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Pulmonary changes induced by *trans*, *trans*-2, 4-decadienal, a component of cooking oil fumes

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ABSTRACT: Cooking oil fumes (COF) are known to be associated with respiratory diseases and risk of lung cancer. Involvement of *trans*, *trans*-2,4-decadienal (*tt*-DDE), a major component in COF, is suspected.

Male imprinting control region mice were intratracheally instilled with either 8 or 24 mg·kg^{-1} *tt*-DDE weekly for 8 weeks. Total numbers and types of cells in bronchoalveolar lavage fluid (BALF), as well as pathological changes, and inflammatory gene modulations in the lung tissues were assessed.

We demonstrated that the number of alveolar macrophages in the BALF was significantly increased in *tt*-DDE-exposed animals. Histologically, there was a dose-correlated increase in epithelial hyperplasia and granulomatous nodules at the bronchioloalveolar junctions (BAJ). Although both Clara and alveolar type II cells were present in the BAJ lesion, only Clara cells were actively proliferative. However, only alveolar type II cells were found in the BAJ granulomatous nodules. Enhanced accumulation of phosphorylated signal transducer and activator of transcription 3 (pSTAT3), a known pro-carcinogenic factor, was also detected in many alveolar type II cells at the BAJ lesions.

As both BAJ hyperplasia and enhanced pSTAT3 accumulation are known risk factors associated with increased lung adenocarcinoma development, these findings suggest that *tt*-DDE may pose a risk in lung carcinogenesis.

KEYWORDS: Bronchioloalveolar junctions, cooking oil fumes, lung adenocarcinoma, signal transducer and activator of transcription 3, *trans*, *trans*-2, 4-decadienal

Lung cancer is a leading cause of cancer death worldwide [1]. Although cigarette smoking is the major risk factor for lung cancer [2], the incidence of lung adenocarcinoma in nonsmoking females is unusually high among Chinese populations [3]. Recent epidemiological studies have demonstrated that exposure to cooking oil fumes (COF) is strongly associated with female lung adenocarcinoma in China, Hong Kong, Singapore and Taiwan [4, 5]. Upon heating, cooking oil undergoes thermal and oxidative decomposition for aldehyde production. Increased mutagenic metabolites in the urine and enhanced abnormal cell proliferation (hyperplasia) have been reported in the oesophagus of rats exposed to oils that have been heated [6]. Besides its associations with lung carcinoma in nonsmoking females in Asia [4, 5], COF exposure has also been reported to be associated with various respiratory diseases in kitchen workers in Norway [7], strongly suggesting a potential link between COF exposure and respiratory diseases in humans.

COF are a complex mixture of chemicals, in which fatty acids in the cooking oils, especially polyunsaturated fatty acid, decompose readily to yield aldehydes upon heating or oxidation [8]. Among these aldehydes, *trans*, *trans*-2, 4-decadineal (*tt*-DDE) is the most abundant and cytotoxic [8]. *tt*-DDE is a dienaldehyde readily detected in heated oils [8] and stored food products [9], as well as in restaurant and kitchen emissions [10]. It has been recently reported that the *tt*-DDE concentration is extremely high in COF: more than 100-fold higher than that of polycyclic aromatic hydrocarbon [11]. Similar to the findings by HAGEMAN *et al.* [6] that oxidised oils induced epithelial hyperplasia in rat oesophagus, a study by the National Toxicology Program at NIEHS also demonstrated pathological cell growth (epithelial hyperplasia) in rat stomachs when these animals were chronically fed with *tt*-

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DDE [12]. Thus, the potential carcinogenicity of *tt*-DDE is highly suspected. With the abundance of *tt*-DDE in COF and its potential association with lung cancer in humans, the adverse effects of *tt*-DDE in the lung tissues or cells, *in vivo*, deserve specific investigation.

tt-DDE has been reported to interact with calf thymus DNA, inducing DNA breaks and damages [13]. It has also been reported that *tt*-DDE induced oxidative stress and genotoxicity in A549 cells (a human lung cancer cell line) [14]. In a previous study, we reported that *tt*-DDE exposure increased cell proliferation, expression and release of pro-inflammatory cytokines such as interleukin (IL)-1β and tumour necrosis factor-α, with a reduction of p27 in human bronchial epithelial cell BEAS-2B [15, 16]. Our present study is designed to evaluate the pathological impact and tissue alterations in lung tissues induced by *tt*-DDE *in vivo*. We believe that such *in vivo* information is lacking and is very much needed. We further believe that this information will certainly contribute to the understanding of the role of *tt*-DDE in the induction of lung carcinogenesis.

MATERIALS AND METHODS

Animal study protocol and design

In this study, 54 6-week-old male Institute for Cancer Research (ICR) mice were used. All animals were purchased from BioLASCO (Taiwan) and were housed at the Animal Facility Center at the National Health Research Institutes in Taiwan in accordance with standard and approved protocols at the facility ($23\pm1^{\circ}C$, 39–43% relative humidity; water and food were available *ad libitum*).

ICR mice were used in this study because the general information on their pulmonary status (anatomy, histology and pathology) is well established [17], and we have previous experience using this strain of mouse. In the present study, mice were divided into three groups: 21 mice in the vehicle group (controls), 10 in the low-dose (8 $mg \cdot kg^{-1}$ body weight *tt*-DDE) and 23 in the high-dose group (24 $mg \cdot kg^{-1}$ body weight *tt*-DDE). Mice in the control group were intratracheally instilled with 50 μL vehicle solution, which was prepared as 7.5% v/v tricaprylin (Sigma Chemical, St. Louis, MO, USA) dissolved in 0.9% sodium chloride. Mice in the *tt*-DDE-exposed groups were intratracheally instilled with either 8 or 24 $mg \cdot kg^{-1}$ body weight *tt*-DDE per week for 8 weeks under isoflurane anaesthesia.

The selection of a working dose regimen for our present study was based on a prior dose–response study. A dosage of $\leqslant 24$ $mg \cdot kg^{-1}$ *tt*-DDE was found to be relatively safe for ICR mice during the period of 8-week exposure. All animals survived with relatively good health with good body weight gain compared to the controls. The duration of exposure for our present study was determined in accordance with our previous *in vitro* studies, which lasted ~8 weeks [15, 16]. In the present *in vivo* study, we aimed to expose the animals for approximately the same duration of time in order to compare the *in vitro* and *in vivo* studies, if needed. To ensure that the final dosage was biologically effective, animals were sacrificed 1 week after final intratracheal administration (ninth week of the experiment). The overall experimental design and treatment scheme are shown in figure 1.

Preparations and evaluations of bronchoalveolar lavage fluids and necropsy specimens

Animals were sacrificed *via* isoflurane inhalation to ensure no undue suffering. At necropsy, whole lung was dissected out surgically and was lavaged with 1 mL saline. The recovered amount of lavagate was recorded and saved in individually labelled bottles.

To assess the inflammatory response in lung tissues induced by *tt*-DDE treatment, the total cell numbers and cell types in the bronchoalveolar lavage fluid (BALF) from the animals were determined with a cell counter (Coulter Inc., Miami, FL, USA). The BALF was cytospun at $450 \times g$ for 15 min using a Shandon Cytospin 4 (Thermo Scientific, Waltham, MA, USA). The cytospin smear was then prepared and Liu's staining (Tonyar Biotech, Tao Yuan, Taiwan) was performed to distinguish different cell types. Cells in the BALF were classified into two main categories: epithelial cells and leukocytes (including macrophages, neutrophils and lymphocytes). The cytology was separately evaluated and scored by two independent pathologists. Data presented represent the mean of two scorings.

Measurement of reduced glutathione and glutathione disulfide in mouse lungs

Glutathione (GSH) was quantified by reacting with o-phthalaldehyde, which produced a fluorescence product with excitation and emission at 365 and 430 nm, respectively [18]. In brief, lung tissues were homogenised with homogenisation buffer (154 mM KCl, 5 mM diethylenetriaminepentaacteic acid and 0.1 M potassium phosphate buffer, pH 6.8), diluted with equal volume of redox quenching buffer (10% trichloroacetic acid, 40 mM HCl, 10 mM diethylenetriaminepentaacteic acid and 20 mM ascorbic acid) and centrifuged at $14{,}000 \times g$ for 15 min at 4°C. For quantification of GSH, the supernatant was incubated with 2.5 $mg \cdot mL^{-1}$ o-phthalaldehyde in redox quenching buffer. For quantification of total GSH, the supernatant was incubated with 2.5 $mg \cdot mL^{-1}$ o-phthalaldehyde in redox quenching buffer containing 100 mM dithionite. Samples were incubated at room temperature for 30 min and the fluorescence was measured at excitation and emission at 365 and 430 nm, respectively [18]. The amount of GSH disulfide (GSSG) was calculated according to the following formula: GSSG = (total glutathione-GSH)/2.

Quantifications of pathological changes at the bronchioloalveolar junctions induced by tt-DDE

Formalin-fixed and paraffin-embedded lung tissues were serially sectioned at 3-μm thickness. The first, sixth and eleventh consecutive sections from each mouse lung were randomly selected and stained with haematoxylin and eosin for histological examinations. Masson's trichrome stain was also performed to demonstrate and confirm fibrotic status on bronchioloalveolar junctions (BAJ) lesions.

The BAJ lesions in each group were assessed in two parameters: frequency (percentage of occurrence) and extent of involvements (size of the lesions). The fibrotic status on BAJ lesions was quantified as the percentage of BAJ lesions with increased fibrous (collagen) deposit. A minimum of three tissue sections from each animal per study group were examined and evaluated. The percentage of BAJ lesions was

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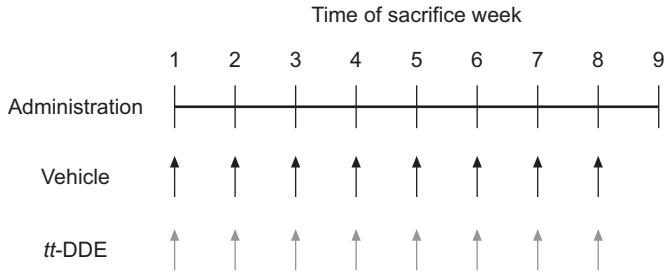

FIGURE 1. Experimental design and schedule for intratracheal instillation treatment in the animal study. All animals (vehicle (n=21) and *trans*, *trans*-2, 4-decadineal (*tt*-DDE) at a dose of 8 mg·kg^{-1} (n=10) and 24 mg·kg^{-1} (n=23)) were treated weekly for 8 weeks. Animals were sacrificed 1 week (ninth week) after the last treatment.

calculated as the average number of BAJ lesions observed *versus* the total number of BAJs found in each study group (BAJ lesion/BAJ $\times$ 100). The extensiveness or severity of the BAJ lesions were estimated by the ''size'' of the BAJ lesions that developed. The perimeters (area of each lesion) were traced and auto-evaluated quantitatively *via* a computer-assisted morphometric microscope with MetaMorph software (Molecular Devices, Downington, PA, USA). The percentage of the BAJ lesions with increased collagen deposit *versus* the total number of BAJ lesions found in each study group was calculated as the number of BAJ lesions with increased collagen deposit/total BAJ lesions $\times$ 100. Statistical analysis was then performed *via* standard statistical methods as described in the Statistical analysis section.

Immunohistochemistry

In order to identify the specific cell type and protein expression involved in the BAJ pathology, immunohistochemistry was performed as previously described [19]. The Universal LSAB2 kit and Chromogen DAB+ system (DakoCytomation, Glostrup, Denmark) was used for detecting the immunoreactivity with a single antibody. The following specific antibodies were used: anti-cytokeratin (CK; Chemicon, Huissen, the Netherlands) for bronchiolar epithelial cells; anti-Clara cell secretory protein (CCSP; Millipore, Billerica, MA, USA) for bronchiolar Clara cells; and anti-prosurfactant protein C (proSP-C; Chemicon) for alveolar type II cells. Cells involved in proliferation or growth (hyperplasia) were assessed by the immunostaining of proliferating cell nuclear antigen (PCNA; BD Transduction Laboratories, Lexington, KT, USA). The immunostaining of phosphorylated signal transducer and activator of transcription 3 (pSTAT3; Cell Signaling, Danvers, MA, USA) was used to detect the localisation of pSTAT3. Normal sera instead of primary antibodies were used as negative controls.

Double immunostaining

Double immunostainings were performed to determine the associations of proSP-C (alveolar type II cells) and PCNA/pSTAT3, or CCSP (Clara cells) and PCNA/pSTAT3 in the BAJ lesions. A multivision polymer detection system (Thermo Fisher Scientific, Fremont, CA, USA) was used in our study. The double immunostaining protocol was based on manufacturer's procedures with minor adjustments on incubation conditions for primary antibodies. The incubation times for proSP-C, or CCSP and pSTAT3 were adjusted to 2 h and 16 h at room temperature, respectively, for optimal results for mice lung tissues.

Analysis of real-time RT-PCR array

RNA was extracted from lung tissues preserved in TriReagent (Life Technologies, Rockville, MD, USA). Synthesis of cDNA was performed using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Six representative lung cDNA samples were selected from vehicle- and 24 mg·kg^{-1} *tt*-DDE-exposed mice. Between both groups, the relative expression of inflammatory genes was measured using mouse inflammatory cytokine and receptor PCR array (PAMM-011; Superarray, Frederick, MD, USA). The pathway analysis of the inflammatory gene expression was performed using Metacore™ (Genego, St Joseph, MI, USA).

Statistical analysis

Cell numbers/populations in BALF from vehicle, 8 mg·kg^{-1} *tt*-DDE or 24 mg·kg^{-1} *tt*-DDE-treated mice were compared *via* one way ANOVA. An unpaired t-test was used to compare data of PCR array from vehicle and 24 mg·kg^{-1} *tt*-DDE-exposed groups. The linear regression analysis method was used to analyse the dose correlations on the severity or size of the BAJ lesions induced by *tt*-DDE. All statistical analyses were performed using the SPSS Base 10.0 software (SPSS, Chicago, IL, USA).

RESULTS

Changes in total cell numbers and differential cell populations in the BALF from animals exposed to tt*-DDE*

In the BALF, the total cell counts in mice exposed to 24 mg·kg^{-1} *tt*-DDE were significantly increased by approximately two-fold when compared to that of controls (fig. 2a). Differential cell counts of the BALF also showed that both epithelial and nonepithelial components (leukocytes) in 24 mg·kg^{-1} *tt*-DDE-exposed mice were 2.3-fold of those in controls (fig. 2b). The BALF of 24 mg·kg^{-1} *tt*-DDE-treated mice showed a 2.5-fold increase in macrophages without significant elevation in neutrophil (polymorphonuclear cells) and lymphocyte counts (fig. 2c). These parameters were not found to be significantly changed in the BALF of 8 mg·kg^{-1} *tt*-DDE-exposed mice (fig. 2).

The oxidative stress status determined by the GSH/GSSG ratio

The GSH/GSSG ratio was used as a criterion to reflect the general oxidative stress status in mouse lungs. In vehicle groups, the GSH/GSSG ratio was 1.33 ± 0.68. In 8 mg·kg^{-1} and 24 mg·kg^{-1} *tt*-DDE exposed groups, the GSH/GSSG ratios were 1.21 ± 0.81 and 1.28 ± 0.62, respectively. No significant alternation in the GSH/GSSG ratio was found in lung tissues of *tt*-DDE-exposed animals.

Pulmonary pathological changes induced by tt*-DDE exposure*

Two prominent lesions were found in the lungs of mice exposed to *tt*-DDE: epithelial hyperplasia and granulomatous nodules (fig. 3b and c). Both lesions were located at the BAJ and were much more remarkable in the high dose (24 mg·kg^{-1}) treated mice rather than the low dose (8 mg·kg^{-1}) treated

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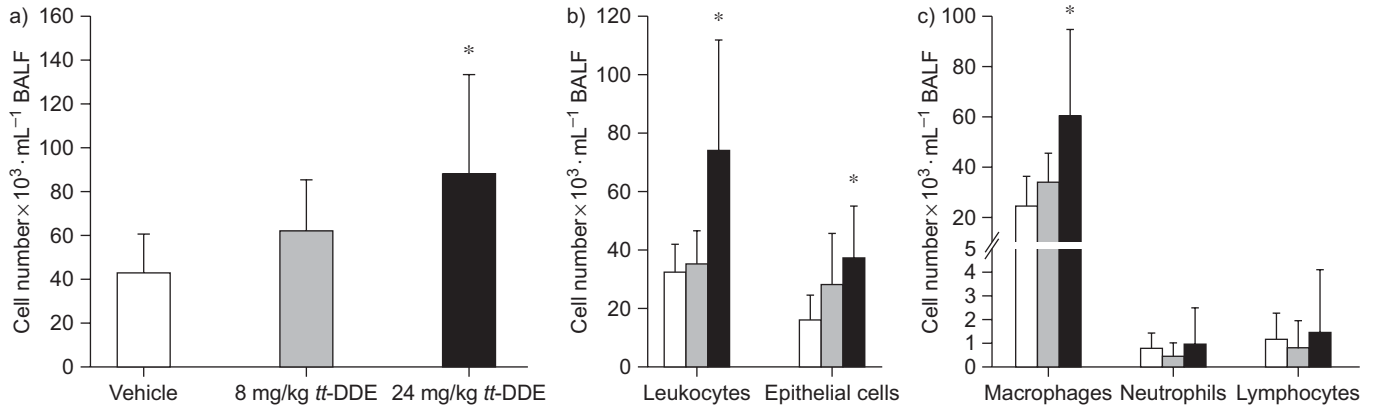

FIGURE 2. Changes in cell numbers among different cell populations in the bronchoalveolar lavage fluids (BALF) from mouse lungs at the ninth week of *trans*, *trans*-2, 4-decadineal (*tt*-DDE) exposure. a) Total cell numbers in the BALF. b) Leukocytes and epithelial cells represented the two major populations of cells increased in the BALF. c) Among the leukocytic cell populations (macrophages, neutrophils and lymphocytes) in the BALF, only macrophages showed the most significant elevation in number. □: vehicle (n=17); ■: 8 mg·kg^{-1} *tt*-DDE (n=11); ■: 24 mg·kg^{-1} *tt*-DDE (n=23). *: $p<0.05$, represents significance and was analysed by the one way ANOVA when compared with vehicle group.

animals (table 1). Careful examination of these lesions revealed that the BAJ hyperplasia was characterised by nonciliated bronchiolar epithelial hyperplasia, lymphocytic infiltration and mild peri-lesional interstitial thickening possibly representing fibrosis (fig. 3b inset). Some of the BAJ hyperplasia could be seen to extend and develop into focal tissue mass or granulomatous nodules, which primarily comprised epithelial hyperplasia, lympohocytic infiltrates, epitheloid cell and macrophage aggregations, and fibroblasts (fig. 3c). Special histochemical stain (Masson's trichrome) also confirmed increased collagen deposits (mild fibrosis) in the interstitial areas of these BAJ hyperplastic sites and in the granulomatous nodules (fig. 3d and e). Linear regression analysis of the collected data revealed dose-correlated increases in collagen deposit, as well as in number and size of the BAJ lesions (hyperplasia and granulomatous nodules) in mice exposed to *tt*-DDE (table 1).

It is also important to affirm the occurrence of hyperplastic activity and to characterise the specific cell types of involvement in the BAJ lesions. Special histochemical stains were employed for such affirmation and characterisation. Immunohistochemically, the cells at BAJ hyperplasia demonstrated both CK and PCNA reactivities (fig. 4a and b), verifying that they were indeed of epithelial origin (CK positive) and hyperplastic (proliferative) in nature (PCNA positive). By means of CCSP (Clara cell marker) and proSP-C (alveolar type II cell marker) staining, both Clara cells and alveolar type II cells could be identified in the BAJ lesions (fig. 4c and d). However, by means of double staining with PCNA antibody (a proliferating cell marker), together with the other two cell markers (CCSP or pro-SP-C), only Clara cells, but not alveolar type II cells, were found to be actively proliferative in these BAJ lesions (fig. 4e and f). It is interesting to note that similar cell marker staining procedures revealed that only alveolar type II cells, not Clara cells, were present in the BAJ granulomatous nodules (fig. 5c and d).

Effects of tt*-DDE exposure on gene expression of inflammatory cytokines and chemokines*

Utilising the real-time RT-PCR array, we further identified modulation in several inflammatory cytokines and chemokines in lung tissues of animals exposed to *tt*-DDE. Chemokine (C-C motif) ligand (CCL)1, CCL11, CCL12, chemokine (CXC motif) ligand (CXCL)13 and cytokine IL-10 were significantly upregulated, whereas CCL2 and IL-1β were downregulated in the *tt*-DDE-treated mice (table 2). In contrast, three receptors, CCLR1, IL-R1-II and IL-8R were downregulated by *tt*-DDE exposure (table 2). To further clarify the potential downstream regulator or factor that could be affected by these *tt*-DDE-induced inflammatory genes, we used Metacore™ (Genego) to perform pathway analysis on these inflammatory genes. pSTAT3 was identified as the common downstream factor activated by CCL1, CCL11, CCL12, CXCL13 and IL-10, which were all significantly elevated by *tt*-DDE.

Enhancement of pSTAT3 in cells in the BAJ lesions

To confirm the involvement of pSTAT3 in the BAJ lesions, we further performed an immunohistochemical staining for pSTAT3 protein in lung tissues from *tt*-DDE-treated mice. Strong nuclear staining of pSTAT3 was demonstrated in many cells in the BAJ lesions (fig. 5a). *Via* double immunostainings of pSTAT3 with CCSP (marker for Clara cells) or proSP-C (marker for alveolar type II cells), we further demonstrated that the enhanced pSTAT3 accumulation was not associated with Clara cells (fig. 5b), but was strongly associated with alveolar type II cells (fig. 5c) in the BAJ lesions. It is also important to note that, although Clara cells were abundant in the BAJ hyperplasia lesion (fig. 5b), they were not found in the BAJ granulomatous nodules (fig. 5d).

DISCUSSION

Recent epidemiological studies have suggested that exposure to COF was strongly associated with lung adenocarcinoma in nonsmoking Chinese females [4, 5]. Chinese cooking style (open-wok cooking with strong COF generation) was suggested as a cause of unusual exposures [4, 5]. However, increased respiratory and pulmonary diseases among kitchen

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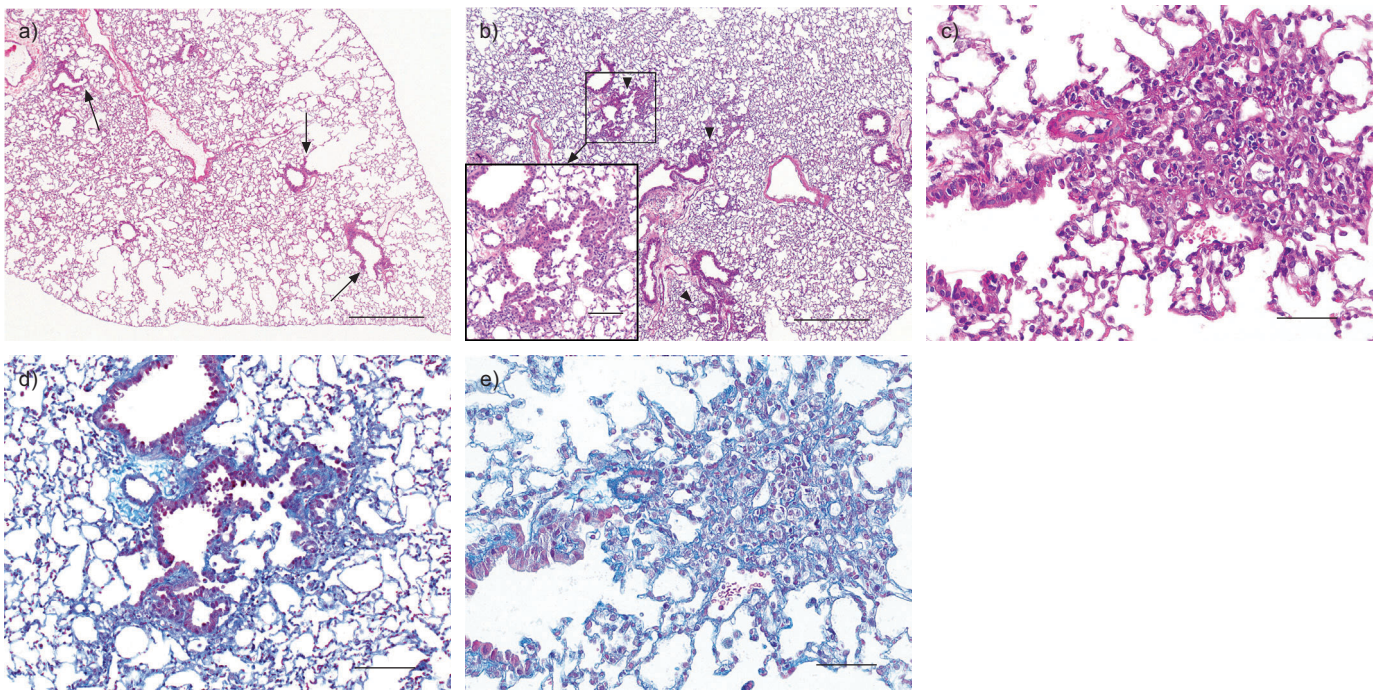

FIGURE 3. Pathological changes induced by intratracheal *trans*, *trans*-2, 4-decadineal (*tt*-DDE) instillation in mouse lungs. a) Controls. Normal lung histology with bronchioloalveolar junctions (BAJs; arrows). b) Lung section from animal treated with *tt*-DDE (24 mg·kg^{-1}). Increased cellularity around many BAJs was noted (arrowheads). Inset: higher magnification of a BAJ from demonstrating BAJ hyperplasia, with increased cellular proliferation extending from the bronchiolar site into the alveolar area. Lymphocytic infiltration and mild fibrosis were also evident. c) A granulomatous nodule at the BAJ. This lesion can be seen to be physically continuous with the BAJ and was probably a tissue outgrowth from the BAJ hyperplasia. Careful examination revealed epithelial hyperplasia, epithelioid cell/macrophage aggregations, lymphocytic infiltration and fibroblasts in this lesion. d, e) Increased collagen deposits (blue colour) in the BAJ hyperplasia area (d) and in a bronchioalveolar granulomatous nodule (e) were demonstrated confirming mild fibrosis was present in these lesions. a–c) Haematoxylin and eosin stain; d, e) Masson's trichrome stain. Scale bars: a, b) 500 μm; inset, d) 100 μm; c, e) 50 μm.

workers in Europe have also been reported [6], denoting that COF is a general rather than isolated health concern in Asia. Chemical analysis revealed that *tt*-DDE is a major component in COF [8]. *tt*-DDE is considered to be a potently toxic aldehyde. Increased oxidative stress and cellular proliferation have been demonstrated in both cultured human lung cells *in vitro* [15] and in stomach epithelium of rats *in vivo* [12]. Although there are ample epidemiological indications that COF exposure is associated with pulmonary diseases and lung adenocarcinoma [4, 5, 7, 20], scientific validations and demonstrations of actual pathological or pro-cancerous changes in lung tissues from animals exposed to *tt*-DDE are still lacking. The present study was designed to provide both pathological and molecular evidence of such associations *via* strategically planned *in vivo* studies.

Aldehydes, when introduced into the lungs *via* intratracheal instillation, certainly may provoke tissue irritations and inflammatory responses. The increased macrophages in the BALF reflect such irritation and inflammatory process. Alveolar macrophages are the major defensive cells against toxicants and pathogens at the alveolar level of the lungs [21]. An increase in alveolar macrophages, therefore, may serve as a biomarker for pulmonary response in the removal of invading pathogens or other irritants. Elevation of various inflammatory cytokines, chemokines and proteinases by macrophages has also been reported [22]. Indeed, an elevation in pulmonary macrophages has been noted in kitchen workers exposed to COF, and alveolar macrophages were suggested to be used as a biomarker for pulmonary irritation in humans [20].The increased alveolar macrophage in BALF of *tt*-DDE exposed animals in this study was consistent with that observed in kitchen workers exposed to COF [20]. However, it seems paradoxical in the present study that while there was a decrease in CCL2 levels in the lung tissues at 8 weeks, the number of alveolar macrophages in the BALF of *tt*-DDE-treated animals was elevated. It must be pointed out that while CCL2 is an important factor in the recruitment of monocytes to tissue sites (to become macrophages), CCL2 is by no means a macrophage marker [23]. In fact, it has been demonstrated that CCL2 modulation is a time-dependent process [24]. The CCL2 are usually elevated in the early or acute phase of inflammation [25], but decline at a later stage when macrophage recruitment has subsided and fibrosis/repair has been initiated. The time point for the present study (8 weeks) probably represents the latter condition.

In our previous *in vitro* study, we observed an increase in IL-1β in *tt*-DDE-treated BEAS-2B cells [15]. However, the IL-1β level was found to be less in the present *in vivo* study. This *in vitro versus in vivo* discrepancy may be explained by the presence of other cell types, such as lymphocytes, *in vivo* but not *in vitro*. The release of IL-1β is known to be modulated by lymphocytes [26]. In *tt*-DDE-exposed lungs, lymphocytic infiltration was

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TABLE 1 Quantifications of the bronchioloalveolar junctions (BAJ) lesions in lungs of mice treated with vehicle or *trans*, *trans*-2, 4-decadineal (*tt*-DDE)

Mouse group	BAJ with increased collagen deposit[#]	BAJ lesion occurrence[¶]	BAJ lesion size[+,***]
Vehicle	0.59	0.46	1.04±0.21
8 mg·kg^{-1} *tt*-DDE	7.92	7.40	1.20±0.63
24 mg·kg^{-1} *tt*-DDE	22.37	29.50	1.91±0.84

Data are presented as % or mean±SD. [#]: the percentage of BAJ with increased collagen deposit was calculated as the number of BAJ with increased collagen staining increased collagen deposit observed over total number BAJ in the tissue sections. A dose-correlated increase in the number of BAJ with fibrosis was observed; [¶]: the percentage of lesion occurrence was calculated as the number of BAJ lesions observed over total number BAJ in the tissue sections. A dose-correlated increase in the number of lesions was observed; [+]: average size of the BAJ lesion was measured *via* computerised morphometric quantitative analyser (an average of three sections per animal were analysed). Dose-correlated increase in size of lesion was also demonstrated with *tt*-DDE exposures; [***]: $p<0.001$, data were analysed by linear regression testing.

prominent in the BAJ lesions. Although induction of IL-1β may occur in lung tissues during early phases of *tt*-DDE exposure, suppression of such activity by increasing numbers of lymphocytes could occur at a later time. IL-1β elevation may only represent an acute or early phase of tissue or cellular response to *tt*-DDE, such as that observed in the *in vitro* situation [15]. The present study was an 8-week long study. Prominent lymphocytic infiltration in the lung tissues has already occurred during this time period as demonstrated in our histopathology. Thus, a reduction in IL-1β level at this late

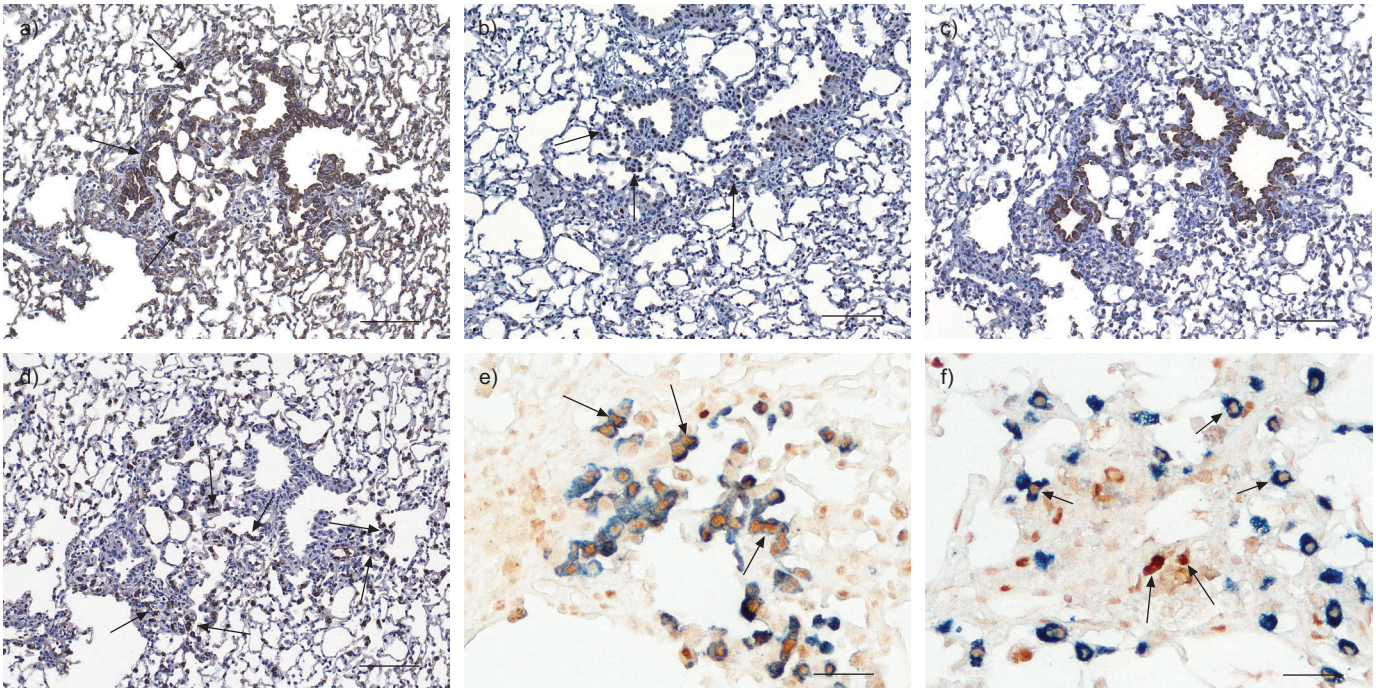

FIGURE 4. Identification of specific epithelial cell types involved in the *trans*, *trans*-2, 4-decadineal (*tt*-DDE)-induced bronchioloalveolar junctions (BAJ) hyperplasia using immunohistochemical techniques. a) Cytokeratin immunostaining is a specific stain for epithelial cells. It demonstrates that all the hyperplastic cells involved in the BAJ hyperplasia were epithelial cells (arrows). b) Proliferating cell nuclear antigen (PCNA) immunostaining is a specific stain in nuclei of cells with hyperplastic activity. We have demonstrated that the increased epithelial cell mass observed in the BAJ were indeed hyperplastic (arrows). c) Clara cell secretory protein (CCSP) immunostaining is a specific stain for Clara cells. It demonstrates that many Clara cells (a member of bronchiolar epithelium) were involved in the BAJ hyperplasia. d) Prosurfactant protein C (proSP-C) immunostaining is a specific stain for alveolar type II cells. Proliferation of alveolar type II cells, which were more heavily stained than normal alveolar area and tended to form rows or clusters of cell aggregations (arrow), were demonstrated in the BAJ hyperplasia lesions. e) Double immunostaining with CCSP (marker for Clara cells) and PCNA (marker for proliferating cells) in a BAJ hyperplasia lesion showed that many cells with blue cytoplasm (Clara cells) also demonstrated positive PCNA staining (brownish stained nuclei; arrows), indicating that many Clara cells in the BAJ hyperplasia were actively proliferative. f) Double immunostaining for proSP-C and PCNA in a BAJ granulomatous nodule did not show an association of PCNA positivity (proliferating activity) and alveolar type II cells (cells with blue cytoplasm). This finding indicates that although alveolar type II cells were involved in BAJ granulomatous development, they were not actively proliferating in the granulomas. a–d) Counterstaining was performed with haematoxylin. Scale bars: a–d) 100 μm; e, f) 20 μm.

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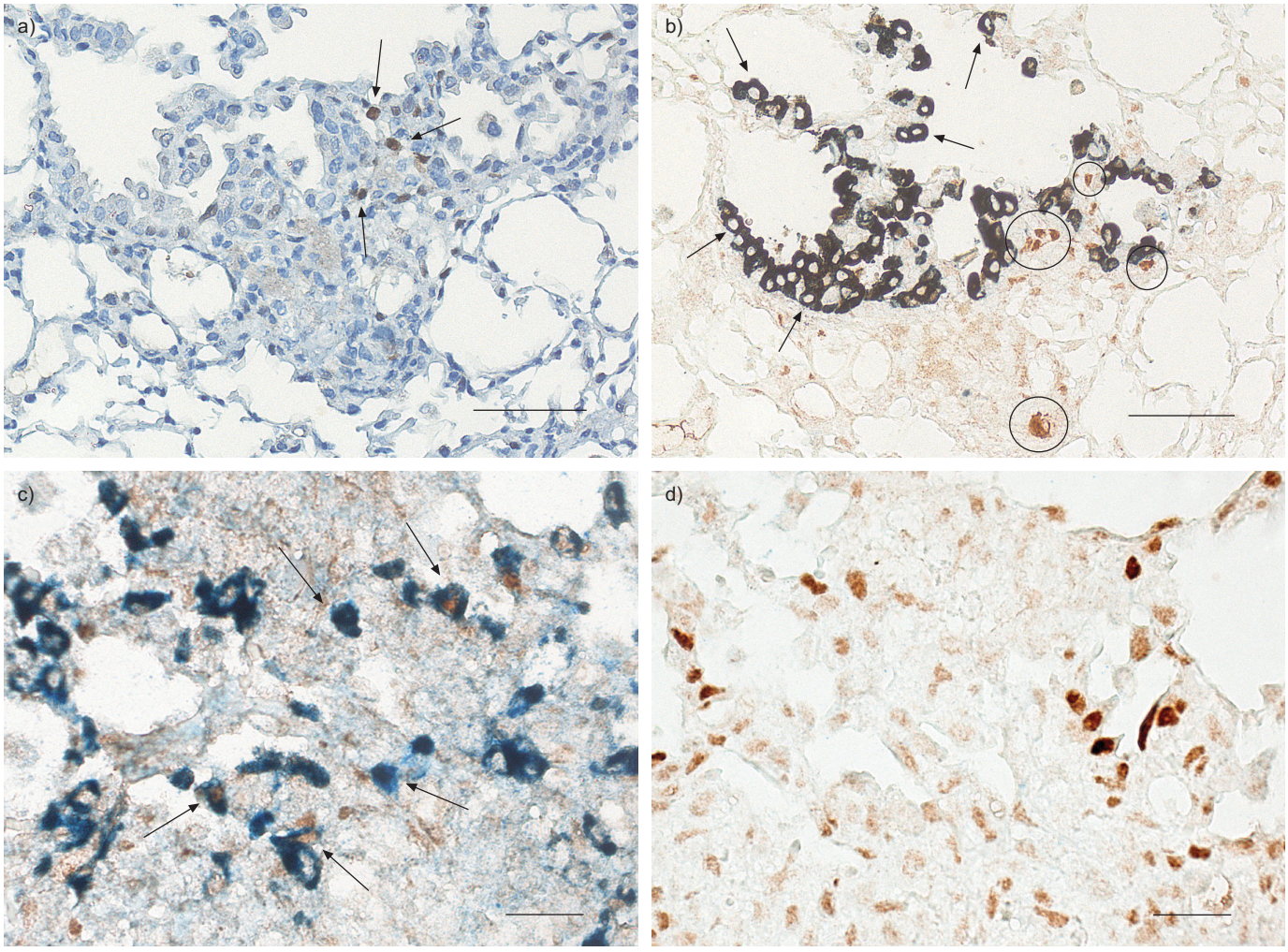

FIGURE 5. The immunohistochemical staining of phosphorylated signal transducer and activator of transcription 3 (pSTAT3) in the *trans*, *trans*-2, 4-decadineal (*tt*-DDE)-induced bronchioloalveolar junctions (BAJ) hyperplasia. a) Some cells in the BAJ hyperplasia displayed positive immunoreactivity (light brown colour) of pSTAT3 in the nuclei (arrows). The counterstain was haematoxylin. b) Double immunostainings for Clara cell secretory protein (CCSP) (Clara cells) and pSTAT3. All the Clara cells (dark blue-brown cytoplasm) were shown to have no brownish nuclear staining (arrows) indicating that they were not associated with pSTAT3 accumulation. pSTAT3 stained cells appeared to be isolated light brown nuclei without dark blue-brown cytoplasmic staining (circles). c) Double immunostainings for prosurfactant protein C (proSP-C) (alveolar type II cells) and pSTAT3. Many alveolar type II cells (dark blue cytoplasm) in a BAJ granulomatous nodule showed brown staining nuclei (arrows) indicating enhanced pSTAT3 in these cells. d) Double immunostainings for CCSP (Clara cells) and pSTAT3 in a BAJ granulomatous nodule. All pSTAT3 stained cells only showed brownish nuclei without dark blue cytoplasmic staining indicating that cells with enhanced pSTAT3 accumulation were not Clara cells. The pSTAT3-positive cells without cytoplasmic staining were probably alveolar type II cells (as shown in c.) No Clara cell (cells with positive CCSP staining) was in fact identified in the BAJ granulomatous nodule. Scale bars: a, b) 50 μm; c, d) 20 μm.

time period is not totally surprising. It may also be pointed out that there is an interesting inter-relationship between IL-1β levels with granuloma and fibrosis in lung diseases [26]. Our present pathological findings (granulomatous nodules development with mild fibrosis) are accompanied by a reduced IL-1β level. These observations are consistent with those found in some chronic lung diseases, such as sarcoidosis, which also showed characteristic lung granuloma formation and low-graded fibrotic changes [26]. However, IL-1β provides a positive signal to fibroblasts [26]. Thus, elevated IL-1β levels are frequently associated with lung diseases with extensive interstitial fibrosis, such as idiopathic pulmonary fibrosis [26].

In the present study, we demonstrated that epithelial hyperplasia at the BAJ was one of the most characteristic pathological changes induced by *tt*-DDE. Classically, BAJ epithelial hyperplasia has been described as bronchiolisation or outgrowth of bronchiolar cells (predominately Clara cells) into the alveoli [27]. In the present study, we found that proliferation of Clara cells (bronchiolar origin) occurred in the BAJ zones. There are indications that both bronchiolar Clara cells and alveolar type II cells can behave as local ''stem cells'' in the lung with abilities to proliferate and to differentiate [28]. Local bronchiolar or alveolar injuries induced by toxic agents, such as cigarette smoke or other environmental toxicants, can induce proliferations and differentiations of these local stem cells as part of the tissue repair process [29]. Furthermore, there

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TABLE 2 Significant changes of *trans*, *trans*-2, 4-decadineal (*tt*-DDE)-induced inflammatory gene expression in mouse lung

Gene function	Gene name	Fold change*
Chemokines	CCL1	2.09
	CCL11	1.96
	CCL12	2.52
	CXCL13	2.09
	CCL2	0.44
Chemokine receptors	CCLR1	0.49
ILs	IL-1β	0.38
	IL-10	2.04
ILRs	ILR1-Il	0.38
	IL-8Rβ	0.31

CCL: chemokine (C-C motif) ligand; CXCL: chemokine (CXC motif) ligand; CCLR: chemokine (C-C motif) receptor; IL: interleukin; ILR: interleukin receptor.
*: $p<0.05$, using an unpaired t-test when compared with vehicle group.

is increasing evidence showing that both bronchiolar Clara cells and alveolar type II cells can potentially be cells of origin for lung tumourigenesis, especially for adenocarcinoma development [30]. Thus, occurrence of bronchioloalveolar hyperplasia does not only reflect tissue repair but is also suggestive of a putative early pre-cancerous lesion in lung tumourigenesis. The continued development of some of the BAJ hyperplasia tissues into focal growths (granulomatous nodules) at the BAJ, as observed in our present study, makes the latter event highly plausible [27].

Of special interest is the development of granulomatous nodules at the BAJ. These tissue growths are not typical granulomas or granulomatous inflammation foci seen in many chronic lung diseases, such as tuberculosis or sarcoidosis [26]. The granulomatous nodules induced by *tt*-DDE are not foci of tissue reaction, but rather appear to be extensions or outgrowths from the adjacent epithelial hyperplasia. Our present study demonstrated that BAJ hyperplasia induced by *tt*-DDE only involved bronchiolar Clara cells. Furthermore, although Clara cells proliferated actively in the BAJ hyperplasia area, it is apparent that the proliferation of Clara cells at the BAJ is self-limiting and does not extend into the BAJ outgrowths (granulomatous nodules). This phenomenon is consistent with that seen in BAJ epithelial hyperplasia in some lung diseases or injuries [31] where epithelial hyperplasia is predominantly represented by bronchiolar Clara cells. This type of epithelial (Clara cell) hyperplasia is primarily reparative in nature and is self-limiting [27].

During the inflammatory process induced by *tt*-DDE, several chemokines (such as CCL1, 11 and 12, and CXCL13) and cytokines (such as IL-10) were found to be upregulated in the lung tissues. A known common downstream factor to be activated by these chemokines and cytokines during the inflammatory process is pSTAT3 [32, 33]. When constitutively activated, pSTAT3 can not only serve as a critical mediator for inflammation and repair in lungs [34], but also can mediate cell transformation leading to carcinogenesis [35]. Indeed, a study of lung-specific STAT3 in transgenic mice indicated that the activation of over-expressed STAT3 in alveolar type II cells would lead to the development of bronchioloalveolar adenocarcinoma in the animals [36]. In our study, we have demonstrated the association of enhanced nuclear pSTAT3 accumulation of many alveolar type II cells in the BAJ hyperplasia and nodules. The continued proliferation of alveolar type II cells with enhanced accumulation of pSTAT3 in these cells would certainly increase carcinogenic risk in lung tissues.

Although our previous *in vitro* study suggested that a reduction in GSH/GSSG status may be the underlying mechanism for *tt*-DDE-induced cell proliferations in BEAS-2B cells [15], the present *in vivo* study failed to detect significant changes in the glutathione redox status (GSH/GSSG ratio) in the lung tissues of *tt*-DDE-treated animals. Such discrepancy between *in vitro* and *in vivo* findings may be attributed to the timing of measuring the GSH redox status in the lungs after *tt*-DDE exposure. The redox response may have a narrow window and be short-lived *in vivo*. Measurements at a later time period, as that in our study, would fail to demonstrate such response. Another possibility is that *tt*-DDE-induced oxidative stress only occurred within the microenvironment of BAJ lesions associated with Clara cell proliferation. Other cells, including Clara cells, outside of BAJ lesions were not affected by the *tt*-DDE. As the areas of BAJ lesions were relatively small in comparison to the whole lung area, this specific redox change in BAJ lesions might not be significantly detected by measuring the whole lung. These speculated possibilities will need to be confirmed by future investigations.

In sum, we have provided an animal model for studying the impact of *tt*-DDE, a major component in COF, in lungs. We have demonstrated site-specific (BAJ) pathological changes by *tt*-DDE, including chronic inflammatory reaction, BAJ epithelial hyperplasia, and outgrowths of the BAJ hyperplasia to form granulomatous nodules at the BAJ. We further demonstrated that only Clara cells were involved in BAJ hyperplasia. Furthermore, enhanced pSTAT3 accumulation was only found to be associated with alveolar type II cells in the BAJ lesions. Induction of Clara cell proliferation, outgrowth nodules at the BAJ and an enhanced pSTAT3 accumulation in alveolar type II cells, which involved BAJ granulomatous nodules, are all factors that would increase risks for lung adenocarcinoma development. We believe that our present study has not only provided a strong scientific validation for the epidemiological observations on the association of COF and lung adenocarcinoma development, but also provided new and important information on the pathological impacts of *tt*-DDE on lung tissues *in vivo*.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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