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

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Short running head: tt-DDE-induced lung lesions in mouse

Abstract
Cooking oil fume (COF) is known to be associated with respiratory diseases and risk of lung cancer. The involvement of Trans, trans-2,4-decadienal (tt-DDE), a major component in COF, is suspected. Male ICR mice were intratracheally instilled with either 8 or 24 mg/kg tt-DDE weekly for eight weeks. Total numbers and types of cells in bronchoalveolar lavage fluids (BAL), as well as pathologic changes, and inflammatory gene modulations in the lung tissues were assessed. We demonstrated that the number of alveolar macrophages in the BAL fluids was significantly increased in tt-DDE-exposed animals. Histologically, there was a dose-correlated increase in developments of epithelial hyperplasia and granulomatous nodule at the bronchioloalveolar junctions (BAJ). While both Clara and alveolar type II cells were present in the BAJ lesion, only Clara cells were found to be actively proliferative. On the other hand, only alveolar type II cells were found in the BAJ granulomatous nodules. Furthermore, enhanced accumulation of pSTAT3, a known pro-carcinogenic factor, was also detected in many alveolar type II cells at the BAJ lesions. Since 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 risk in lung carcinogenesis.

**Key words:** Lung adenocarcinoma, cooking oil fume, tt-DDE, BAJ hyperplasia and granulomatous nodule, signal transducer and activator of transcription 3 (STAT3)

**Introduction**

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 non-smoking females is unusually high among Chinese populations [3]. Recent epidemiological studies have demonstrated that exposure to cooking oil fume (COF) was 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 productions. Increased mutagenic metabolites in the urine and enhanced abnormal cell proliferation (hyperplasia) have been reported in esophagus and of rats exposed to oils which has been heated [6]. Besides its associations with lung carcinoma in non-smoking women 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 is a complex mixture of chemicals, in which fatty acids, especially the polyunsaturated fatty acids, in the cooking oils 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], stored food productions [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 a hundred fold higher than that of polycyclic aromatic hydrocarbon [11]. Similar to the findings by Hageman et al. [6] that oxidized 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*-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 have reported that *tt*-DDE exposure increased cell proliferation, expression and
release of pro-inflammatory cytokines such as interleukin-1 beta (IL1β) and tumor necrosis factor alpha (TNFα), 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 \textit{tt-DDE in vivo}. We believe that such \textit{in vivo} information is lacking and is very much needed. We further believe that this information will certainly contribute to the understanding on the role of \textit{tt-DDE} in the induction of lung carcinogenesis.

\textbf{Materials and Methods}

\textbf{Animal study protocol and design}

54 six-week-old male ICR mice were used in this study. 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 to standard and approved protocols at the facility (23 ± 1 °C, 39-43% relative humidity; water and food were available \textit{ad libitum}). Because the general information on the pulmonary status (anatomy, histology, and pathology) of ICR mice were well established [17] and our previous experience with this strain of mouse, ICR mice were used in this study. In the present study, mice were divided into three groups: 21 mice in the vehicle group (controls), 10 in the low dose group (8 mg/kg body weight \textit{tt-DDE}), and 23 in the high dose group (24 mg/kg body weight \textit{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) dissolved in 0.9 % sodium chloride. Mice in the \textit{tt-DDE}-exposed groups were intratracheally instilled with either 8 or 24 mg/kg body weight \textit{tt-DDE} per week for 8 weeks under isoflurane anesthesia.

The selection of a working dose regimen for our present study was based on a prior dose-response study. The dosage of 24 mg/kg \textit{tt-DDE} or less was found to be relatively safe for ICR mice during the period of eight-week exposure. (All animals survived with relatively good health with good body weight gain as compared to the controls). The duration of exposure for our present study was determined in accordance with our previous \textit{in vitro} studies which lasted approximately 8 weeks [15, 16]. In present \textit{in vivo} study, we intend to expose the animals to approximately the same duration of time so that we may compare between these \textit{in vitro} and \textit{in vivo} studies, if needed. To ensure the last dosing would be a biologically effective one, animals were sacrificed one week after the last intratracheal administration (9\textsuperscript{th} week of the experiment).
The overall experimental design and treatment scheme are presented graphically in Figure 1.

**Preparations and evaluations of BAL 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 labeled bottles.

To assess the inflammatory response in lung tissues induced by \( \mu \)-DDE treatment, the total and cell numbers and cell types in the BAL fluids from the animals were determined with a cell counter (Coulter, Inc., Miami, FL). The bronchoalveolar larvage (BAL) fluid was first cytopspined and collected at 450 × g for 15 min using a Shandon Cytospin 4 (Thermo Scientific, Waltham, MA). The cytopsin smear was then prepared and Liu’s staining (Tonyar Biotech, Taiwan) was performed for distinguish different cell types. Cells in the BAL fluids 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 (GSH) and glutathione disulfide (GSSG) in mouse lungs**

GSH was quantified by reacting with o-phthalaldehyde which produced a fluorescence product with excitation and emission at 365 and 430 nm [18]. In brief, lung tissues were homogenized with homogenization buffer (154 mM KCl, 5 mM diethylenetriaminepentaacetic 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 diethylenetriaminepentaacetic acid and 20 mM ascorbic acid) and centrifuged at 14,000 x g for 15 min at 4 °C. For quantification of GSH, the supernatant was incubated with 2.5 mg/ml o-phthalaldehyde in redox quenching buffer. For quantification of total glutathione, the supernatant was incubated with 2.5 mg/ml 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 Ex/Em 365/430 nm [18]. The amount of GSSG was calculated according to the following formula: GSSG = [Total glutathione-GSH]/2.

**Quantifications of pathological changes at the BAJ induced by \( \mu \)-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 sections were randomly selected and stained with hematoxylin and eosin for histological examinations. Masson’s trichrome stain was also performed to demonstrate and confirm fibrotic status on 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. Minimal three tissue sections from each animal per study group were examined and evaluated. The percentage of BAJ lesion was calculated as the average number of BAJ lesions observed vs. the total number of BAJs found in each study group (BAJ lesion/BAJ x 100). The extensiveness or
severity of the BAJ lesions were estimated by the “size” of the BAJ lesions 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). The percentage of the BAJ lesions with increased collagen deposit vs. the total number of BAJ lesions found in each study group was calculated as number of BAJ lesions with increased collagen deposit/total BAJ lesions x 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 single antibody. The specific antibodies used were: anti-cytokeratin (CK; Chemicon, Huissen, Netherlands) for bronchiolar epithelial cells, anti-Clara cell secretory protein (CCSP; Millipore, Billerica, MA) for bronchiolar Clara cells and anti-prosurfactant protein C (proSP-C; Chemicon, Huissen, Netherlands) for alveolar type II cells. Cells involved in proliferation or growth (hyperplasia) was assessed by the immunostaining of proliferating cell nuclear antigen (PCNA; BD Transduction Laboratories, Lexington, KT). The immunostaining of phosphorylated signal transducer and activator of transcription 3 (pSTAT3; Cell Signaling, Danvers, MA) was used to detect the localization 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. Multivision Polymer Detection System (Thermo Fisher Scientific, Fremont, CA) 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 time for proSP-C, or CCSP, and pSTAT3 were adjusted to 2 hr and 16 hr at room temperature, respectively for optimal results for mice lung tissues.

Analysis of real-time reverse transcription polymerase chain reaction (RT-PCR) array
RNA was extracted from lung tissues preserved in TriReagent (Life Technologies, Rockville, MD). Synthesis of cDNA was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Six representative lung cDNA samples were selected from vehicle- and 24 mg/kg tt-DDE-exposed mice. Between both groups, the relative expression of inflammatory genes was measured using mouse inflammatory cytokine & receptor PCR Array (PAMM-011, Superarray, Frederick, MD). The pathway analysis of the inflammatory gene expression was done with the software MetaCore™ (Genego, St Joseph, MI).

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

**Results**

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

In the BAL fluids, the total cell counts in mice exposed to 24 mg/kg *tt*-DDE was significantly increased by about 2 folds when compared to that of controls (Figure 2A). Differential cell counts of the BAL fluids also showed that both epithelial and non-epithelial components (leukocytes) in 24 mg/kg *tt*-DDE-exposed mice were 2.3 folds of those in controls (Figure 2B). The BAL of 24 mg/kg *tt*-DDE-treated mice showed a 2.5-fold increase in macrophages without significant elevation in neutrophil (PMN) and in lymphocyte counts (Figure 2C). These parameters were not found to be significantly changed in the BAL fluid of 8 mg/kg *tt*-DDE-exposed mice (Figure 2).

**The oxidative stress status determined by 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 and 24 mg/kg *tt*-DDE exposed groups, the GSH/GSSG ratios were 1.21 ± 0.81 and 1.28 ± 0.62 respectively. No significant alternation in GSH/GSSG ratio was found in lung tissues of *tt*-DDE-exposed animals.

**Pulmonary pathologic changes induced by *tt*-DDE exposure**

Two prominent lesions were found in lungs of mice exposed to *tt*-DDE: epithelial hyperplasia and granulomatous nodules (Figure 3B and C). Both these lesions were located at the bronchioloalveolar junctions (BAJ) and were much more remarkable in the high dose (24 mg/kg) treated mice than in the lower dose (8 mg/kg) treated animals (Table 1). Careful examination of these lesions revealed that the BAJ hyperplasia was characterized by non-ciliated bronchiolar epithelial hyperplasia, lymphocytic infiltration and mild peri-lesional interstitial thickening possibly representing fibrosis (Figure 3B insert). Some of these BAJ hyperplasia could be seen to extend and develop into focal tissue mass or granulomatous nodules which primarily comprised of epithelial hyperplasia, lymphocytic infiltrates, epitheloid cell and macrophage aggregations, and fibroblasts (Figure 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 (Figures 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 characterize the specific cell types of involvement in the BAJ lesions. Special histochemical stains were employed for such affirmation and characterization. Immunohistochemically, the cells at BAJ hyperplasia demonstrated both CK and PCNA reactivities (Figure 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) stainings, both Clara cells and alveolar type II cells could be identified in the BAJ lesions (Figures 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 (Figure 4E and F). It is interesting to note that similar cell marker staining procedures revealed that only alveolar type II cells, but not Clara cells, were present in the BAJ granulomatous nodules (Figure 5C and D).

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

Utilizing 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. Chemokines CCL1, CCL11, CCL12, CXCL13, and cytokine IL10 were significantly up-regulate, whereas CCL2 and IL1β were down-regulated in the tt-DDE treated mice (Table 2). In contrast, three receptors, CCLR1, ILR1-II and IL8R were down-regulated by tt-DDE exposure (Table 2). To further clarify the potential downstream regulator or factor which could be affected by these tt-DDE-induced inflammatory genes, we used the software (Metacore™) to do a pathway analysis on these inflammatory genes. pSTAT3 was identified as the common downstream factor activated by CCL1, CCL11, CCL12, CXCL13 and IL10 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 developed, 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 (Figure 5A). Via double immunostainings of pSTAT3 with CCSP (marker for Clara cells) or with proSP-C (marker for alveolar type II cells), we further demonstrated that the enhanced pSTAT3 accumulation was not associated with Clara cells (Figure 5B), but was strongly associated with alveolar type II cells (Figure 5C) in the BAJ lesions. It is also important to note that although Clara cells were abundant in the BAJ hyperplasia lesion (Figure 5B), they were not found in the BAJ granulomatous nodules (Figure 5D).

**Discussion**

Recent epidemiological studies have suggested that cooking oil fumes (COF) exposure was strongly associated with lung adenocarcinoma in non-smoking Chinese females [4, 5]. Chinese cooking style (open-wok cooking with strong COF generation) for unusual exposures were suggested [4, 5]. However, increased respiratory and pulmonary diseases among kitchen workers in Europe were also 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]. While 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 is still lacking. Our 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 BAL fluids reflect such irritation and inflammatory process. Alveolar macrophage is the major defensive cells against toxicants and pathogens at the alveolar level of the lungs. An increase of 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 have 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 BAL of *tt*-DDE exposed animals in our present study was consistent with that observed in kitchen workers exposed to COF [20]. However, it seems paradoxical in our present study that while there was a decreased CCL2 levels in the lung tissues at 8 weeks, the number of alveolar macrophages in the BAL fluid 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 would decline in a later stage when the macrophage recruitment has subsided and fibrosis/repair has initiated. Time point for our present study (8 weeks) probably represents the latter condition.

In our previous *in vitro* study, we observed an increased IL1β in *tt*-DDE treated BEAS-2B cells [15]. However, the IL1β level was found to be lowered in our present *in vivo* study. This *in vitro* vs. *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 IL1β is known to be modulated by lymphocytes [26]. In *tt*-DDE exposed lungs, lymphocytic infiltration was prominent in the BAJ lesions. Although induction of IL1β may occur in lung tissues during early phase of *tt*-DDE exposure, suppression of such activity by increasing number of lymphocytes could occur in a later time. IL1β elevation may only represent an acute or early phase of tissue or cellular response to *tt*-DDE, as that we have observed in the *in vitro* situation [15]. Our present study was an 8 weeks 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 IL1β level at this late time period is not totally surprising. It may also be pointed out that there is an interesting interrelationship between IL1β levels with granuloma and fibrosis in lung diseases [26]. Our present pathological findings (granulomatous nodule development with mild fibrosis) are accompanied with a reduced IL1β level. These observations are consistent with that found in some chronic lung diseases, such as sarcoidosis, which also showed characteristic lung granuloma formation and low-graded fibrotic changes [26]. On the other hand, IL1β provides positive signal to fibroblasts [26]. Thus, elevated IL1β 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 bronchiolization or out-growth of bronchiolar cells (predominately Clara cells) into the alveoli [27]. In our 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 are increasing evidence showing that both bronchiolar Clara cells and alveolar type II cells can potentially be cells of origin for lung tumorigenesis, 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 tumorigenesis. The continued development of some of the BAJ hyperplasia tissues into focal growths (granulomatous nodules) at the BAJ, as those 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 extension 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, 12 and CXCL13), and cytokines (such as IL10) were found to be up-regulated 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 not only can 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 the activation of over-expressed STAT3 in alveolar type II cells would lead to the development of bronchioalveolar 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], our 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 glutathione redox status in the lungs after *tt*-DDE exposure. The redox response may have a narrow window and 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 lesion associated with Clara cells proliferation. Other cells, including Clara cells, outside of BAJ lesion were not affected by the *tt*-DDE. Since the areas of BAJ lesion were relatively small in comparison to the whole lung area, this specific redox change in BAJ lesion 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 in 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 found to be associated only with alveolar type II cells in the BAJ lesions. Induction of Clara cells proliferation, outgrowth nodules at the BAJ, and an enhanced pSTAT3 accumulation in alveolar type II cells which involved in BAJ granulomatous nodules, are all factors which would increase risks for lung adenocarcinoma development. We believe that our present study not only has 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*.

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References

**Figure Legends**

**Figure 1:** Experimental design and schedule for intratracheal instillation treatment in the animal study. All animals (Vehicle control, n = 21; tt-DDE at 8 mg/kg dose, n = 10; tt-DDE at 24 mg/kg, n = 23) were treated weekly for 8 weeks. Animals were sacrificed one week (9th week) after the last treatments.

**Figure 2:** Changes in cell numbers among different cell populations in the bronchoalveolar larvage (BAL) fluids from mouse lungs at 9th week of tt-DDE exposure (8 or 24 mg/kg dose). **A.** Total cell numbers in the BAL fluids. **B.** Leukocytes and epithelial cells represented the two major populations of cells increased in the BAL fluids. **C.** Among the leukocytic cell populations (macrophages, neutrophils and lymphocytes) in the BAL fluids, only macrophages showed the most significant elevation in number. Results were presented as mean ± SD in vehicle group (n = 17) and in 8 or 24 mg/kg tt-DDE-exposed group (n = 11 or 23 respectively). *p < 0.05 represented significant and was analyzed by the one way ANOVA when compared with vehicle group.
Figure 3: Pathologic changes induced by intratracheal tt-DDE instillation in mouse lungs. A. Controls. Normal lung histology with BAJs (arrows). Hematoxylin & eosin (H&E) stain. B. Lung section from animal treated with tt-DDE (24 mg/kg). Increased cellularity around many BAJs was noted (arrow heads). H&E stain. B-insert. Higher magnification of a BAJ from Figure 3B demonstrating BAJ hyperplasia, with increased cellular proliferation extending from the bronchiolar site into the alveolar area. Lymphocytic infiltration and mild fibrosis were also evident. H&E stain. C. A granulomatous nodule at the BAJ. This lesion can be seen 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. H&E stain. D. and E. Increased collagen deposits (blue color) in the BAJ hyperplasia area (D) and in a BA granulomatous nodule (E) was demonstrated confirming mild fibrosis was present in these lesions. Masson’s trichrome stain. Scale bar in A and B: 500 µm; B-insert and D, 100 µm; C and E: 50 µm.
Figure 4: Identification of specific epithelial cell types involved in the \( \mu \)-DDE-induced BAJ hyperplasia using immunohistochemical techniques. **A.** CK 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.** PCNA immunostaining is a special 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.** 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.** 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. Counterstaining for Figure 4A to 4D was hematoxylin. Scale bar, A to D: 100 µm; E and F: 20 µm.
Figure 5: The Immunohistochemical stainings of pSTAT3 in the tt-DDE-induced BAJ hyperplasia. A. Some cells in the BAJ hyperplasia displayed positive immunoreactivity (light brown color) of pSTAT3 in the nuclei (arrows). The counterstain was hematoxylin. B. Double immunostainings for CCSP (Clara cells) and pSTAT3. All the Clara cells (dark blue-brown cytoplasm) were shown to have no brownish nuclear staining (cells with hollow centers, 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 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 demonstrated in Figure 5C). No Clara cell (cells with positive CCSP staining) was in fact identified in the BAJ granulomatous nodule. Scale bar: A and B, 50 µm; C and D, 20 µm.
# Table 1: Quantifications on the BAJ lesions in lungs of mice treated with vehicle or \( tt \)-DDE (8 mg/kg or 24 mg/kg)

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<th>Mouse group</th>
<th>% of BAJ with increased collagen deposit ( ^a )</th>
<th>% of BAJ lesion occurrence ( ^b )</th>
<th>BAJ lesion size (Mean ± SD) ( ^c, ^d )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.59</td>
<td>0.46</td>
<td>1.04 ± 0.21</td>
</tr>
<tr>
<td>8 mg/kg ( tt )-DDE</td>
<td>7.92</td>
<td>7.40</td>
<td>1.20 ± 0.63</td>
</tr>
<tr>
<td>24 mg/kg ( tt )-DDE</td>
<td>22.37</td>
<td>29.50</td>
<td>1.91 ± 0.84</td>
</tr>
</tbody>
</table>

\( ^a \), The % of BAJ with increased collagen deposit was calculated as 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. 

\( ^b \), The % of lesion occurrence was calculated as number of BAJ lesion observed over total number BAJ in the tissue sections. A dose correlated increase in the number of lesion was observed. 

\( ^c \), Average size of BAJ lesion was measured via computerized morphometric quantitative analyzer (Average 3 sections per animal were analyzed.). Dose correlated increase in size of lesion was also demonstrated with \( tt \)-DDE exposures. 

\( ^d \), \( P < 0.001 \), data were analyzed by linear regression testing.
<table>
<thead>
<tr>
<th>Gene function</th>
<th>Gene name</th>
<th>Fold change*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokines</td>
<td>Chemokine (C-C motif) ligand 1 (CCL1)</td>
<td>2.09</td>
</tr>
<tr>
<td></td>
<td>Chemokine (C-C motif) ligand 11 (CCL11)</td>
<td>2.96</td>
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<tr>
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<td>Chemokine (C-C motif) ligand 12 (CCL12)</td>
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<tr>
<td></td>
<td>Chemokine (C-C motif) ligand 13 (CCL13)</td>
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<tr>
<td></td>
<td>Chemokine (C-C motif) receptor 1 (CCLR1)</td>
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<td>Chemokine receptor 1 (CCL2)</td>
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<tr>
<td>Interleukins</td>
<td>Interleukin 1 beta (IL1β)</td>
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<td></td>
<td>Interleukin 10 (IL10)</td>
<td>2.04</td>
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<td></td>
<td>Interleukin receptor, type II (ILR1-II)</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>Interleukin receptor, beta (IL8Rβ)</td>
<td>0.31</td>
</tr>
</tbody>
</table>

* represented significant by Student t-test when compared with vehicle group (P < 0.05).