), iNOS, chemokines and inflammatory cytokines [56]. Exposure to high and low doses of 2,4-D with or without LPS increased the mRNA expression of NF-κB1, increased concentration in BAL fluid and protein expression in the lung. NF-κB expression increases during SCLCs as compared to NSCLCs [25] and in lung damage induced by hyperoxia [57], oxidative stress [58] and in number of pulmonary disease including cystic fibrosis, pulmonary hypertension and cancer [59]. The data taken together suggest that high or low dose of 2,4-D alone or in combination with LPS caused a significant increase in the NF-κB1 expression in lungs and BAL fluid.

Taken together, we provide the first evidence of activation of genes, Itgb1, Cdk6, NF-κB1, p53 and Apaf1, involved in SCLC signaling pathway and induction of lung inflammation in animals exposed to 2,4-D. These data are important because SCLC is the dominant cause of patient death [60] and 2,4D is linked to pulmonary cancer. These descriptive data set the stage for mechanistic studies involving methods such as gene-knockout mice and in vitro methods such as siRNA.

5. Conclusion

We conclude that long-term exposure to high (9.58 mg kg^{-1}) and low (5.12 mg kg^{-1}) dose of 2,4-D alone or in combination with LPS alter the histoarchitecture and transcriptome profile of lungs. The data are significant because it describes the involvement of the SCPC pathway by dysregulating the pulmonary expression of key genes associated with this pathway viz. Itgb1, Cdk6, NF-κB1, p53 and Apaf1 during 2,4-D induced lung damage.

6. Declarations

- Ethics approval and consent to participate: All procedures performed in studies involving animals were in accordance with the ethical standards of the Institutional Animal Ethics Committee, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana.

- Consent for publication: Manuscripts reporting studies do not involve human participants.
- Availability of data and materials: The datasets during and/or analysed during the current study are available from the corresponding author on reasonable request.
- Competing interests: None
- Funding: None
- Authors' contributions: Its PhD work of GK under the supervision of RSS, BVSK helped to perform ELISA on BALF samples, BS contributed in the writing of manuscript.
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Figures

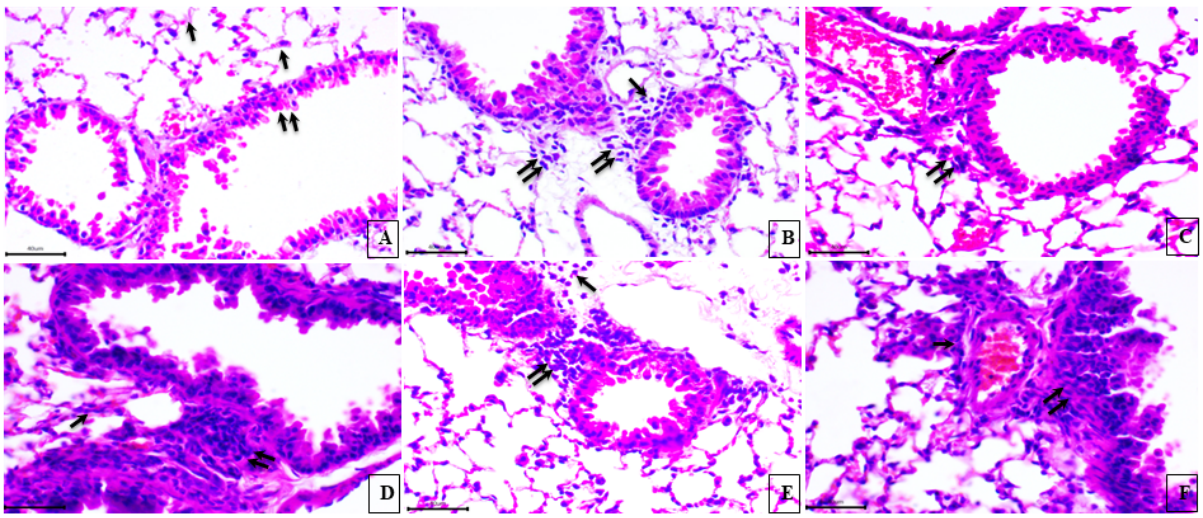

Fig 1: H&E staining: Paraffin section of lung showing normal alveolar epithelium (double arrows) and alveolar septa (single arrow) in control group (A). Perivascular (single arrow) and peribronchial (double arrows) infiltration of mononuclear cells following exposure of LPS (B), high dose of 2,4-D alone (C) or in combination with LPS (D), low dose of 2,4-D alone (E) or in combination with LPS (F). Haematoxylin and Eosin staining. Original magnification: 40X.

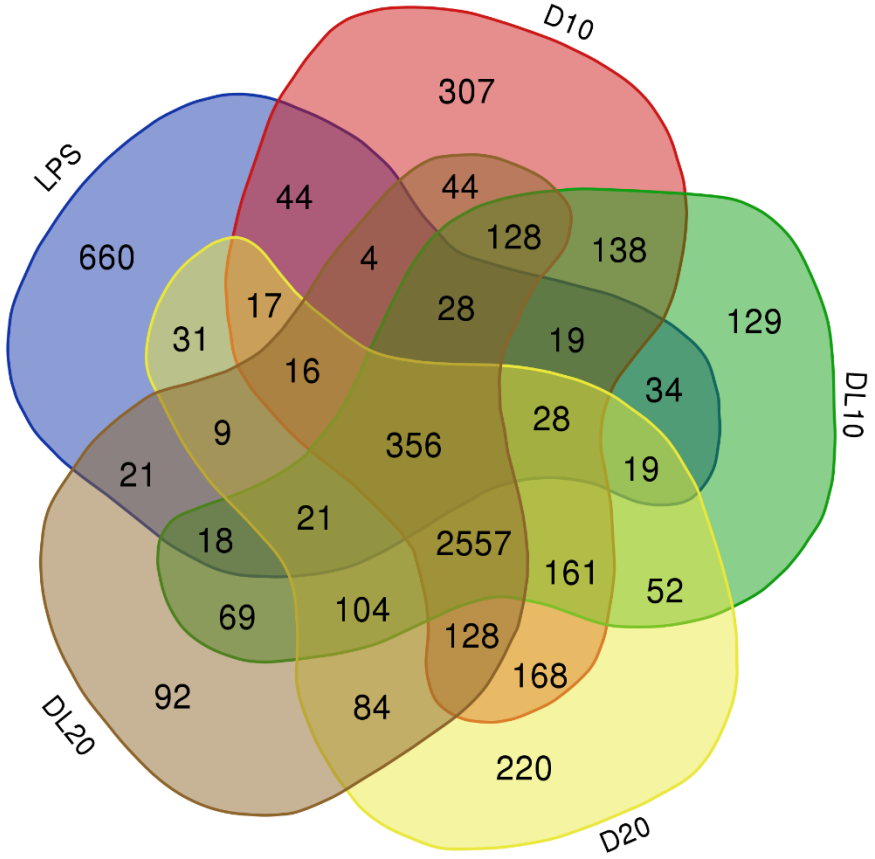

Fig 2: Hierarchical clustering and overlap of DEGs: Venn diagram showing the overlap of DEG's involved across LPS, high dose of 2,4-D alone (D10) or in combination with LPS (DL10), low dose of 2,4-D alone (D20) or in combination with LPS (DL20) group.

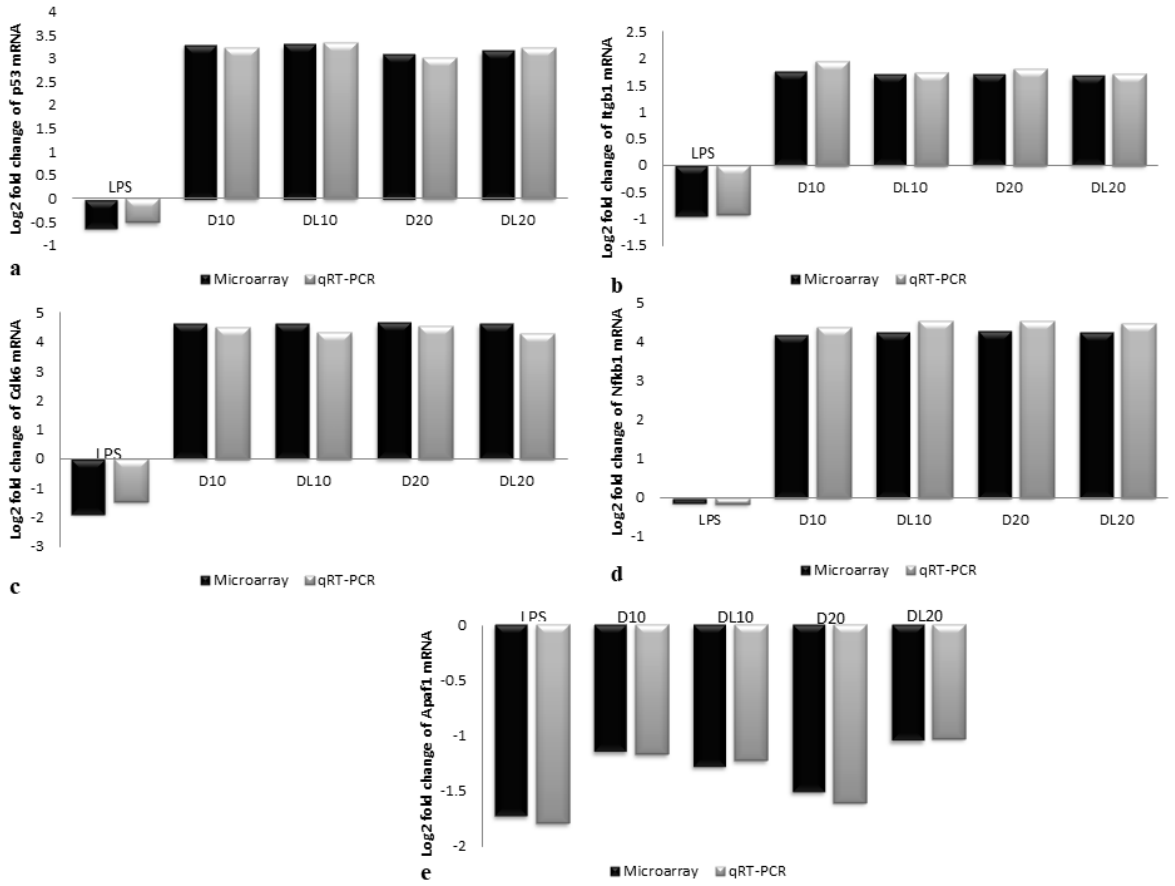

Fig 3: mRNA expression of qPCR and microarray data: Fold change concordance of p53 (a), Itgb1 (b), Cdk6 (c), Nfkb1 (d) and Apaf1 (e) by Real time PCR (qPCR). Log2 fold change is plotted with the standard error of the difference.

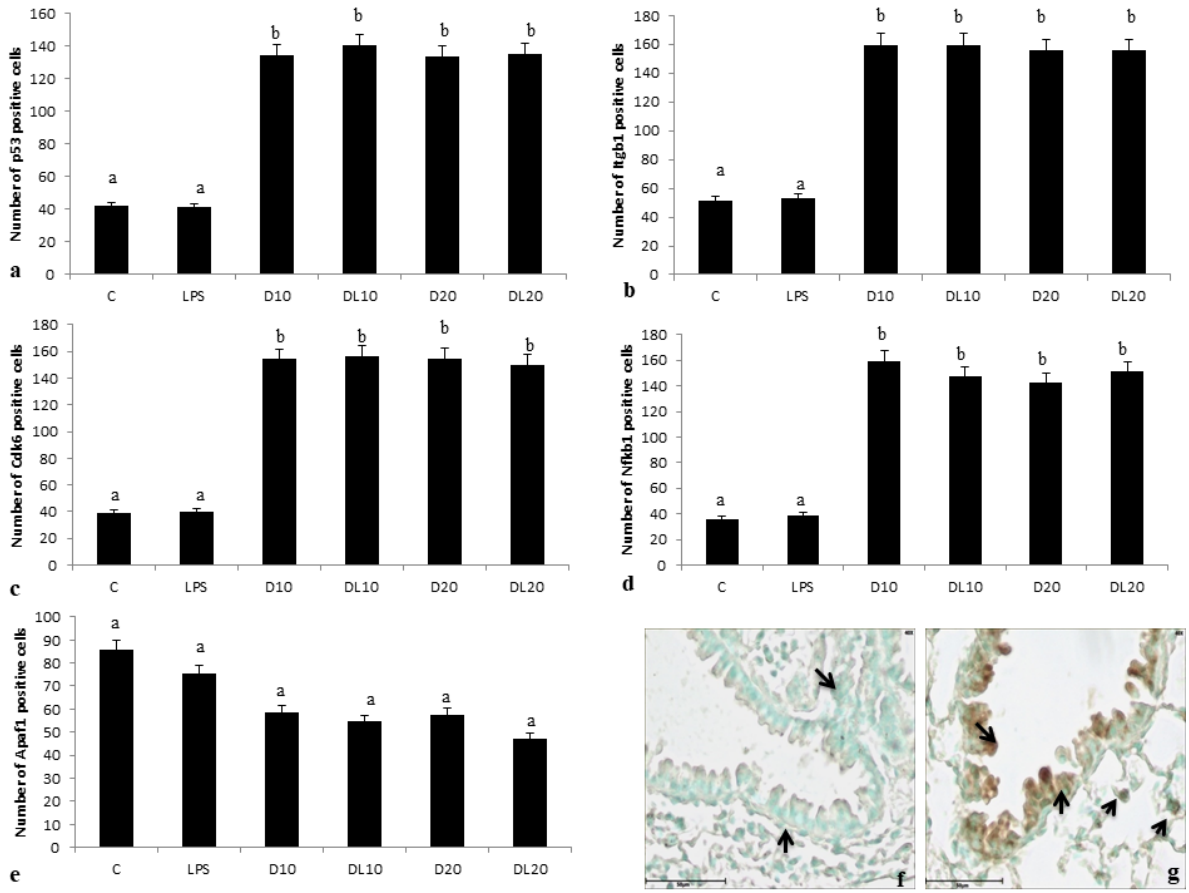

Fig 4: Quantification of immunopositive cells a) p53, b) Itgb1, c) Cdk6, d) Nfkb1 and e) Apaf1 in control (C), LPS, high dose of 2,4-D alone (D10) or in combination with LPS (DL10) and low dose of 2,4-D alone (D20) or in combination with LPS (DL20) group. Lung section stained without primary antibody (f) does not show any colour development in airways epithelium and staining with p53 (g) showed immunopositive reaction in airway epithelial (arrow) and septal cells (arrow head).

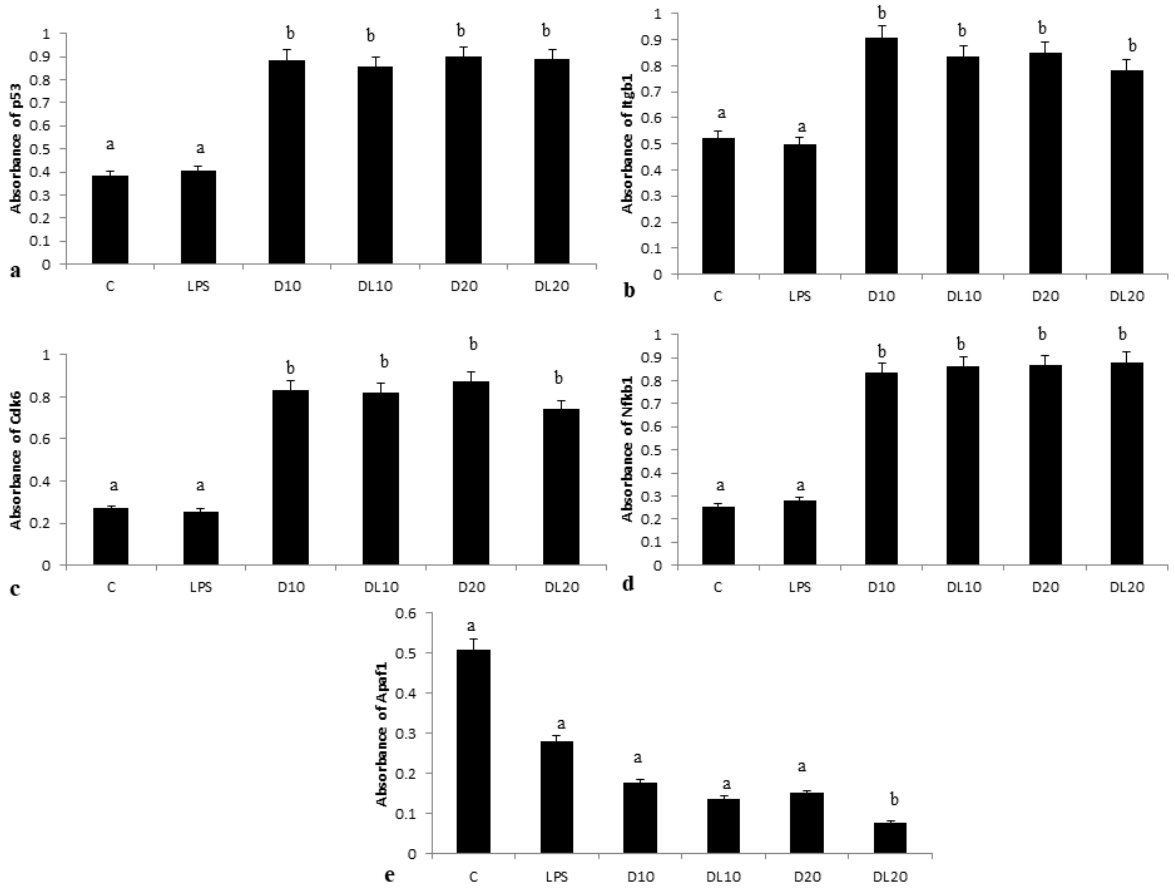

Fig 5: ELISA: Absorbances reflecting concentration of p53 (a), Itgb1 (b), Cdk6 (c), Nfkb1 (d) and Apaf1 (e) in BAL fluid in control, LPS, high dose of 2,4-D alone (D10) or in combination with LPS (DL10), low dose of 2,4-D alone (D20) or in combination with LPS (DL20) group.

Legends to Supplementary Figures

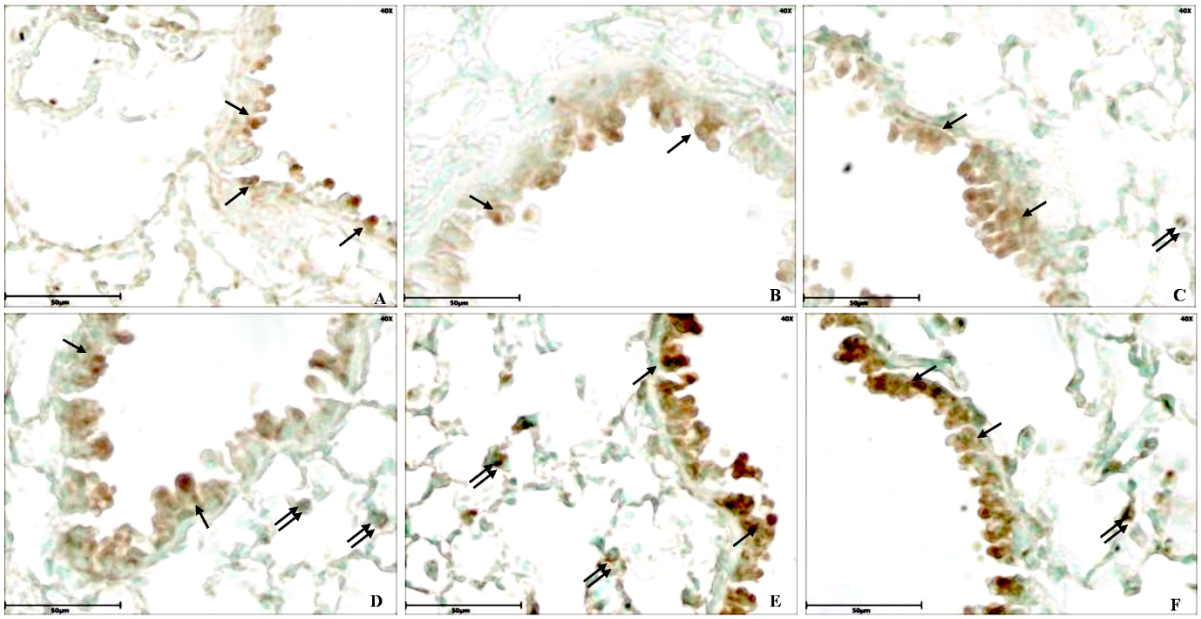

Fig 1: Immunohistochemistry for expression of p53: Immunopositive reactivity for p53 in alveolar septal cell (single arrow) and epithelium cells (double arrow) in control (A), LPS (B), high dose of 2,4-D alone (C) or in combination with LPS (D), low dose of 2,4-D alone (E) or in combination with LPS (F) group. Original magnification: 40X

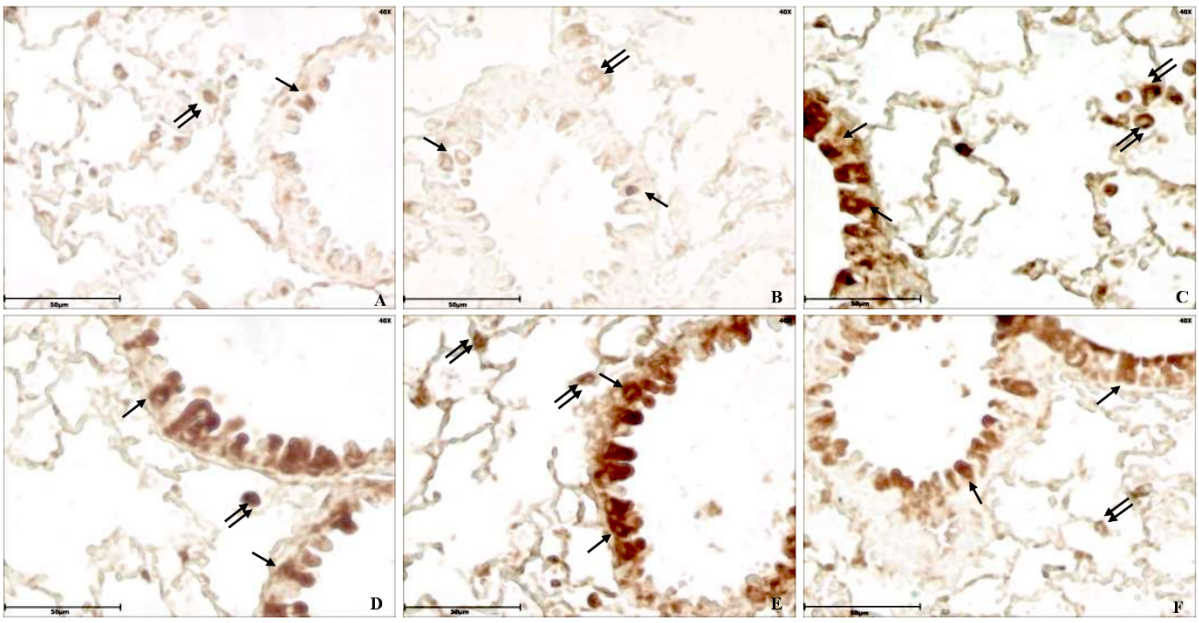

Fig 2: Immunohistochemistry for expression of Itgb1: Immunopositive reactivity for Itgb1 in alveolar septal cell (single arrow) and epithelium cells (double arrow) in control (A), LPS (B), high dose of 2,4-D alone (C) or in combination with LPS (D), low dose of 2,4-D alone (E) or in combination with LPS (F) group. Original magnification: 40X

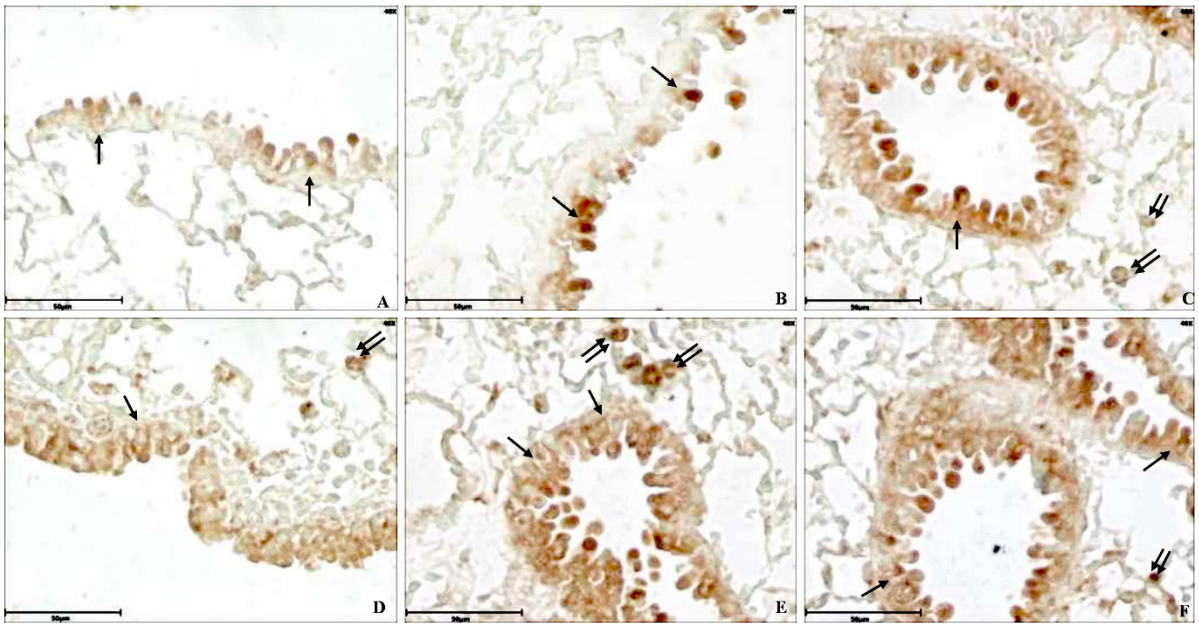

Fig 3: Immunohistochemistry for expression of Cdk6: Immunopositive reactivity for Cdk6 in alveolar septal cell (single arrow) and epithelium cells (double arrow) in control (A), LPS (B), high dose of 2,4-D alone (C) or in combination with LPS (D), low dose of 2,4-D alone (E) or in combination with LPS (F) group. Original magnification: 40X.

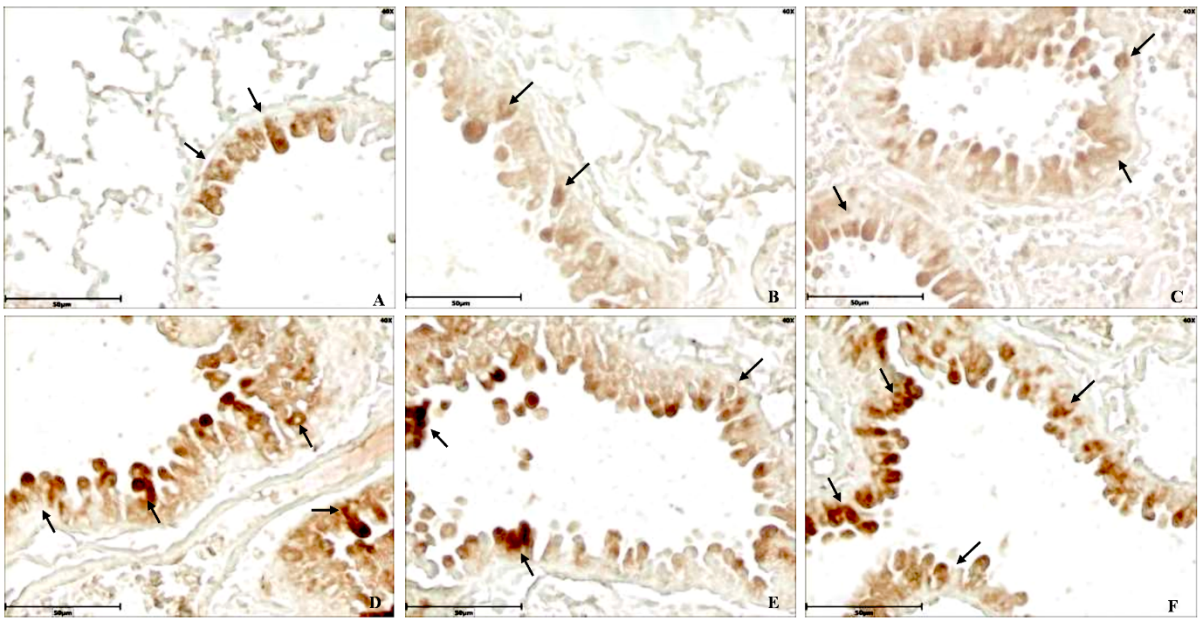

Fig 4: Immunohistochemistry for expression of Nfkb1: Immunopositive reactivity for Nfkb1 in alveolar septal cell (single arrow) and epithelium cells (double arrow) in control (A), LPS (B), high dose of 2,4-D alone (C) or in combination with LPS (D), low dose of 2,4-D alone (E) or in combination with LPS (F) group. Original magnification: 40X

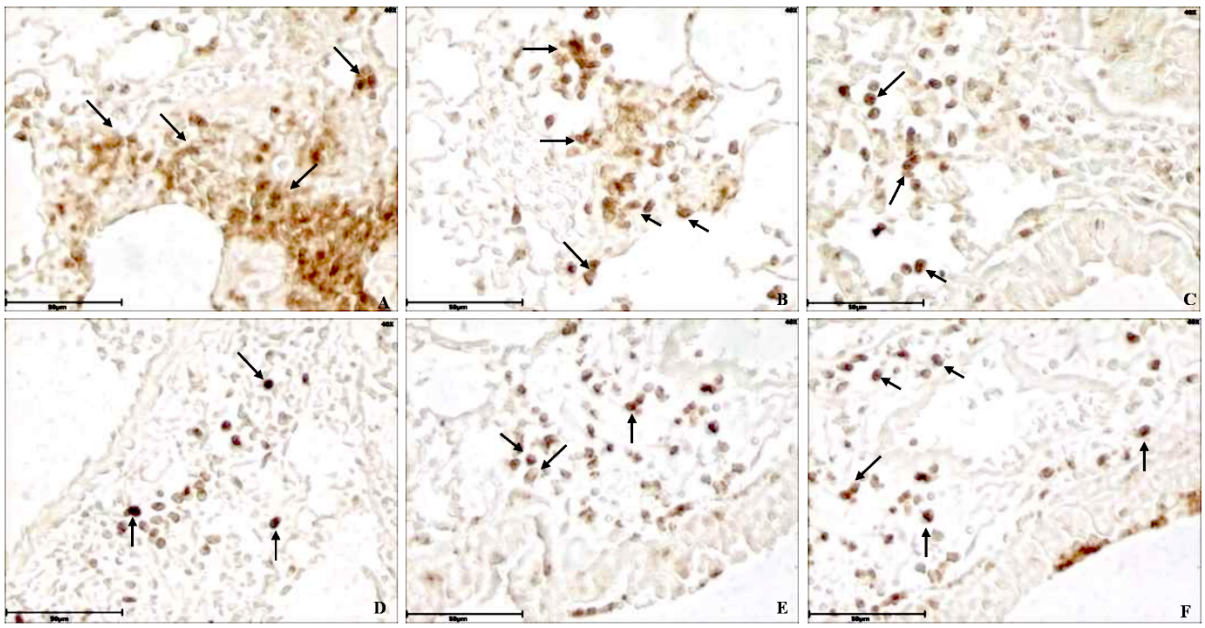

Fig 5: Immunohistochemistry for expression of Apaf1: Immunopositive reactivity for Apaf1 in alveolar septal cell (single arrow) and epithelium cells (double arrow) in control (A), LPS (B), high dose of 2,4-D alone (C) or in combination with LPS (D), low dose of 2,4-D alone (E) or in combination with LPS (F) group. Original magnification: 40X

Table 1: Total leukocyte count (TLC) and Differential Leukocyte count (DLC) of blood and BAL fluid (per µl) following exposure to 2,4-D with or without endotoxin.

TLC and DLC expressed as Mean±SE

Experimental Groups	Blood			BAL Fluid	
	TLC (per µl)	Absolute number of Neutrophils (%)	Lymphocytes (%)	TLC (per µl)	Absolute number of Neutrophils (%)
Control	3699.16±199.1[a]	28±1.06[a]	72±1.06[a]	157.83±3.49[a]	21.83±1.92[a]
LPS	4951.66±219.7[b]	39.5±1.05[b]	60.5±1.05[b]	437±15.52[b]	35.33±1.72[b]
High dose of 2,4-D	4748.83±289.8[b]	36.16±1.83[b]	63.83±1.83[b]	329.83±28.72[c]	30.16±1.16[a]
High dose of 2,4-D+LPS	5826.66±160.5[c]	51.83±0.98[b]	48.16±0.98[b]	492.16±15.61[b]	42.16±1.66[c]
Low dose of 2,4-D	4483.33±162.6[a]	34.66±2.18[b]	65.33±2.18[b]	303.83±12.1[c]	30.5±1.64[a]
Low dose of 2,4-D+LPS	5100.83±136.9[b]	45.66±3.52[b]	54.33±3.52[b]	432.33±17.8[b]	38.83±2.18[c]

[a,b]no common superscript between two levels of an effect indicates significant difference ($p<0.05$).

Supplementary Table 1: Total Histology Score (THS) for HE-stained lung tissue sections in various groups

Experimental Groups	Mean THS
Control	0.61±0.08[a]
LPS	1.97±0.14[b]
High dose of 2,4-D	2.16±0.12[b]
High dose of 2,4-D+LPS	2.36±0.15[b]
Low dose of 2,4-D	2.0±0.11[b]
Low dose of 2,4-D+LPS	2.27±0.12[b]

Total histology score is expressed as Mean±SE

[a,b]no common superscript between two levels of an effect indicates significant difference ($p<0.05$).

Supplementary Table 2: Top five biological processes with enrichment score

S.No.	Biological processes	Enrichment score	P value
1	Response to oxygen-containing compound (GO:1901700)	1.3	3.59E-02
2	Tissue development (GO:0009888)	1.29	3.85E-03
3	Regulation of protein modification process (GO:0031399)	1.29	1.52E-03
4	Regulation of cell population proliferation (GO:0042127)	1.26	3.37E-02
5	Nucleic acid metabolic process (GO:0090304)	1.25	2.31E-02