Air Pollution Particles Hijack Peroxidasin to Disrupt
Immunosurveillance and Promote Lung Cancer

Zhenzhen Wang\textsuperscript{1,2}, Ziyu Zhai\textsuperscript{1}, Chunyu Chen\textsuperscript{1}, Xuejiao Tian\textsuperscript{1}, Zhen Xing\textsuperscript{1}, Panfei Xing\textsuperscript{2}, Yushun Yang\textsuperscript{1}, Junfeng Zhang\textsuperscript{1,*}, Chunming Wang\textsuperscript{2,*}, Lei Dong\textsuperscript{1,3,*}

1. State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, Jiangsu 210023, China.
2. State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Taipa, Macau SAR.
3. Chemistry and Biomedicine Innovative Center, Nanjing University, Nanjing, Jiangsu 210023, China.

* Corresponding Authors:
L.D., Email: leidong@njlu.edu.cn; C.W., Email: cmwang@umac.mo; J.Z., Email: jfzhang@njlu.edu.cn.

Keywords
Inhaled Fine Particulate Matter, Cancer Immunosurveillance, Migration of Immune Cells, Collagen Crosslinking, Lung Tumorigenesis
Abstract

Although fine particulate matter (FPM) in air pollutants and tobacco smoke is recognized as a strong carcinogen and global threat to public health, its biological mechanism for inducing lung cancer remains unclear. Here, by investigating FPM’s bioactivities in lung carcinoma mice models, we discover that these particles promote lung tumor progression by inducing aberrant thickening of tissue matrix and hampering migration of anti-tumor immunocytes. Upon inhalation into lung tissue, these FPM particles abundantly adsorb peroxidasin (PXDN) – an enzyme mediating type IV collagen (Col IV) crosslinking – onto their surface. The adsorbed PXDN exerts abnormally high activity to crosslink Col IV via increasing the formation of sulfilimine bonds at the NC1 domain, leading to an overly dense matrix in the lung tissue. This disordered structure decreases the mobility of cytotoxic CD8+ T lymphocytes into the lung and consequently impairs the local immune surveillance, enabling the flourishing of nascent tumor cells. Meanwhile, inhibiting the activity of PXDN abolishes the tumor-promoting effect of FPM, indicating the key impact of aberrant PXDN activity on the tumorigenic process. In summary, our finding elucidates a new mechanism for FPM-induced lung tumorigenesis and identifies PXDN as a potential target for treatment or prevention of the FPM-relevant biological risks.
Introduction

Inhalable fine particulate matter (FPM) with a diameter less than 1 micron in air pollutants and tobacco smoke has been recognized as a Group 1 carcinogen and substantial threat to global health (1). About 10 μg/m³ increase in its concentration was correlated with an 8% rise in lung cancer mortality (2). However, its carcinogenic mechanism remains unclear. During lung tumorigenesis, both the growth of cancer cells per se and the supporting microenvironment are crucial (3). Earlier studies propose that the particles directly induce gene mutations and carcinogenesis (4). Clinical data suggests smoking as the factor for the highest prevalence of somatic mutation among lung cancers (5; 6). However, despite its mutagenic potential, recent investigations reveal that FPM does not directly promote (and even inhibit) the proliferation of lung cancer cells. These inconsistent findings suggest that FPM might have unidentified targets other than cancer cells in promoting tumorigenesis, such as immune cells that play key roles in tumor development (7). Under normal circumstances, the immune system rapidly detects and suppresses the tumor progression at the initial stage (8). Especially, the mostly ‘informed’ defender immunocyte - cytotoxic CD8⁺ T lymphocytes (CTLs) protect against potential cancer through efficient migration and cytotoxic contact with transformed or tumorigenic cells that have emerged in the lung interstitial space (9). Once this crucial immunosurveillance and defense process of CTLs were compromised, the tumorigenesis would be uncontrollable (10).

Under the chemotaxis of biochemical signals, the mobility of immunocytes depends on not only its intrinsic capacity but also the microstructure of the interstitial extracellular matrix (ECM), that is, the way paved for immune cells (11). For the former, evidence about the direct
effect of FPM on the immune cell’s migration capacity was validated. It is estimated that tobacco smoke particulates (TSP) could impair the migration function of macrophages to mycobacteria and lead to increased susceptibility to tuberculosis in smokers (12). For the latter, clinical evidence links a dense collagen matrix surrounding the tumor with the restriction of T cells’ access (13; 14). Based on these reports and analysis, we speculated that FPM could disturb the migration and distribution of T cells in lung tissue, thus impairing CLTs’ immune defense capacity to cancerous cells and consequently promoting tumor progression.

To test this hypothesis, we set out to study the effect of FPM inhalation on CTLs’ immune response and tumor development, by employing both transplantation (Lewis lung carcinoma, LLC) and transgenic ($K\text{ras}^{G12D}Trp53^{-/-}$) mouse models of lung carcinoma (15). First, we validated that FPM promotes tumorigenesis by impairing CTLs’ migration towards cancerous cells. The defect was attributed to denser collagen structure induced by FPM on CTLs’ migration path, generating the physical isolation around tumor cells. More interestingly, we found that FPM exerts this effect by adsorbing peroxidasin (PXDN) – a crucial enzyme specifically mediating collagen crosslinking at NC1 domain – and increasing this enzyme’s activity to over-crosslink ECM and prevent CTLs migration, which eventually tolerates tumor progression.

Results

FPM Promotes Lung Cancer Development by Hampering CTLs’ Migration

To analyze the effect of fine particulate matter (FPM) on lung tumorigenesis, first, we
collected and prepared particulate matter in air pollutants with diameter < 1 µm (PM1) from 7
locations in China and the tobacco smoke particle (TSP) respectively. Given that these
particles displayed diverse morphology and physicochemical characteristic (Figure 1—figure
supplement 1 and Figure 1—source data 1), which is consistent with the material of
particles in other reports (16), we mixed PM1 with the equal proportion from each collection
to eliminate the interference of sampling resources. Then, mice exposed to mixed PM1
(mixture) or TSP were analyzed on two cancer animal models: the syngeneic Lewis lung
carcinoma (LLC) inoculation model (LLC-model) or the transgenic mouse model
(Kras<sup>G12D</sup>Trp53<sup>−/−</sup>) as illustrated in Figure 1—figure supplement 2A. Gross view (Figure 1—
figure supplement 2B) and histological hematoxylin and eosin (H&E) analysis (Figure 1A)
indicated that the FPM treatment markedly increased the tumors’ multiplicity and progression.
As Kras<sup>G12D</sup>Trp53<sup>−/−</sup> mice could generate multifocal tumors corresponding to different grades
of lung carcinoma (17; 18), the histological grade of this model was further analyzed. Tumors
in FPM-exposed lung tissue were mainly classified as grade II and the ones of grade III and
IV were significantly higher, whereas the majority of tumors in the phosphate buffer saline
(PBS) group were of grade I, showing FPM lead more advanced lung tumorigenesis (Figure
1B). Statistical analysis suggested that the number of tumors in the FPM-treated group was
significantly higher than that in the PBS group (about 3–5 folds higher in LLC model and 2
folds higher in Kras<sup>G12D</sup>Trp53<sup>−/−</sup> model) (Figure 1C). Furthermore, the scenario was further
validated by the corresponding tumor burden, based on the percentage of the area of tumor
regions versus that of the total lung (about 7 folds more in LLC model and 10 folds more in
Kras<sup>G12D</sup>Trp53<sup>−/−</sup> model). Moreover, in both models, PM1 and TSP exposure significantly
shortened the survival of mice (Figure 1D). These results validated the correlation between FPM exposure and lung cancer development, in agreement with the epidemiological studies (19).

**Figure 1.** FPM accelerated lung tumorigenesis by inhibiting cytotoxic T cells lymphocytes (CTLs) infiltration. A. Representative hematoxylin and eosin (H&E) staining images of different model 20 days or 50 days after FPM-exposed mice were stimulated with LLC or Cre-inducible adenovirus (AdCre). Scale bar = 100 μm; B. Tumor stage (stages 1 to 4) of lungs in Kras^{G^{12D}/Trp53^/-} model (left) and representative tumors H&E staining images with higher magnification (right). P values are for comparisons of the percentage of stage 3 and 4 tumors in FPM-treated and control mice. n=5; C. Quantitative analysis about tumor number and tumor burden in lung tissue of different models treated as for panel A. n=5; D. Survival analysis of mice exposed to FPM and subsequently stimulated with LLC or AdCre. n=7; E. Representative EdU staining images to analyze the proliferation rate of tumor site in
l lung tissue of LLC model 20 days after tumor initiation (DAPI, blue; EdU positive cells, green). Scale bar = 100 μm; F. The statistical analysis of CTLs (IFN-γ' CD8+/CD45+CD3+) in lung tissue based on flow cytometry at indicated day (0 d, 1 d, 3 d, 5 d, 10 d and 20 d) after intravenous injection of LLC. n=3. The inserted results in the dashed boxes indicated the fold change of CTLs in lung tissue 1 day and 3 days after the LLC stimulation, relative to that under the physiological condition; G. The statistical analysis of CTLs (IFN-γ' CD8+/CD45+CD3+) in lung tissue of KrasG12DTrp53+/- mice based on flow cytometry 4 weeks after tumor initiation with the intratracheal injection of AdCre. n=3; H. IFN-γ enzyme-linked immunospot assay (ELISPOT) in the lung tissue of OT-1 TCR transgenic mice 1 day after OVA-LLC stimulation (upper) and representative immunospot images (lower). n=5; I. Representative H&E staining images of lung tissue in Foxn1mu nude mice exposed to FPM 20 days after intravenous injection of LLC. Scale bar = 100 μm; J. Quantitative analysis about tumor number and tumor burden in lung tissue of Foxn1mu nude mice treated as for panel I. n=5. Images are representative of three independent experiments. Results are shown as mean ± SD. *p<0.05 after ANOVA with Dunnett's tests.

**Figure 1—figure supplement 1.** A. Representative scanning electronic microscope (SEM) for particles detained on the fiber filter membrane, which were collected from airborne pollution in 7 different locations, including Nanjing City (Qixia, Jiangning, Pukou, Gulou, and Gaochun) in Jiangsu Province, Suzhou City in Anhui Province and Tieling City in Liaoning Province (named as: QX, JN, PK, GL, GC, SZ and TL) and tobacco smoke particle (TSP). Scale bar = 5 μm; B. Representative transmission electron microscopy (TEM) images for detached particles from filter membrane in panel A. Scale bar = 100 nm. Images are representative for three independent experiments.

**Figure 1—figure supplement 2.** A. Schematic diagram of Lewis lung carcinoma (LLC)-stimulated or KrasG12DTrp53+/-transgenic lung cancer model with FPM exposure; B. Gross lung tissue images in the FPM-exposed mice of LLC model or KrasG12DTrp53+/- model 20 days or 50 days after mice were stimulated with LLC or Cre-inducible adeno virus (AdCre). The tumor sites were labelled with black asterisk.

**Figure 1—figure supplement 3.** Cell cytotoxic analysis of LLC cells stimulated with a serial concentration of FPM (0, 5, 10, 30, 50, 100 and 500 μg/mL) for 24 h and 48 h. n=3. Results are shown as mean ± SD.

**Figure 1—figure supplement 4.** The quantified analysis of EdU-positive cells in tumor site of lung tissue of LLC model 20 days after tumor initiation. n=3. Results are shown as mean ± SD. n.s. indicates no statistical significance.

**Figure 1—figure supplement 5.** A. Gating strategy for the quantification of cytotoxic T lymphocytes CTLs cells (IFN-γ' CD8+/CD45+CD3+) in the lung tissues. B. Representative flow cytometry analysis of CTLs in FPM-exposed lung tissue of mice under the physical conditions (0 d) or at indicated days (0d, 1 d, 3 d, 5 d, 10 d and 20 d) after intravenous injection with LLC.

**Figure 1—figure supplement 6.** A. Representative immunofluorescence images of CTLs’ infiltration into the FPM-exposed lung tissue at indicated days (1 d, 3 d, 5 d, 10 d and 20 d) after intravenous injection with LLC. The CTLs were labelled with CD8 and shown in green. Images are representative for three independent experiments. Scale bar = 200 μm.

**Figure 1—figure supplement 7.** Representative flow cytometry analysis of CTLs (IFN-γ' CD8+/CD45+CD3+) in FPM-exposed lung tissue of KrasG12DTrp53+/- mice 4 weeks after tumor initiation.
initiation with the intratracheal injection of AdCre.

**Figure 1**—**figure supplement 8.** Representative flow cytometry analysis of CTLs (IFN-γ^+^CD8^+^/CD45^+^CD3^+^) in FPM-exposed lung tissue of OT-1 TCR transgenic mice 1 day after OVA-LLC stimulation.

**Figure 1**—**figure supplement 9.** Gross lung tissue images in FPM-exposed Foxn1nu nude mice 20 days after they were intravenously injected with LLC.

**Figure 1**—**figure supplement 10.** A. Scheme of analyzing the effect of T cell chemokines IP-10 on the CTLs’ infiltration into the FPM-exposed mice; B. Statistical flow cytometry analysis of CTLs cells (IFN-γ^+^CD8^+^/CD45^+^CD3^+^) in lung tissue 2 hours after the mice were stimulated with 5 μg/kg IP-10 through intratracheal injection. n=3. Results are shown as mean ± SD. *p<0.05 after ANOVA with Dunnett’s tests.

**Figure 1**—**Source data 1.** Physicochemical characteristic of fine particulate matter collected from airborne pollution in 7 different locations.

**Figure 1**—**Source data 2.** The Excel spreadsheet source file for Figure 1C, D, F, G, H, and J.

Next, we explored the reason for FPM promoting tumorigenesis. The conditions of the seeds and soil – the uncontrollably proliferative cancer cells and a tolerable immune microenvironment – are both crucial for tumor development (20). We analyzed the effect of FPM on the tumor cells and their congenial microenvironment, respectively. Interestingly, FPM hardly promoted the growth of tumor cells and even inhibited their proliferation at higher concentrations (**Figure 1**—**figure supplement 3**). EdU incorporation assay was further employed to determine the impact of FPM exposure on tumor cells’ proliferation *in vivo*. The result showed that the tumor site displayed similar replication capacity regardless of its size and advancement in these groups (**Figure 1E and Figure 1**—**figure supplement 4**), casting the doubt of FPM’s direct promotion on tumor growth. These results inspired us to assess the effect of FPM on the immune microenvironment. Among these immunocytes related to immune surveillance, cytotoxic T lymphocytes (CTLs) as the most “informed” defender are critical for locally extinguishing the nascent tumor. The efficacy of these cells determines the fate of transformed cells – to death or flourish. Thus, we examined the change of CTLs’
response in different groups during tumor progression (Figure 1—figure supplement 5). In the LLC model, CTLs in PBS group were efficiently recruited into lung tissue to defend LLC stimulation, increasing up to about 9-fold than that under the physiological condition at the initial stage (1-3 days). Conversely, the lung tissue with FPM exposure displayed blunt and insufficient early immune defense, with slight CTLs infiltration, decreasing by more than 60% relative to that of PBS group, though there was no dramatic difference in CTLs accumulation among these groups at late stage (5-20 days). The immunofluorescence (IF) images also showed that FPM-exposed lung tissue was infiltrated with lower CTLs at the initial stage (Figure 1—figure supplement 6). These results indicated that the CTL’s early immune response might not be normal in FPM-exposed mice and be decisive for the lung tumorigenesis, which is consistent with the reports in transgenic autochthonous lung tumors (9). Then we detected the CTLs’ infiltration in Kras$^{G12D}$Trp53$^{-/-}$ model 4 weeks after tumor initiation, during which the immune response was reported to reach to the peak (9). The results displayed similarly insufficient CTLs’ defense in the FPM-exposed group (Figure 1G and Figure 1—figure supplement 7).

To further determine whether CTLs’ reaction was specific to tumor cells, we further evaluated CD8+ T cell activation in an OT-1 TCR transgenic mouse model, in which the CD8+ T cells express a T cell receptor recognizing the SIINFEKL peptide of ovalbumin (OVA) (21). Upon the stimulation of OVA-expressing LLC (OVA-LLC) cells, the flow cytometry analysis of activated CTLs in lung tissue showed consistent tendency (Figure 1—figure supplement 8). OVA-LLC specific immunity response in lung tissue was also tested by IFN-γ enzyme-linked immunospot assay (ELISPOT) (Figure 1H). The result further demonstrated that the
antigen-specific activated CTLs was significantly impaired by FPM exposure, decreasing to about 25% of that in PBS group. Furthermore, to testify the indispensable role of CTLs on the tumor development, LLC cells were intravenously injected into FPM-exposed athymic Foxn1nu nude mice with T cell deficiency. As expected, the difference of lung tumorigenesis in PBS and FPM-exposed groups was abolished (Figure 1 I, J and Figure 1—figure supplement 9), highlighting that the influences of FPM to tumor development were mediated by the CTLs. Additionally, without using tumor cells, we treated mice with a T cell chemokine – C-X-C motif chemokine ligand 10 (CXCL10) (22), or named as interferon-inducible protein-10 (IP-10) – intrabronchially for 2 h, as illustrated in Figure 1—figure supplement 10A, and found that the proportion of CTLs in FPM-treated mice (about 8%) significantly decreased compared with that of the PBS group (about 18%; Figure 1—figure supplement 10B). This finding is consistent with the scenario observed in the tumor (LLC and KrasG12DTrp53−/−) model and strengthens the conclusion that FPM exposure delays the CTLs’ instantaneous defense response. The above results, taken together, indicate that FPM accelerates lung tumorigenesis via impairing CTLs infiltration into the lung tissue.

FPM Hinders CTLs Migration by Crosslinking Type IV Collagen and Thickening Tissue

Matrix

Next, we explored the reason for the impaired early response of CTLs under FPM exposure. CTLs distribution in the lung interstitial tissue depends on its migration ability (23), which is related to both the intrinsic activity of cells and the structure of the interstitial space formed by the local extracellular matrix (ECM) on its migrating path (24). We analyzed which
factor was mainly affected by FPM. First, T cells treated with FPM in vitro or the CTLs
separated from FPM-exposed lung tissue were respectively analyzed. Integrin-1 (ITGB-1), C-
X-C motif chemokine receptor 3 (CXCR 3) and Rho-associated kinase (ROCKi) (25-27), the
biomarkers related to CTLs’ migration were detected with quantitative real-time polymerase
chain reaction (qRT-PCR). These results showed that FPM stimulation had little effect on the
migration potential of CTLs (Figure 2—figure supplement 1). Second, we analyzed the
change of the lung tissue structure after FPM exposure for 7 days. From SEM images and
quantitative analysis about the pore size of interstitial matrix (Figure 2A and Figure 2—
figure supplement 2), we noticed that the FPM exposure dramatically compressed the
structure and crushed the interstitial space of the lung tissue. Further, Masson’s trichrome
staining indicated a higher density of collagen (Figure 2B). These data implied that FPM
inhaled into the lung tissue condensed the native framework of ECM, which could block the
path of CTLs migrating to the tumor site.
Figure 2. FPM impaired CTLs migration by increasing Col IV crosslinking in the lung tissue. A. Scanning electronic microscope (SEM) images of the interstitial matrix in the lung tissue exposed to FPM for 7 days. Scale bar = 100 μm; B. Representative Masson’s trichrome histological analysis of lung tissue exposed to FPM for 7 days. Images are representative of three independent experiments. Scale bar = 100 μm; C. Representative trajectory of CTLs’ migration in lung tissue slice of FPM-exposed mice or PBS group; D. The quantified analysis of migration distance, displacement and velocity of tracked CTLs vs. time (min) based on panel C; E. Proportion of high crosslink Col IV in lung tissue of mice exposed to FPM or PBS.
for 7 days, which was calculated by the ‘high-cross’ Col IV fragment divided by the sum of different fractions (‘low-cross’ ones and ‘high-cross’ ones). The content of each part was detected by ELISA. n=5; F. Western blotting analysis of ‘low-cross’ collagen and ‘high-cross’ collagen in lung tissue of mice exposed with FPM or PBS for 7 days; G-J. The in-depth analysis of representative Col IV immunofluorescence images of lung tissue in the mice exposed to FPM for 7 days through Image J: G. Look-up tables (LUTs) analysis of Col IV fluorescence intensity; H. Surface plot analysis of Col IV distribution based on invert binary distance maps; I. Binary images of Col IV network generated by ridge detection plugin; J. Quantification analysis of junction number and density in Col IV network based on panel I. n=3; K. Representative immunofluorescence images of Col IV and SEM images of FMP-exposed lung tissue treated with collagenase D (50 μg/ml). Scale bar = 10 μm; L. The trajectory of CTLs migrating in FPM-exposed lung tissue slice treated as for panel K; M. Average migration distance, displacement and velocity of tracked CTLs vs. time (min) in lung tissue slice treated as in panel K. Images are representative for three independent experiments. Results are shown as mean ± SD. *p<0.05 after ANOVA with Dunnett’s tests.

**Figure 2**—figure supplement 1. Transcriptional level of typical markers related to CTLs’ migration, integrin-1 (ITGB-1), C-X-C motif chemokine receptor 3 (CXCR 3) and Rho-associated kinase (ROCK)I, in Jurkat T cells after they were stimulated with FPM for 48 h (A) or in the CTLs separated from lung tissue exposed to FPM for 7 days (B). n=3. Results are shown as mean ± SD. n.s. indicates no statistical significance. p values were listed underneath.

**Figure 2**—figure supplement 2. The quantified analysis of pore diameter of interstitial matrix in the lung tissue, based on the scanning electron microscope (SEM) images and analyzed by Image J. n=3. Results are shown as mean ± SD. *p<0.05 after ANOVA with Dunnett’s tests.

**Figure 2**—figure supplement 3. Schematic diagram of analyzing of CTLs’ migration in lung tissue slice of FPM-exposed mice or PBS group.

**Figure 2**—figure supplement 4. Schematic diagram of separating collagen fraction with different crosslink level. The part separated by neutral salt, acetic acid and pepsin was defined as ‘low-cross’ and the residual ones was regarded as ‘high-cross’, according to the reported literature (28).

**Figure 2**—figure supplement 5. The relative intensity of high-crosslink Col IV to low-crosslink ones according to the WB results of lung tissue exposed to FPM for 7 days, based on the Image J analysis. n=3. Results are shown as mean ± SD. *p<0.05 after ANOVA with Dunnett’s tests. n.s. indicates no statistical significance.

**Figure 2**—figure supplement 6. Representative Col IV immunofluorescence images of lung tissue in the mice exposed to FPM for 7 days, with the blue DAPI staining images shown in the inserted box. Scale bar = 100 μm.

**Figure 2**—figure supplement 7. A. Proportion of high-crosslink Col I (left) and Col III (right) in lung tissue of mice exposed to FPM or PBS for 7 days, which was calculated by the ‘high-cross’ fragment divided by the sum of different fraction (‘low-cross’ ones and ‘high-cross’ ones). The content of each part was detected by enzyme linked immunosorbent assay (ELISA). n=5.; B. Western blotting analysis of ‘low-cross’ collagen and ‘high-cross’ Col I (left) and Col III (right) in lung tissue of mice exposed to FPM or PBS for 7 days. Their relative intensity analyzed by Image J was shown underneath. n=3; C. Representative Col I and Col III immunofluorescence images of lung tissue in the mice exposed to FPM for 7 days. Scale bar = 100 μm. Results are shown as mean ± SD. n.s. indicates no statistical significance.
**Figure 2—figure supplement 8.** A. The integrated optical density (IOD) of Col IV immunofluorescence images of lung tissue exposed to FPM for 7 days, based on Image J analysis. n=3. Results are shown as mean ± SD. *p<0.05 after ANOVA with Dunnett’s tests. B. Pearson’s correlation line of the migration index (migration distance, displacement and velocity) of different groups with integrated optical density (IOD) of Col IV in corresponding lung tissue slice as panel A. n=3. Results are shown as mean ± SD. *p<0.05 after ANOVA with Dunnett’s tests.

**Figure 2—figure supplement 9.** Schematic diagram about CTLs’ migration on the lung tissue exposed to FPM.

**Figure 2—video 1-3.** Dynamic migration videos of T cells in the slice of lung tissue exposed to FPM. Jurkat T cells were pre-stained with Calcein-AM and labelled as green in videos.

**Figure 2—video 4-7.** Dynamic migration videos of T cells on FPM-exposed lung tissue pre-treated with collagenase D (0.05 mg/mL) for 5 min. Jurkat T cells were pre-stained with Calcein-AM and labelled as green in videos. Collagen IV in lung tissue were labelled as red.

**Figure 2—Source data.** The Excel spreadsheet source file for Figure 2D, E, J, and M.

Consequently, we investigated in greater detail the movement of CTLs in an *ex vivo* model. The migration of CTLs in the slice of lung tissue (native or FPM-exposed) was analyzed by dynamically visualizing the cells’ movement (**Figure 2—figure supplement 3**). According to the outcomes from time-lapse sequential images and trajectory analysis of CTLs’ migration (**Figure 2C and Figure 2—video 1-3**), in the lung tissue exposed to FPM, CTLs struggled to migrate, while those of PBS group displayed quick migration pattern. Statistical analysis further validated that compared with the PBS group, the FPM-treated lung tissue severely hindered the migration of CTLs (**Figure 2 D**), which were weakly motile and showed insufficient displacement, distance and velocity (29). Therefore, the change in the interstitial space, rather than attenuated migrating potential of CTLs *per se*, is responsible for the weakened infiltration of these cells in the FPM-treated lung tissue.

Then, we asked what caused the change of the lung structure after FPM exposure. Collagens, the main ECM components (30), especially 3 kinds of ones enriched in lung tissue, including the type I, III and IV ones (Col I, Col III and Col IV) were focused on (31). First,
enzyme-linked immunosorbent assay (ELISA) was performed after different fragments of collagens were respectively harvested and divided into 2 categories by a reported protocol (28), that is, ‘low-crosslinked’ ones (low-cross) – containing freshly secreted collagens, procollagens and moderately crosslinked collagens – and the other remainder ‘high-crosslinked’ ones (high-cross) (Figure 2—figure supplement 4). The results showed both PM1 and TSP exposure significantly elevated the high-crosslink proportion of Col IV, 2-fold higher than that in PBS group, based on the separate examination of low-cross and high-cross ones (Figure 2 E). Next, the relative quantification of high-crosslinked collagens compared with low-crosslinked ones based on western blotting analysis showed consistent changes (Figure 2 F and Figure 2—figure supplement 5). Besides, the IF images indicated that FPM exposure induced Col IV in the lung tissue to generate enhanced crosslink and denser distribution, leading the collagen network with more junction site and higher junction density (Figure 2 G-J and Figure 2—figure supplement 6). However, the other two types of collagens (Col I and Col III) showed no obvious change (Figure 2—figure supplement 7), demonstrating an increased Col IV crosslinking accounted for the change in the lung ECM structure. Furthermore, based on the related integrated optical density (IOD) of Col IV in lung tissue slices and related CTLs’ migration index (migration distance, displacement and velocity) of different groups in Figure 2D, we performed the Pearson’s correlation analysis and found an inverse relationship between Col IV density and CTLs’ migration potential (Figure 2—figure supplement 8). Furthermore, we pre-treated the FPM-exposed lung tissue with collagenase D to reduce the Col IV crosslink and alleviate Col IV density (Figure 2 K). The trajectory images and related quantification analysis showed CTLs’ migration was
effectively reversed (**Figure 2L, M and Figure 2—video 4-7**), further validating the crucial role of Col IV crosslink on the CTLs’ movement. These data together suggested that FPM exposure blocked CTLs migration and trapped these cells mainly through increasing Col IV crosslinking and consequently generating a denser ECM in the lung tissue, which might isolate the tumor cells from the CTLs’ attack (**Figure 2—figure supplement 9**).

**FPM Increases Col IV Crosslinking through Promoting Sulfilimine Bond Formation**

We then investigated why FPM exposure led to increased Col IV crosslinking (30). According to recent discovery, protein adsorbed onto the nanoparticles surface would endow them with new activities (32; 33). As the median size of the both PM1 and TSP is about 100-200 nm, it is possible that the collagen-crosslinking activity of FPM is derived from the proteins adsorbed onto their surface from lung tissue. To elucidate this, we separately incubated FPM in PBS or lung homogenate (LH) to simulate the scenario of FPM per se (FPM group) or the complex of FPM and its surface proteins (LH-FPM group, including LH-PM1 and LH-TSP). Then, according to an established experimental model with a slight modification (34), soluble Col IV was generated by stimulating mouse bone marrow fibroblasts M2-10B4 cells, which highly express Col IV, with the inhibitor of collagen crosslink (**Figure 3—figure supplement 1**). Next, the effect of FPM on the crosslink of soluble Col IV in the cellular system and acellular system was respectively analyzed as shown in **Figure 3A**. For the cellular system, Col IV immunostaining result of M2-10B4 cells showed FPM itself could not induce the crosslinking of soluble Col IV (**Figure 3B**).

Relatively, the LH-FPM initiated the crosslinking and reinforced the network to a greater
extent than that induced by LH per se, which could be validated by intensive crosslink

intensity and a denser Col IV distribution, and collagen network with more junction site and

higher junction density (Figure 3C-F). Meanwhile, for the acellular experiment, the cell

lysate of M2-10B4 enriched soluble Col IV was incubated with FPM or LH-FPM mixture.

Western blotting result showed a similar scenario – the naked FPM had little devotion to Col

IV crosslink, but the LH-FPM dramatically enhanced the high-crosslinked Col IV fragment

(Figure 3G and Figure 3—figure supplement 2).
Figure 3. FPM increased Col IV crosslink via promoting sulfilimine bond formation at the NCI1 domain. A. Schematic representation of the procedures to generate soluble Col IV and analyze the effect of FPM on its crosslink. Briefly, the M2-10B4 cells highly expressing Col IV were treated with crosslink inhibitor for 1 day or 7 days and then treated with FPM or
the mixture of LH-FPM, stimulating the scenario of FPM per se or its interface with LH, to initiate the crosslink. The crosslink level of Col IV was analyzed with diversity methods; B. Representative immunofluorescence capture of Col IV in M2-10B4 cells stimulated with FPM or LH-FPM for 24 h after pretreated with crosslink inhibitor for 1 day. Scale bar = 20 μm. Images are representative of three independent experiments; C-F. The in-depth analysis of Col IV immunofluorescence images in panel B through Image J: C. Look-up tables (LUTs) analysis of Col IV fluorescence intensity; D. Surface plot analysis of Col IV distribution based on invert binary distance maps; E. Binary images of Col IV network generated by ridge detection plugin; F. Quantification analysis of junction number and density in Col IV network based on panel E; G. Western blotting of ‘low-cross’ and ‘high-cross’ Col IV fraction in M2-10B4 cells lysate enriched with soluble collagen after their treatment with FPM or LH-FPM; H. Schematic diagram of separating fragments containing the NC1 domain crosslink site in Col IV. The general crosslink network generated by Col IV was displayed on the left, with the important crosslink sites (7S domain and NC1 domain) respectively labelled in the yellow and red dotted box; I. High-resolution mass spectrum depicting tryptic peptides containing sulfilimine bond (-S=N-), with magnified spectrum displayed on the bottom. The formation of -S=N- and the known peptide sequence with different oxidation containing the sulfilimine bond were shown on the upper right.

**Figure 3—figure supplement 1.** Western blotting analysis of ‘soluble’ and ‘crosslinked’ Col IV fraction in M2-10B4 cells lysate after the cells were treated with different concentration of crosslink inhibitor phloroglucinol (PHG) for 7 days.

**Figure 3—figure supplement 2.** The relative intensity of high-crosslink Col IV to low-crosslink ones according to the WB results in M2-10B4 cells lysate enriched with soluble collagen after their treatment with FPM or the mixture of lung homogenate (LH) and FPM, that is, LH-FPM. n=3. Results are shown as mean ± SD. *p<0.05 after ANOVA with Dunnett’s tests.

**Figure 3—figure supplement 3.** Structure of sulfilimine bond formed at the covalent crosslinks of NC1 domains, shown in the lilac box.

**Figure 3—figure supplement 4.** A. The potential crosslink site formed at 7S domain, containing methylenimine bond (-C=N-, left) or pyridinium crosslink (right), shown in the lilac box; B. The allylsine generated during the Col IV crosslinking after soluble Col IV were incubated with LH per se or the mixture of LH-FPM (LH-PM 1 and LH-TSP) for 30 min, detected with specific probes for allylsine, with a serial content of oxidized bovine serum albumin containing known aldehydes as the internal standard (35). n=3. Results are shown as mean ± SD. n.s. indicates no statistical significance.

**Figure 3—Source data.** The Excel spreadsheet source file for Figure 3F, and I.

These data raised the question of how LH-FPM increased Col IV crosslinking. During crosslinking, the triple-helical protomer of Col IV, as the building block, form network through two key types of crosslinking sites (36): NC1 domains including sulfilimine bond (-S=N-) formed at the C-terminal (37) and 7S tetramers, including aldehyde group formed at
the N-terminal (38) (Figure 3H). To distinguish which one is mainly disturbed by the FPM, we detected their changes under FPM stimulation respectively: 1) For the NC1 domain, sulfilimine bond (-S=N-) is formed by two juxtaposed Col IV protomers at residues methionine 93 (Met93) and hydroxyllysine 211 (Hyl211) (Figure 3—figure supplement 3) (34). Based on indicated theoretical mass of crosslinked tryptic peptides containing -S=N- in NC1 domain, we performed high-resolution liquid chromatograph-mass spectrometer (LC-MS) analysis to differentiate these peptides. For the NC1 domain separated from the crosslinked Col IV as illustrated in Figure 3H, we found significantly more sulfilimine-containing peptides in LH-FPM-treated soluble Col IV than that in LH group according to the total ion chromatography (TIC) diagram (Figure 3I). 2) For the 7S domain crosslinking site, it was derived from the oxidation of one lysine residue in the N-terminal to the aldehyde (39). The generated allysine would subsequently undergo a series of condensation reactions with other amino acids, mainly the other lysine or lysines on neighboring C-terminus, forming methylenimine bond (-C=N-), pyridine or others to stabilize crosslink (Figure 3—figure supplement 4A). During the process, the detection of primary product allysine could reflect the level of 7S domain crosslink. With the reported specific and efficient probes to allysine (35), the allysine yielded during the crosslinking of the soluble Col IV incubated with LH or LH-FPM were respectively analyzed. The result showed a slight difference, indicating the 7S domain would not be interfered by FPM (Figure 3—figure supplement 4B). Summarily, FPM gained a catalyzing activity from the proteins adsorbed from the tissue, which mediated the cross-linking through forming excessive -S=N- bonds among Col IV molecules.
Phase Transition of PXDN on FPM Surface Increases Its Activity for Col IV

Crosslinking

Although the above findings demonstrated that LH-FPM increased sulfilimine bond formation to enhance Col IV crosslinking, it remained unclear how FPM gained the activity from LH to mediate this biochemical process in vivo. To elaborately dissect this process, following a standard procedure (Figure 4—figure supplement 1) (40), we separated the biomacromolecules from LH-FPM, the majority of which are proteins and also known as ‘protein corona’ (32). Unexpected corona formation can trigger serious pathological reactions (33; 41). Thus we speculated whether FPM could recruit certain proteins related to Col IV crosslink into its corona, thereby enriching and empowering this protein – to influence the crosslinking of collagen IV.

Given that the sulfilimine bond is uniquely catalyzed by peroxidasin (PXDN) enzyme in animal tissue (34; 42), we focused and detected the PXDN in FPM’s protein corona. The LC-MS result showed that PXDN was listed in the component profile of protein corona on both PM1 and TSP (Figure 4—source data 1), reflecting the interaction of PXDN and FPM. We further analyzed PXDN adsorbed on FPM and its time evolution with Western blotting (33). The data showed that PXDN was not only adsorbed on the FPM (Figure 4A), but also stably tethered to FPM as the incubation time increased (Figure 4B), underlying it might affect FPM’s biological behavior durably. To further assess the adsorption of FPM to PXDN, we injected rhodamine fluorescence-labelled particles (R-FPM) via the trachea into the lung tissue and detected PXDN therein. The IF co-localization of FPM and PXDN in vivo was clearly presented, confirming the recruitment of this enzyme to FPM (Figure 4C), which
could be further validated by the dramatically similar distribution of PXDN and FPM on M2-10B4 cells (Figure 4D). Taken together, these data indicate that FPM enriches and stabilizes PXDN in its surface corona.

**Figure 4.** FPM increased PXDN activity by triggering the enzyme’s phase transition. A. Quantitative western blotting analysis of PXDN harvested from FPM corona formed in LH after incubation for 2 h, with a serial content of recombinant PXDN protein as the standard control; B. Western blotting analysis of PXDN at indicated time points to identify its time evolution in FPM’s protein corona; C. Representative confocal microscopic photographs showing the co-localization of rhodamine-labelled FPM (shown in red) and PXDN in lung tissue. PXDN was indicated as
green. Scale bar = 100 μm); D. Representative fluorescent photographs of M2-10B4 cells treated
with rhodamine-labelled PXDN and FITC-labelled FPM for 1 h. Rhodamine-labelled PXDN was
shown in red, FITC-FPM in green, and DAPI staining for the nuclei in blue. Scale bar = 10 μm; E.
Schematic representation of the procedure about the formation of sulfilimine bond catalyzed by
PXDN. Aimed at the substrate H₂O₂, the intermediates HOBr and the final NC1 domain with
sulfilimine bond, different experimental analyses were respectively performed; F and G. Fold
change of enzyme activity (F) and enzyme kinetics (G) of PXDN stimulated with FPM,
determined by Amplex Red Hydrogen Peroxidase Assay Kit; H. Ration of NADH bromohydrin
relative to NADH based their intensity of peaks detected by LC-MS. The analysis was performed
after PXDN was incubated with FPM for 30 min and then catalyzed in the presence of 100 μM
H₂O₂ and 200 μM NaBr at 37°C for 30 min; I. SDS-PAGE and Coomassie staining of NC1
domain 4 h after they were incubated with PXDN, following the latter’s incubation with PBS or
FPM for 30 min. The crosslinked dimeric (NC1_d) and un-crosslinked monomeric subunits
(NC1_mo) were respectively labelled. Images are representative of three independent experiments;
J. The confocal microscopy of FITC-labelled PXDN was incubated with rhodamine-labelled FPM
in LH for the indicated time (0 min and 30 min). Shown at the right were images with higher
magnification for the assemblies of PXDN's liquid-like droplets on the FPM at 30 min. Scale bar =
5 μm; K. Representative images from FRAP experiments showing the dynamic and reversible
characteristics of PXDN-droplets. The rhodamine-labelled PXDN was shown in red and FITC-
labelled FPM in green. The bleached region of interest (ROI) was indicated with white triangles
and the unbleached control ROI was labelled with dotted white ones; L. Quantification of
fluorescence recovery percentage in the ROI regions of PXDN's liquid-like droplets. The black
arrow indicates the initiation of laser bleach treatment; M. Interactive docking model on the effect
of PXDN's phase separation on its enzymatic performance at the catalytic interface of NC1
domain. The structure of PXDN solution state (PDB ID: 5MFA.1) and its assembly (PDB ID:
4C1M; created through homology modeling) were respectively displayed as the lateral stereo view
of transparent chain model (left) and Dand swiss model (right). The interface site of contact
between NC1 domain (PDB ID: 5NAY) and PXDN was labelled with the red dotted ellipses. n=3.
Results are shown as mean ± SD. *p<0.05 after ANOVA with Dunnett’s tests.

Figure 4—figure supplement 1. Schematic diagram of separation and preparation of FPM’
protein corona in lung homogenate (LH).

Figure 4—figure supplement 2. Liquid chromatography-mass spectrometry (LC-MS) spectrum
for NADH (dotted line) and the bromohydrin (line), according to the reported literature (43). The
analysis was performed after the enzyme PXDN was incubated with FPM for 30 min and then
catalyzed in the presence of 100 μM H₂O₂ and 200 μM NaBr at 37°C for 30 min.

Figure 4—figure supplement 3. HOCl production induced by PXDN measured with HClO
detecting fluorescent probes, after the enzyme was incubated with FPM for 30 min and then
catalyzed in the presence of 100 mM H₂O₂ and 200 mM NaCl, with a serial concentration of
HClO as internal control. n=3. Results are shown as mean ± SD. *p<0.05 after ANOVA with
Dunnett’s tests.

Figure 4—figure supplement 4. Effect of FPM on PXDN’s phase separation. A. Phase contrast
microscopy of PXDN per se or incubated with FPM for 30 min in LH; B. The fluorescence
distribution profiles of the cross-sectional region of liquid-like droplets on the FPM after FITC-
labelled PXDN was incubated with rhodamine-labelled FPM in LH for 30 min.
**Figure 4—figure supplement 5.** Intrinsically disordered regions (IDRs) analysis of PXDN domains by the IUPred algorithm. The low complexity domains and enzymatic activity site were respectively labelled with dotted green circles. PXDN’s template crystal structures were shown on the upper. Besides, bioinformatics analysis of the amino acid sequence of full-length PXDN shown underneath. The kinds of amino acids with order or disorder potential were listed with different color and the superfamily of PXDN was shown on the lower right (44).

**Figure 4—Source data 1.** List of protein component identified by LC-MS for PM 1’s and TSP’s protein corona.

**Figure 4—Source data 2.** The Excel spreadsheet source file for Figure 4F, G, H and I.

The adsorption of FPM might disturb the activity of proteins, especially for the enzyme therein. Thus, we examined the activity of FPM-recruited PXDN shown as illustrated in **Figure 4E.** Aimed at the substrate H$_2$O$_2$, the intermediates hypohalous acids and the final NC1 domain with sulfilimin bond, different experimental analyses were respectively performed (34). First, peroxidase activity of PXDN incubated with FPM was measured through Amplex Red molecular probes (43). The relative fluorescence intensity showed that FPM incubation raised the enzymatic activity of PXDN up to 5-10 folds (**Figure 4F**). Next, PXDN’s enzyme kinetic behaviors were investigated according to the Michaelis–Menten model. Based on the generated Line weaver–Burk representative plot, the Michaelis constant (Km) and turnover number (Kcat) were calculated, which respectively reflects the enzyme-substrate binding efficiency and the enzymatic efficiency. The result showed that PXDN incubated with FPM revealed significantly decreased Km (PM1: 7.36 μM; TSP: 13.10 μM versus PBS: 15.92 μM) and increased catalytic efficiency about 1.4-to-1.8-fold than the enzyme per se (**Figure 4G**). Also, the secondary product hypohalous acids (34; 45), for instance, HOBr and HOCl, generated by PXDN from bromide and chloride, were respectively analyzed. For HOBr, bromohydrin formed by the bromination of NADH was measured based
on its close relation with HOBr production as reported in the literature (43; 46). The LC-MS detection showed that the ratio of bromohydrin to NADH in the group of PXDN incubated with FPM was 3-5-fold higher than that of the enzyme per se group, indicating more HOBr production (Figure 4H and Figure 4—figure supplement 2). Besides, we measured HOCl in consideration of the vast excess of Cl\(^-\) over Br\(^-\) in most animals (47), although PXDN uses Br\(^-\) to catalyze the formation of sulfilimine crosslinks with greater efficiency (45). With the detection of a hypohalous acid-detecting fluorescent probe (48), we found HOCl produced by the FPM-incubated PXDN was about 2-to-4-fold higher than that of PBS group (Figure 4—figure supplement 3). Furthermore, to analyze the effect of FPM’s adsorption on the catalytic product, after the commercial non-crosslinking NC1 domain (NC1\(_{\text{no}}\)) was reacted with FPM-incubated PXDN, the generated crosslinked dimeric with sulfilimine bond (NC1\(_{\text{d}}\)) therein were detected by SDS-PAGE (34; 49). The result suggested that FPM incubation significantly enhanced PXDN’s enzymatic performance, with a higher yield of crosslinked NC1 dimeric subunits (Figure 4I). Taken together, these results suggest that the pro-crosslink potential of FPM is attributed to the aberrant enzyme activity of PXDN adsorbed on its surface.

Next, we gave an insight into the mechanism of PXDN’s tampered catalysis. Recent emerging evidence suggests that phase transition (or separation) is a common way to regulate proteins’ activity (50). Phase transition refers to the process that macromolecular solution condenses into liquid droplets, solids, or gels (51; 52), in response to certain physicochemical stimuli, sharply increasing the macromolecule’s concentration and separating them from the surrounding compartments (53; 54). Such enrichment affects the subsequent biochemical
reactions. Thus, we asked whether the FPM’s adsorption could induce the PXDN’s phase transition, thus disturbing the latter’s enzymatic activity. Taking PM1 as an example, with a series of microscopic observations, we found the formation of PXDN’s liquid-like droplets in LH after its incubation with PM1 with both confocal fluorescence and phase-contrast microscope (Figure 4J, Figure 4—figure supplement 4A). However, for the PXDN alone in LH under the same processing time and imaging parameters, no assemblies are observed. Moreover, the profiles of fluorescence distribution further indicated the phase-separating PXDN’s accumulation was initialized on the PM1, based on their evident colocalization (Figure 4—figure supplement 4B). Besides, to characterize the dynamic nature of the droplets, we performed fluorescence recovery after photobleaching (FRAP) experiments. FRAP studies revealed that fluorescence recovery of the bleached region of PXDN droplets could be partially recovered in minutes after photobleaching (Figure 4K and L). The reversible characteristic observed for PXDN droplets on PM1 further validated that PXDN underwent phase separation. More interestingly, the droplet-like accumulation of PXDN on FPM could also be observed in M2-10B4 cells (Figure 4D). Overall, these results indicated that the increased activity for PXDN’s crosslinking Col IV was triggered by its phase transition on the FPM surface.

Furthermore, we theoretically inferred the relationship of PXDN’s phase separation with its enzymatic performance. First, we focused on the low complexity domains in PXND sequence, which could drive phase transition and be predicted by intrinsically disordered regions (IDR; Figure 4—figure supplement 5). The analysis indicated PXDN’s phase separation might occur at sequence 200-400 aa, which shows a higher IDR score (53). More
importantly, it’s away from PXDN’s activity center (800-1200 aa) according to the spatial structure in SWISS-MODEL, giving us a hint that PXDN’s phase transition on FPM surface might not lead to deformation of PXDN's catalytic center and loss of its function. Next, protein-protein interactive docking simulation were performed at the interface of PXDN with its substrate NCI domain through ZDock protocol (Figure 4M). As a homotrimeric multidomain enzyme, PXDN exists as trimerization in solution, and three monomers of PXDN are linked by disulfide bonds at the indicated flexible linker region in the residual non-catalytic domains (43). According to the reported modelled structure of PXDN (55), its trimeric peroxidase domain displayed a triangular arrangement. However, oversized trimerization of PXDN might not get in contact thoroughly with its substrate (56). So we chose the PXDN monomer with the exposed enzymatic surface contacting with NCI domain to simulate its function at solution state. The computational result indicated that once PXDN triggered phase separation, which transferred from the solution state to the aggregation one (the putative assembly structure created through homology modelling) (44), the interactive area at the NCI interface would be notably increased, thus facilitating the enzymatic catalysis.

Inhibiting PXDN Ameliorates FPM-Induced Tumorigenesis

Based on the above findings, we speculated that inhibiting PXDN could abolish ECM change and recover CTLs migration in the lung. To testify our hypothesis, the effect of the plasmids capable of ectopically expressing PXDN specific short hairpin RNA (PXDN shRNA, shPXDN) was detected. After validating its interference efficiency (Figure 5—figure supplement 1), shPXDN mixed in the in vivo-jetPEI gene transfer regent
was delivered into murine lung tissue through trachea injection, as illustrated in

**Figure 5—figure supplement 2.** The assessment of Col IV with a different fraction

(‘low-cross’ ones and ‘high-cross’ ones) measured by ELISA revealed the shPXDN

diminished crosslinked level of Col IV (**Figure 5A**). Masson's trichrome staining

images and SEM observation also confirmed that the shPXDN effectively decreased

collagen density and expanded interstitial space (**Figure 5—figure supplement 3**).

More importantly, the ameliorative Col IV network induced by shPXDN further

recovered the migration and accumulation of CTLs in lung tissue 1 day after the LLC

stimulation, revealed as the CTLs’ migration trajectory images and the

immunofluorescent staining (**Figure 5B and C**). Flow cytometry analysis further

demonstrated that shPXDN efficiently reversed the CTLs’ infiltration in the FPM-

exposed lung tissue (**Figure 5D and Figure 5—figure supplement 4**).
Figure 5. PXDN inhibitor alleviated FPM-induced lung tumorigenesis. A. Proportion of high crosslink Col IV in lung tissue of FPM-exposed mice pretreated with PXDN specific short hairpin RNA (PXDN shRNA, shPXDN), which was calculated by the ‘high-cross’ Col IV fragment divided by the sum of different fractions (‘low-cross’ ones and ‘high-cross’ ones) based on ELISA; B. Migration distance, displacement and velocity vs. time (min) of tracked CTLs in lung tissue slice of FPM-exposed mice administrated with PXDN shRNA (shPXDN); C. Representative immunofluorescence images of CTLs’ infiltration into the FPM-exposed lung tissue pretreated with shPXDN 1 day after intravenous injection of LLC. Scale bar = 100 μm; D. The statistical analysis of CTLs (IFN-γ⁺CD8⁺/CD45⁺CD3⁺) based on flow cytometry in lung tissue of mice treated as in panel C. n=5; E. Representative H&E staining images of lung tissue (the lower ones:
capture with higher magnification) yielded from LLC model and Kras\textsuperscript{G12D}\textsuperscript{Trp53\textsuperscript{-/-}} model after mice pretreated with shPXDN. The scale bar = 200 µm (upper) and 100 µm (lower). Tumor stage (stages 1 to 4) in lungs of Kras\textsuperscript{G12D}\textsuperscript{Trp53\textsuperscript{-/-}} mice was shown on the right. p values are for comparisons of the percentage of stage 3 and 4 tumors in different groups. n=5; F. Statistical analysis about tumor burden of mice in LLC model and Kras\textsuperscript{G12D}\textsuperscript{Trp53\textsuperscript{-/-}} model administrated with shPXDN. n=5; G-J. Statistical analysis about number and burden of tumors and survival curve of mice in LLC model (G and H) and Kras\textsuperscript{G12D}\textsuperscript{Trp53\textsuperscript{-/-}} model (I and J) administrated with methimazole (MMZ) or phloroglucinol (PHG). Images are representative of three independent experiments. n=3. Results are shown as mean ± SD. *p<0.05 after ANOVA with Dunnett’s tests.

**Figure 5—figure supplement 1. A and B.** The transcriptional level analysis (A) and Western blotting analysis (B) of PXDN in M2-10B4 cells after they were transfected with plasmids capable of ectopically expressing PXDN specific short hairpin RNA (PXDN shRNA, shPXDN) or control shRNA (shNC) for 48 h; C and D. The transcriptional level analysis (C) and Western blotting analysis (D) of PXDN in lung tissue after the mice were administrated with 4 µg plasmids capable of ectopically expressing shPXDN mixed in the in vivo-jetPEI gene transfer regent through trachea injection every 3 day for 4 times.

**Figure 5—figure supplement 2.** Schematic diagram of analyzing the effect of PXDN specific short hairpin RNA interference (PXDN shRNA, shPXDN) on the structure of FPM-exposed lung tissue. **Figure 5—figure supplement 3. A.** Representative Massons’ trichrome histological analysis of lung tissue in FPM-exposed mice administrated with shPXDN (PXDN shRNA). Scale bar = 100 µm; B. Representative SEM images of interstitial matrix in the lung tissue in FPM-exposed mice administrated with shPXDN. Scale bar = 50 µm.

**Figure 5—figure supplement 4.** Representative flow cytometry analysis of CTLs’ infiltration (IFN-γ CD8\textsuperscript{+}/CD45\textsuperscript{+}/CD3\textsuperscript{+}) into lung tissue of FPM-exposed group pretreated with shPXDN 1 day after they were stimulated with the LLC.

**Figure 5—figure supplement 5. A.** Schematic diagram of analyzing the therapeutic effect of shPXDN on LLC-stimulated or Kras\textsuperscript{G12D}\textsuperscript{Trp53\textsuperscript{-/-}}-transgenic lung cancer model with FPM exposure. **Figure 5—figure supplement 6. A and B.** Gross lung tissue images in LLC-stimulated (A) or Kras\textsuperscript{G12D}\textsuperscript{Trp53\textsuperscript{-/-}}-transgenic lung cancer model (B) administrated with shPXDN; C and D. Statistical analysis of tumor number in LLC-stimulated (C) or Kras\textsuperscript{G12D}\textsuperscript{Trp53\textsuperscript{-/-}}-transgenic lung cancer model (D) administrated with shPXDN. n=5. Results are shown as mean ± SD. *p<0.05 after ANOVA with Dunnett’s tests.

**Figure 5—figure supplement 7.** Representative H&E staining images of lung tissue with intratracheal injection with different concentration of methimazole (MMZ) and phloroglucinol (PHG) for 3 days. Scale bar = 100 µm. The concentration at the indicated group with red dotted lines was chosen as the subsequent administration dose.

**Figure 5—figure supplement 8. A.** Schematic diagram of analyzing the therapeutic effect of PXDN inhibition (MMZ or PHG) on LLC-stimulated or Kras\textsuperscript{G12D}\textsuperscript{Trp53\textsuperscript{-/-}}-transgenic lung cancer model with FPM exposure; B and C. Gross images of lung tissue yielded from LLC-induced model (B) or Kras\textsuperscript{G12D}\textsuperscript{Trp53\textsuperscript{-/-}}-transgenic lung cancer model (C) 20 days or 50 days after mice pretreated with MMZ or PHG were stimulated with LLC or AdCre; D and E. Representative H&E staining images of lung tissue yielded from LLC-induced model (D) or Kras\textsuperscript{G12D}\textsuperscript{Trp53\textsuperscript{-/-}}-transgenic lung cancer model (E) as panel B and C. Images are representative for three independent experiments.
These results encouraged us to assess the effect of shPXDN in suppressing the tumor progress induced by FPM, as illustrated in Figure 5—figure supplement 5. Encouragingly, our data showed that the shPXDN significantly suppressed tumor growth, diminished tumor grade, and reduced tumor number and burden (Figure 5E, F and Figure 5—figure supplement 6) in both \( \text{Kras}^{G12D}\text{Trp53}^{-/} \) transgenic model and LLC-model. These results suggested the feasibility of inhibiting PXDN as a potential therapeutic target for FPM-associated lung cancer. Moreover, the efficacy of small molecule PXDN inhibitors, including methimazole (MMZ) and PHG (34), were similarly studied with optimal therapeutic doses (Figure 5—figure supplement 7). To be satisfactory, both MMZ and PHG also effectively suppressed lung tumorigenesis (Figure 5G-J and Figure 5—figure supplement 8), further expanding the strategy for lung cancer treatment.

Discussion

Although inhalable particles from air pollutants and tobacco smoke have clearly been identified as a potent carcinogen to humans, its pathological mechanism remains unclear, which hampers the design of therapeutic approaches. Existing findings, though supporting that fine particulate matter (FPM) induces lung cancer, provide insufficient and inconsistent explanations for the underlying mechanism. In the present study, we have discovered an unexpected mechanism that, as shown in Figure 6, apart from directly targeting tumor cells, the inhaled FPM changes the formation of
l lung tissue matrix to prevent the infiltration of T lymphocytes and their anti-tumor
immunosurveillance, which consequently accelerates lung tumorigenesis.

Figure 6. Schematic diagram of the mechanism underlying how FPM promotes lung
tumorigenesis. The adsorption of FPM triggers the phase separation of PXDN to generate an
aberrant catalytic activity and induce a disordered crosslink of Col IV. Reinforced Col IV network
impaired CTLs migration and local immunosurveillance, which considerably increases
tumorigenesis in lung tissue.

Our study was inspired by an unanswered, fundamental question – which
component in the lung tissue is first affected by FPM? Most previous studies suggest
that FPM directly act on cells, by inducing gene mutation and increasing malignant
‘seed cells’ in the affected lung (57). They further suggest that the particles carry chemical reagents, such as acrolein, nicotine, oxidants and reactive nitrogen moieties (58), into the lung to exert such effects. However, the respiratory tract is exposed to various environmental substances (e.g. O₂ or O₃) that are much more powerful in damaging DNA, more abundant in the air, and more soluble in the tissue than the above chemicals. It requires further explanation of how these assumed mutagens, delivered in a trace amount by the particles into the lung tissue, could significantly increase lung cancer incidence. Moreover, FPM from different locations carries distinct types of those carcinogens exiting in the local environment, but these highly various particles exert the same carcinogenic effect (16), which is unexplainable. Additionally, in vitro, FPM is considered more capable of entering the cell than larger particles (59) (such as the PM10, that is, the particulate matter with the diameter of 1-10 µm); but in vivo, particles are difficult to directly contact with cells, due to the barrier effects from sticky body fluids and gel-like extracellular matrix (ECM) proteins (60). Since inhaled particles are mostly accumulated in the ECM (61), there is no reason to ignore the impact of ECM and only focus on the interaction between particles and cells. Thus, we suspected that the lung tissue ECM, apart from the cells, could also be affected by the particles. Indeed, ECM abnormality is closely correlated with cancer development. On the one hand, the clinical samples showed the developed tumor was surrounded by a dense collagen matrix; on the other hand, one typical example is that tissue fibrosis often precipitates tumor development in the same tissue – such as liver cirrhosis progressing into hepatoma and pulmonary
fibrosis leading to lung cancer (62; 63). These clinical and experimental proven reports prompt us to investigate the pathological change of FPM-stimulated lung interstitial ECM and its role in lung tumorigenesis.

Our experimental findings validated this assumption, by demonstrating – and elucidating the mechanism of – FPM-induced alteration in the interstitial lung structure. As soon as we, for the first time, found that FPM in the lung homogenate (LH) dramatically enhanced the crosslinking of soluble type IV collagen (Col IV), we speculated that a specific biomolecule in LH enriched on the FPM surface mediates this action. With proteomics tools, we identified peroxidasin (PXDN); this enzyme, specialized in catalyzing collagen network formation (34), is enriched in the protein corona of FPM, enabling FPM to mediate ECM remodeling. Meanwhile, for PXDN, its adsorption onto FPM triggered its aberrant enzymatic activity through a ‘phase-transition’ process, which further disturbs Col IV crosslinking and the organization of the lung interstitial space. Pre-treatment using PXDN inhibitor could alleviate the tumor-promoting effect of FPM. Therefore, an aberrant ECM remodeling mediated by enriched PXDN in the particle corona is the key pathological change caused by FPM.

Our study further elucidates how such a change in ECM accelerates tumor development. As a consequence of FPM treatment, the denser Col IV and more compacted interstitial space in lung tissue would hinder the migration of CTLs, which are the most ‘informed’ defender to protect against potential cancer and delay malignant progression (9; 11). Once CTLs’ migration track was blocked, the CTLs infiltration would be impaired, thus decreasing the chance of cell-cell cytotoxic
interaction to tumor cells in lung tissue (64). According to the correlation between
tumour mutation burden and immune infiltration in lung tumours (65-67), we
speculated that the deficiency further aggravated the mutation of cancerous cells in
the lung tissue. The insufficiency of CTLs’ early response to tumor cells results in
weakened immune surveillance and unmonitored mutation that in turn accelerate
tumorigenesis. All the evidences recapitulate how FPM accelerated the development
of lung cancer through interfering the lung immune microenvironment, shedding light
on the sequential consequences between particle-induced ECM remodeling, impaired
immunosurveillance, and tumorigenesis.

Taken together, our study reveals a completely new mechanism by which inhaled
fine particles promotes lung tumor development. This mechanism is notable in three
aspects. First, we herein provide direct evidence that protein corona on those foreign
objects can elicit such a significant and adverse effect. Although corona formation on
nanoparticles has been extensively studied in recent years, its involvement in a
pathogenic process related to a global health issue is rare. Our findings here highlight
the importance of the corona-endowed, ‘new’ enzymatic bioactivity of nanomaterials
in vivo – and in a particular tissue. The data suggests that coronas adsorbed from the
environment could also be catalytic, other than simply transforming the cellular and
higher level interactions, in agreement with other literatures (68; 69). Second, PXDN,
or other proteins mainly engaged in ECM modulation, is less expected as a major
player in lung carcinogenesis, especially at the initial stage, until this study reveals it
as a specific and unexpected molecular target for FPM. These investigations enable
the specific design of PXDN-targeted preventive or therapeutic approaches. The relationship between the physicochemical properties of FPM and the affected PXDN activity should be further explored in greater detail. Third, in a specific organ (the lung), our data demonstrate that physical blockage of immunocytes movement directly increases tumorigenesis, which suggests an important previously unconsidered role of the in vivo delivering or deploying the power of the immune system in various immune-oncology processes.

Materials and Methods

FPM Collection and Preparation. Fine Particulate Matter (FPM) samples within sizes of 1 μm were collected using TH-16A multiple-channel atmospheric particulate automatic sampler (Wuhan Tianhong Instrument Ltd, Wuhan, China) and filtered through Whatman PTFE membranes (GE Healthcare Life Sciences, Pittsburgh, USA). For particles in air pollutants (PM1), samples were conducted continuously for 7 days at different representative of Nanjing City (Qixia, Jiangning, Pukou, Gulou, and Gaochun) in Jiangsu Province, Suzhou City in Anhui Province and Tieling City in Liaoning Province (named as: QX, JN, PK, GL, GC, SZ and TL). For TSP samples, the residual smoke of burned tobaccos with filters were collected in customized confined space. Then PTFE filter membranes containing FPM were cut into 0.1 cm × 0.1 cm pieces, immersed in distilled water for 2 days and oscillated ultrasonically for 1 h for 3 times. Detached FPM was separated with filter membranes after centrifugation at 2,000 rpm for 5 min for 3 times. Supernatant enriched FPM were vacuum freeze-dried and then stored at −20 °C. For the preparation of FPM suspension, FPM samples were suspended in sterile 1× PBS (phosphate buffer saline) to achieve 10 mg/mL particles for further analysis.

Characterization of FPM. A series of tests were performed to thoroughly characterize the nanoparticles. First, to analyze morphology of particles, dried PTFE
filter membranes containing FPM was conducted with SEM microscope LEO1530VP (JEOL Ltd., Tokyo, JAPAN). Second, all the particles were characterized for their Zeta potential and particle size using NanoSight NS300 instrument (Malvern Instruments, Malvern, UK). Third, to obtain essential information on the nanoparticles’ size and shape, transmission electron microscopy (TEM) was carried out. After a few drops of deionized water-dispersed nanoparticles was dropped on the 300-mesh carbon-coated copper grid, TEM images of each sample were collected using TEM (JEOL Ltd.). Besides, the elemental analysis was performed on an element analysis instrument (Vario Micro Cube, Elementar, Germany), with the top 15 were listed.

Cell Preparation and Culture.

Cell lines’ culture. Lewis lung cancer cell lines (LLC), mouse bone marrow fibroblasts M2-10B4 and Jurkat T cells were obtained by Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). LLC cells expressing ovalbumin peptide residues 257-264 (OVA<sub>257-264</sub>) in the context of H2K<sup>b</sup> (OVA-LLC) were kindly provided by K. Zeng (Nanjing University, China). Cells were cultured in DMEM or RPMI 1640 medium containing 10% foetal bovine serum (Thermo Fisher Scientific, MA, USA), harvested at ~ 80% confluency, washed twice with phosphate buffer saline (PBS) and subcultured for passage. The short tandem repeat (STR) profiling of these cell lines was authenticated (Beijing Microread Genetics Co., Ltd, Beijing, China). And all cell lines were detected negative for mycoplasma contamination (Corues Biotechnology, Nanjing, China).

Separation of primary cytotoxic CD8<sup>+</sup> T lymphocytes (CTLs). To exact the CTLs from lung tissue, the lung tissues in the FPM-exposed mice stimulated with LLC or OVA-LLC for indicated days, were respectively collected and digested with 2 mg/mL collagenase type I and IV (Thermo Fisher Scientific) for 20 min. Then a single-cell suspension was prepared using the program m_lung_02.01 on the gentleMACSTM Dissociator (Miltennhy Biotec, Bergisch Gladbach, Germany). the CTLs were isolated from this single-cell suspension using the CD8<sup>+</sup> T cell isolation kit with a MidiMACSTM separator (Miltennhy Biotec).

Cloning, expression, and purification of peroxidasin (PXDN)—His-tagged full-length
mouse peroxidasin homolog encoded on indicated vector was generated by GenScript and transfected into HEK 293 F cells using Lipo2000 (Invitrogen) according to standard selection and cultivation procedures with minor modifications (46). On a large scale, cells were cultivated in Expi293™ Met (-) Expression Medium (Thermo Fisher Scientific). The harvested media were stored at 4 °C and eventually purified using Ni-NTA Agarose (Qiagen). Fractions with the best purity number were pooled, concentrated, and desalted using Centricon with a 100-kDa cutoff membrane (Millipore).

**Stimulation of FPM on LLC and Cytotoxic T Lymphocytes (CTLs).** To analyze the effect of FPM on the proliferation of LLC, cell counting kit-8 (CCK-8) test was performed. Briefly, $1 \times 10^4$ LLC were seeded in 96-well culture plates and then simulated with different concentration of FPM (0 μg/mL, 5 μg/mL, 10 μg/mL, 30 μg/mL, 50 μg/mL, 100 μg/mL and 500 μg/mL) and cultured for 24 h and 48 h. CCK-8 kit (DOJINDO LABORATORIES, Kumamoto, Japan) was used to examine the proliferation of LLC at indicated time points, with cells treated with 1×PBS as control. To further analyze the effect of FPM on the migration potential of T cells, the CTLs were stimulated with 10 μg/mL FPM, which showed slight cytotoxic, for 48 h.

**Establishment and Treatment of Lung Tumor Model in FPM-exposed Mice.**

*FPM-exposed mice.* Mice exposed to FPM was generated according to a previously published method (33). Six-to-eight weeks old C57BL/6 mice or Foxn1nu naked mice of the same ground were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). OT-1 T cell receptor transgenic mice [C57BL/6-Tg (TcraTcrb) 1100Mjb/J] were a gift from K. Zeng (Nanjing University, China). These mice were randomly divided into 3 groups (PBS ones, FPM-exposed ones: PM1 and TSP; each group contained at least 3 mice). Mice were anesthetized by intraperitoneal injection of pentobarbital sodium at 45 mg/kg body weight. After the trachea exposed by opening the neck skin and blunt dissection, mice received suspension of 0.2 mg FPM in a total volume of 50 μl of sterile physiological saline by inserting a 7-gauge needle (BD Biosciences, San Jose, CA, USA) into the trachea trans-orally. To be estimated, before intratracheal instillation, FPM suspension was
always sonicated and vortexed. After the site of surgery was sutured and cleaned with penicillin, the mice were allowed to recover until they were sacrificed. As a control, PBS was applied in a similar manner. After being exposed to FPM for 7 days, mice were 1) sacrificed for analyzing the changes of lung tissue structure, or 2) subsequently stimulated with 5 μg/kg T cell chemokine – C-X-C motif chemokine ligand 10 (CXCL10, PeproTech, Rocky Hill, USA) (22), or named as interferon-inducible protein-10 (IP-10) for 2 h through intratracheal injection, to analyze the CTLs’ infiltration into lung tissue, 3) to analyze the location of PXDN and FPM in the lung, mice were exposed to rhodamine-labelled FPM (R-FPM) with intraperitoneal injection. Lung tissue was extracted for immunofluorescence 4 hours later.

**Establishment of lung tumor model in FPM-exposed mice.** For the syngeneic LLC (Lewis lung carcinoma) model, after being exposed to FPM for 7 days, mice were further intravenously injected with 5×10^7 LLC cells for indicated days (0 d, 1 d, 3 d, 5 d, 10 d and 20 d) to create the lung carcinoma model post particle administration. For the LLC-stimulated carcinoma model, 20 days after the injection of LLC, the mice of different groups were sacrificed, and the lung tissue were extracted for analysis. The number of tumors suffered by the mice was examined and evaluated randomly under blindfold conditions. For the transgenic (Kras^{G12DTrp53^/-}) mouse models, mice harboring a Cre-inducible endogenous oncogenic Kras^{LSL-G12DTrp53^fl/fl} allele (GemPharmatech Co., Ltd, Nanjing, China), were treated as above. 7 days after exposed to FPM, mice were allowed to inhale 1×10^7 plaque forming units (PFU) Cre adenovirus (AdCre, OBiO Technology (Shanghai) Corp., Ltd., Shanghai, China) to activate K-ras^{G12D} expression and knock out p53 in lung tissue (Kras^{G12DTrp53^/-} transgenic model). The mice were sacrificed 50 days after tumor initiation.

**Administration of PXDN inhibitor.** A series of plasmids capable of ectopically expressing PXDN specific short hairpin RNA (PXDN shRNA, shPXDN) or control shRNA (shNC) were designed and constructed by GenePharma Biotechnology (Shanghai, China). For transfection, in vivo-jetPEI (Polyplus Transfection, Illkirch, France) was used as a delivery agent (70). The transfection reagent complex (0.16 μL of in vivo-jetPEI per μg plasmid DNA) was prepared and mixed according to the
manufacturer’s instructions in glucose buffer. Then the 20 μl buffer containing 4 μg shPXDN was delivered into murine lung tissue through trachea injection. Besides, to analyze the effect of small molecular PXDN inhibitors, the FPM-exposed mice were administrated with 25 mg/kg methimazole (MMZ, MedChemExpress LLC, Shanghai, China) and 50 mg/kg phloroglucinol (PHG, Aladdin, Shanghai, China) 3 days pre- and post-FPM stimulation respectively. During the experimental procedure, all animal studies were performed under protocols approved by institutional guidelines (Nanjing University Institutional Animal Care and Use Committee). They were also required to be conformed to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health. The mice were housed 5 per cage and fed in a specific pathogen-free (SPF) animal facility with controlled light (12-h light/dark cycles), temperature and humidity, with food and water available.

**Analysis of T Cell Migration on Lung Tissue.**

*Lung tissue slice preparation.* To analyze T cell migration on lung tissue, the lung samples of different groups, including the FPM-exposed mice and the FPM-exposed mice pre-treated with PXDN shRNA (shPXDN) were respectively prepared as the 50 μm frozen section (13). In some experiments, tissue sections were pre-treated with 50 μg/mL collagenase D (Worthington Biochemical Corp., Colorado, USA) in RPMI 1640 for 5 min, then rinsed in complete RPMI 1640 medium.

*Cell preparation.* Jurkat T cells were stained with Calcein-AM (DOJINDO LABORATORIES) for 30 minutes at 37°C and then washed with HBSS (Sangon Biotech) for three times. 1.5 × 10^5 T cells total in 10 – 20 μl were added at one side of the cut surface of each slice. To ensure cells settle down on the slice, slices with T cells were incubated for 1 hour at 37°C, 5% CO_2; gently washed to remove the residual cells that had not entered the tissue; and kept at 37°C, 5% CO_2 before imaging.

*Time-lapse imaging and cell trajectories analysis.* For imaging T cells’ migration on the lung tissue slice, 5 μg/mL IP-10 were added on the other side of the slice and images were then acquired in time-lapse model with a SP5 confocal system (Leica).
every 3 min for 1 h. Imaging was exported and compressed into videos as .avi format.

To quantify T cell trajectories with the surrounding ECM in lung tissue, the cell migration video including image-sequences cell migration was analyzed with TimTaxis Software (https://www.wimasis.com/en/WimTaxis) by identification of the centroids of individual cells at consecutive timepoints. The relationship of statistical data composed of displacement, distance, velocity and acceleration vs. time were respectively further analyzed.

**Extraction of Soluble Collagen IV.** To generate soluble collagen IV, the fused mouse bone marrow fibroblasts M2-10B4 cells were plated at high density and maintained at confluency for 7 days in the presence of 50 µg/mL ascorbic acid (Sangon Biotech, Shanghai, China), with media changes every 24 – 36 h. Crosslinking was inhibited by supplementing the culture conditions with indicated concentration (0, 50, 100, 200, 300 and 500 µM) of PHG. PHG and ascorbic acid treatments were initiated upon confluency. With the 200 µM PHG, which could be sufficient to inhibit the Col IV crosslink, after the M2-10B4 cells stimulated for 7 days and collected through scrape, cultured cells and matrix were homogenized in 1% (w/v) deoxycholate (Aladdin) with sonication, and the insoluble material isolated after centrifugation at 20,000 × g for 15 min. Then the pellet was lysed with RPMI (Beyotime Biotechnology, Shanghai, China) at ice for 30 min. The supernatant containing soluble Col IV was collected after centrifugation at 20,000 × g for 10 min and then incubated with 1 mg/mL FPM per se, 1 mL lung homogenate (LH) or the mixture of FPM-LH (with the volume ratio of 1:10) for 4 h. Then the samples were collected for further Western blotting (WB) analysis to detect the change of Col IV crosslink. Besides, to perform cellular experimental analysis, the M2-10B4 cells were incubated with 200 µM PHG for 24 h and then treated with the same stimulation for 24 h. The cell samples were collected for further immunofluorescence (IF) analysis.

**Analysis of Crosslinking Extent of Different Collagen.** Collagen crosslink was assessed biochemically by separating different collagen fractionation via serial extractions, including neutral salt (freshly secreted collagens and procollagens), acetic acid (more mature collagens), and acid pepsin (fibrillar, moderately crosslinked
collagens) and insoluble high crosslink ones from fresh lung tissue as literature reported (28; 71). Briefly, the whole lung tissue was homogenized in neutral salt buffer (0.5 M NaCl, 0.05 M Tris, pH 7.5; Sangon Biotech) and incubated at 4 °C overnight on a rotary shaker. After centrifugation at 24,000 × g for 30 min, the supernatant was collected (fraction A: neutral salt-soluble collagen). The resulting pellet was then extracted with 0.5 M acetic acid (fraction B: acid-soluble collagen; Sangon Biotech), followed by pepsin (2 mg/mL in 0.5 M acetic acid, fraction C: pepsin-soluble collagen; Sangon Biotech). The remaining insoluble fraction D represents mature, highly crosslinked collagen. Then Type I, III and IV collagens with different extractions were analyzed by corresponding enzyme linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The level of collagen crosslink was calculated as collagen in fraction D divided by total collagen summed by fraction A, B, C and D.

Detection of Crosslinking Sites in Col IV.

Mass spectrometry and identification of sulfilimine bond crosslinked peptides. To analyze effect of FPM on crosslink of collagen IV, the potential reaction site containing sulfilimine bond (NC1 domain formed along with C-terminal aggregation) was detected with liquid chromatography-mass spectrometry (LC-MS) (34; 45). Briefly, 5 mg/mL soluble and commercially available collagen IV (Sigma-Aldrich, St. Louis, MO, USA), which was extracted from murine sarcoma basement membrane, was incubated with LH or LH-FPM mixture, 100 μM H₂O₂ and 200 μM NaBr for 4 h at 37 °C. After centrifugation at 12,000 rpm for 20 min, the crosslink pellet was collected. Then the pellet was digested with collagenase D (50 μg/mL; Worthington) for 30 min at 37 °C to yield the peptide containing the crosslink site. After centrifugation at 12,000 rpm for 20 min, the supernatant containing the crosslinked peptides was collected. After separation by SDS-PAGE, NC1 domain was digested with trypsin (MS Grade, Thermo Fisher Scientific) overnight at 37 °C and then analyzed by LC-MS analysis on a Shimadzu UFLC 20ADXR HPLC system in-line with an AB Sciex 5600 Triple TOF mass spectrometer (AB SCIEX, Framingham, Massachusetts State, USA). To analyze low abundance peptides containing
crosslinked domain, targeted methods were performed with PeakView software (AB SCIEX) based on raw continuum LC-MS data. Briefly, full scan spectra of total ion chromatography (TIC) diagram were acquired, and LC-MS peptide reconstruct with peak finding were provided. According to calculated theoretic mono-isotopic mass of the sulfilimine (the mass of two hydrogen atoms was subtracted from the sum of the masses for Met93-containing peptide and Lys211-containing peptide), corresponding mass spectrum (about 5030.425, 5046.425 or 5062.425) with different oxidation of methionine (M\textsubscript{ox}) were specifically searched. To delineate the difference of crosslink site, the extract ion chromatography (XIC) diagram based on corresponding was displayed and compared.

Detection of allysine at 7S domain crosslinking site. For 7S domain, the detection of primary product allysine could reflect the level of its crosslink. With the reported specific and efficient probes to allysine (35), crosslink of the soluble Col IV incubated with LH or the mixture of LH-FPM was respectively analyzed. Briefly, the 5 mg/mL soluble collagen IV was incubated LH or LH-FPM mixture and 5 mM probes, for 30 min at 37 °C. Then the fluorescence intensity was detected with the exciting wavelengths at 488 nm on the microplate reader (Thermo Fisher Scientific). To be estimated, to quantify the yielded allysine, a serial content of oxidized bovine serum albumin (BSA) containing known aldehydes as the internal standard (oxidized BSA: 16 nM aldehyde/mg; BSA: 1.2 nM aldehyde/mg). For the oxidized BSA, sodium aspartate (13 mg) was added to 50 mg/mL BSA in phosphate buffered saline (PBS), followed by the addition of a solution of ferric chloride (10 μL, 10 mM) and left to stir at room temperature overnight. A BSA protein standard without the addition of ferric chloride was run in parallel as a control.

Analysis of Peroxidasin Enzyme Activity and Reaction.
Measurement of peroxidasin activity. To analyze effect of FPM on PXDN, the enzyme activity was analyzed by Amplex Red hydrogen peroxidase assay kit (72) (Thermo Scientific). Briefly, after incubated with FPM for 30 min at 37 °C, the enzyme activity was detected in reaction containing 50 mM Amplex Red reagent, 1 mM H\textsubscript{2}O\textsubscript{2}, and PXDN (PeproTech) mixed in FPM. After incubation for 30 min at
room temperature, fluorescence was measured at excitation wavelength 590 nm with a fluorescence microplate reader (Thermo Fisher Scientific, MA, USA). To avoid interference of particles per se, the equal FPM was set as negative control.

**Analysis of peroxidasin enzyme kinetics.** To detect the change of PXDN catalytic efficiency after its incubation with FPM, PXDN enzyme kinetic behaviors were investigated according to the Michaelis–Menten model. Briefly, after incubated with FPM for 30 min at 37 °C, 10 mU PXDN was mixed with a serial concentration of H₂O₂(0, 10, 50, 100, 150,200 and 250 μM). The enzymatic reaction was analyzed by adding 50 mM Amplex Red detection reagent (73). The fluorescent reaction was measured every 40 sec for 8 min and subsequent every 5 min for 6 times using a microplate reader (Thermo Scientific) with enzyme kinetics model. Line weaver–Burk representative plot was generated from the relationship of reaction rate and substrate concentration. Based on the plot, the Michaelis constant (Km) reflects the binding efficiency of the enzyme with the substrate and turnover number (Kcat).

**Detection of hypobromous acid.** Based on that NADH could be stably brominated into NADH bromohydrin after its reaction with hypobromous acid, hypobromous acid generated by PXDN and its bromination activity were tested by TripleTOF™ 4600 LC-MS/MS according to the reported literature with a little modification (43). Briefly, 100 nM PXDN or the mixture of PXDN and FPM was incubated at 37 °C in PBS containing 200 μM NADH, and 200 μM NaBr for 30 min. Reactions were started upon addition of 100 μM H₂O₂. Then the supernatant was separated and analyzed to detect NADH and its bromohydrin products. NADH was measured using the transition m/z 664.2 to 408.1, and the bromohydrins by m/z 760.2 and 762.2 both going to m/z 680.2. Intensity of peaks were calculated using PeakView software (AB SCIEX). Then the ration of NADH bromohydrin relative to NADH based their peak intensity was calculated.

**Measurement of hypochlorous acid.** Reactions were initiated with the addition of 100 μM H₂O₂ and 200 μM NaCl after 1 μg PXDN incubated with FPM for 30 min. 5 μM HClO-detecting fluorescent probes (kindly provided by ICMS) were added to react for 30 min and fluorescence intensity was determined at excitation wavelength 488
nm by a fluorescence microplate reader (48). Then HOCl production was calculated according to the standard curve of a serial concentration of HOCl vs. absorbance. To be estimated, HOCl standards should be freshly prepared by adjusting the pH of NaClO to 7.4 to create HOCl solutions before each time.

**Measurement of NC1 crosslink by peroxidasin.** To delineate sulfilimine crosslink of NC1 fragment in the collagen IV, the solubilized NC1 monomer purified from mouse renal basement membrane (Chondrex, Inc., WA, USA) was incubated with PXDN or the mixture of PXDN and FPM. To initiate the reaction, 100 μM H₂O₂ and 200 μM NaBr was respectively added. After incubation for 30 min at 37 °C, to visualize the change of sulfilimine crosslinked dimeric (NC1_d) and non-crosslinked monomeric subunits (NC1_m), the solution underwent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions followed by Coomassie Blue staining.

**Preparation and Separation of FPM’s Protein Corona.** First, lung homogenate (LH) was extracted from the lungs of health mice according to institutional bioethics approval. Briefly, the extracted lung tissue samples were homogenized in equal volume of PBS by a homogenizer (approximately 3 mice/ mL LH), and then centrifuged to remove the debris to obtain LH. 10 mg/mL FPM were incubated with LH at the volume ratio of 1:10 under stirring at 4 °C for indicated time. Then, the mixture was centrifuged through a 0.3 M sucrose cushion for 20 min at 4 °C at 15,300 × g, in order to separate the nanoparticle-corona complexes. Then, after rinsed with 1 × PBS for 3 times, proteins in the corona were eluted by adding RIPM lysis buffer (50 mM Tris pH7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) to the pellet on the ice for 1 h. After centrifugation (20 min at 15,300 × g at 4 °C), the supernatant enriching protein corona was collected and stored at -20 °C.

**LC-MS Analysis and Database Searches of Protein Corona.** The protocol to analyze protein with LC-MS adhered to a method described previously (33; 74). Briefly, after samples were quantified with bicinchoninic acid protein assay (BCA) kit; 100 μg total protein was reduced by adding 1 M DL-Dithiothreitol (DTT) (Sigma-Aldrich) (60 °C, 1 h), and free cysteines were alkylated with 1 M iodoacetamide (IAA)
(Sigma-Aldrich) (room temperature, 10 min in the dark). The alkylated proteins were centrifuged in the 10K ultrafiltration tube (Thermo Fisher Scientific), and the proteins were retained in the 10K ultrafiltration tube. The proteins were further washed with 100 mM tetratetraethylammonium bromide (TEAB) for three times at 4 °C for 20 min by centrifugation at 12,000 rpm. Then the protein was digested with 2 μg porcine sequencing grade trypsin (LC-MS Grade, Sigma-Aldrich) overnight at 37 °C. After digestion, the resulted peptides were collected (12,000 rpm, 20 min, 4 °C), desalted by Zeba Spin Desalting Columns (Thermo Fisher Scientific) and further enriched by C18 reversed-phase columns (Epoch Life Science, Missouri City, Texas, USA). The samples were then subjected to LC-MS analysis. To identify the composition of protein corona, identification of peptides and proteins from continuum LC-MS data was performed with the ProteinPilot™ 4.5 software (AB SCIEX). Proteins were analyzed by searching the mouse taxon of the UniProtKB/SwissProt database (release 2011_11). The proteins with at least one specific high-scoring peptides were detected and exported from ProteinPilot for the final LC-MS data file at the protein level.

**Identification and Characterization of PXDN's Liquid-Liquid Phase Separation.**

*Microscopy analysis of liquid-liquid phase separation (LLPS).* To analyze the droplet formation of PXDN under the stimulation of FPM, 10 mM fluorescein isothiocyanate (FITC) labelled proteins were incubated with 50 μg/mL rhodamine B labelled FPM for 30 min in LH. Then samples of different groups were dropped onto a glass slide and sealed with a coverslip. Phase separation of PXDN and its liquid-like droplets was observed under phase contrast and confocal microscopy with a 100x Oil objective (Nikon). The distribution profile of fluorescence intensity of liquid-like PXDN and FPM were respectively analyzed by the Nikon NIS-Elements software. Besides, to predict the domain that might trigger PXDN’s accumulation, the intrinsically disordered regions (IDR), the domain frequently closed to proteins’ phase-separation, of PXDN is predicted based on the IUPred algorithm (https://iupred2a.elte.hu/).

*Fluorescence recovery after photobleaching (FRAP) assays.* After 10 mM fluorescein isothiocyanate (FITC) labelled proteins were incubated with 50 μg/mL rhodamine B labelled FPM for 30 min in LH, fluorescence recovery after photobleaching
Molecular Docking on The Effect of Phase Separation on Enzymatic Reaction.

Template crystal structures of PXDN and NC1 domain in Col IV were identified and downloaded from Swiss Model Protein Data Bank as the PDB files (PXDN: 5MFA.1A; NC1: 5NAY). Besides, the putative structure under phase transition, which simulated the PXDN assembly, was created through homology modeling based on one experimentally determined structure of peroxidasin-related family member (PDB ID: 4C1M) in the RCSB Protein Data Bank (44). Subsequently, the most similar template conformation with 49.8\% consistency was chosen from among the candidates and named as the PXDN Dimer. Then ZDock protocol was used for molecular docking analysis of the interaction between PXDN and NC1 (respectively for the ‘Solution State’ to the ‘Assembly State’). Docking models of the intuitive contacting interface was outputted after scoring and selection. The interactive area was especially labelled.

Western Blotting. According to the standard protocol, different proteins were separated by SDS-PAGE. To be estimated, the proteins in corona from the nanoparticles were eluted with equal and adequate PAGE sample buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich) and the same volume of eluted corona proteins was analyzed. Besides, to further estimate the content of PXDN adsorbed on the FPM, different amounts of PXDN (100 ng, 500 ng, 1,000 ng and 2,000 ng) together with the corona protein samples was separated by SDS-PAGE and analyzed by western blotting. Then the proteins were transferred onto the polyvinylidine difluoride (PVDF) membranes (Bio-Rad, California, USA). The experiments were performed on rhodamine-labelled PXDN droplets formed in PM1. The photorecovery behavior was tracked using the 564 nm laser line of a 40 \times 1.0NA objective on Zeiss LSM 980 with 2.4-fold magnification. Photobleaching was done with 100\% laser power to 30\% intensity using the bleaching program of the ZEN software and time-lapse images were recorded every 10 s. After bleaching, the fluorescence intensities were measured and collected by mean ROI (photo-bleached region and control region without bleach). The raw data with three bleach treatments are processed and analyzed by GraphPad Prism.
membranes were blocked with skim milk and then incubated with primary antibody – PXDN (Merck Millipore), Type I collagen (Col I, Boster Biological Technology co.ltd, Wuhan, China), Type III collagen (Col III, ABclonal Technology, Wuhan, China), Type IV collagen (Col IV, Abcam, Cambridge, MA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Abcam) at 4 °C with gentle shaking overnight. After being washed with PBST (PBS with 0.1% Tween-20) for 5 times, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat IgG (Life Technologies, Grand Island, NY, USA) at room temperature. After rinse, positive signal was visualized using an enhanced chemiluminescence system (Cell Signaling Technology). The band intensity was quantitated using image J software (http://rsb.info.nih.gov/ij/) and the statistical analysis of three independent experiments was performed.

RNA isolation and quantitative real-time PCR. RNA of cells or lung tissues were extracted by using Trizol reagent (Life Technologies). For mRNA detection, real-time polymerase chain reaction (PCR) was launched in an ABI 7300 Fast Real-time PCR System (Applied Biosystems, FosterCity, CA) using the SYBR Prime Script RT-PCR Kit (Takara Bio, Shiga, Japan). Each sample was analyzed in triplicates and repeated for three or four independent assays with β-actin as internal control. Primers of integrin-1 (ITGB1), C-X-C motif chemokine receptor 3 (CXCR 3), Rho-associated kinase (ROCKi) and peroxidasin (PXDN) are listed as follows (Shanghai Generay Biothech Co., Ltd, Shanghai, China):

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
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<tbody>
<tr>
<td>ITGB1</td>
<td>5’- CGTGGTTGCCGGAATTGTTC -3’</td>
<td></td>
</tr>
<tr>
<td>ITGB1</td>
<td>5’- ACCAGCTTTACGTCATAGTTTG -3;</td>
<td></td>
</tr>
<tr>
<td>CXCR3</td>
<td>5’- TACCTTGAGGTTAGTGGAACGTC -3’,</td>
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</tr>
<tr>
<td>CXCR3</td>
<td>5’- CGCTCTCGTTTTTCCCCATAATC -3’;</td>
<td></td>
</tr>
<tr>
<td>ROCKi</td>
<td>5’- AACATGCTGCTGGATAATCTCTGG -3’,</td>
<td></td>
</tr>
<tr>
<td>ROCKi</td>
<td>5’- TGTATCACATCGTACCAGGCT -3’;</td>
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</tr>
<tr>
<td>PXDN</td>
<td>5’- CCTGTGTTTCCGTACCACCG -3’,</td>
<td></td>
</tr>
<tr>
<td>PXDN</td>
<td>5’- CTCTGATTCTGGACAAGAAGA -3’;</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>5’- GGCTGTATTCCTCCATCG-3’</td>
<td></td>
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β-actin- Reverse: 5'-CCAGTTGGTAACCATGCCTG-3'.

**Flow Cytometry Analysis.** Lung tissue were digested with 2 mg/mL collagenase type I and IV (Thermo Fisher Scientific) for 30 min to generate a single-cell suspension. Cell suspensions were filtered through 70 μm cell strainers, and red blood cells were lysed. For the intracellular staining, 1×10⁶ cells/ml were treated with the cell activation cocktail (BioLegend, San Diego, California, USA) according to the manufacturer’s protocol. After cells were washed with PBS containing 1% BSA, cells were blocked with 1% BSA at 4 °C for 30 min. Zombie Violet™ Fixable Viability Kit was used for live/dead cell determination. Then cells were stained on ice for 30 min with surface-staining antibodies, FITC anti-mouse CD45, BV711 anti-mouse CD3, APC anti-mouse CD8a, and then washed, fixated and permeabilised with the fixation/permeabilization solution kit (BD Biosciences, San Jose, CA, USA) and stained with cytokine PE anti-mouse interferon gamma (IFN-γ) antibodies. in the dark for 30 min at 4 °C. The samples were centrifuged at 400 – 500 × g for 5 min at 4 °C to remove unbound antibody. After rinsing for 3 times, each sample was re-suspended for analysis using a BD Fluorescence Activated Cell Sorter (FACS) Calibur (BD Biosciences). Unconjugated antibodies and IgG controls were run in parallel to set the background. All antibodies and their isotype control antibodies were obtained from BioLegend (San Diego, CA, USA).

**Enzyme-linked Immunospot Assay (ELISPOT).** The lung tissue exposed to PBS or FPM were excised after the intravenous stimulation of LLC-OVA cells for 1 day. After CTLs were separated, 1x10⁵ CTLs were added in each well of IFN-γ antibody pre-coated plate and stimulated by 4x10⁴ irradiated LLC-OVA cells or PMA for 24h in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin. IFN-γ producing CTLs were enumerated by a mouse IFN-γ precoated ELISPOT kit (Dakewe Biotech Co., Ltd.) according to the manufacture instructions. The results were analyzed by AID iSpot (AID-Autoimmun Diagnostika GmbH, Strassberg, Germany).

**Immunofluorescence staining.** Lung tissue samples were collected, frozen at optimal cutting temperature (OCT) medium (Thermo Fisher Scientific) and cut into sections.
The sections or M2-10B4 cells incubated with 10 μg/mL rhodamine-labelled FPM and 1 μg/mL PXDN (at the volume ratio of 1:10) for 1 h were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) and stained with primary antibody at 4 °C overnight. The primary antibodies, including PXDN, Col I, Col III, Col IV and CD8. Next, the sections were incubated with secondary antibody Alexa Fluor (Life Technologies) for 1 h at room temperature, followed by 4,6-diamidino-2-phenylindole (DAPI, Beyotime) for nuclear staining. And then the sections were imaged by LSM 980 with Airyscan 2 confocal microscope (Carl Zeiss; Oberkochen; Germany). To further characterize the crosslink level, based on IF images of Col IV, look-up tables (LUTs) analysis based on the fluorescence intensity, surface plot analysis based on the invert binary distance of fluorescence distribution, were respectively accomplished employing Image J (Image J Software, National Institutes of Health, Bethesda, MD, USA). Using the ‘ridge detection’ plugin in Image J, binary images of Col IV network was generated, and the related quantitative analysis of junction number and junction density were created and compared. Besides, the EdU positive percentage was analyzed by the Bioapps Tools in ZEISS ZEN 3.4 (Carl Zeiss).

**Histological Studies.** The lung tissue fixed in 2.5% paraformaldehyde (PFA) was embedded in paraffin and cut into sections for the hematoxylin and eosin (H&E) and Masson’s trichrome (Abcam) staining (NanJing KeyGen Biotech Co.,Ltd., Nanjing, China) according to the manufacturer’s instructions with slight modification. Stained sections were photographed at different magnification under a microscope. Under blindfold conditions with standard light microscopy, tumor burden (based on the percentage of the area of tumor regions versus that of total lung) according to H&E-stained sections of all five lung lobes was quantified with ImageJ software. Besides, to observe the interstitial ECM structure, lung tissues were fixed with glutaraldehyde at 4 °C for 48 h, dehydrated with an ethanol gradient and dried at the critical point. Then the samples were sprayed with gold particles and observed with SEM (SFEG Leo 1550, AMO GmbH, Aachen, Germany).

**Statistical analysis.** The results are expressed as mean ± standard deviation (SD). Data were statistically analyzed using Prism software (GraphPad) and assessed for
normality or homogeneity of variance. Differences between multiple groups were compared using one-way or two-way analysis of variance (ANOVA) with Dunnett’s tests or, if appropriate, repeated measures ANOVA test with post-hoc Bonferroni correction. Differences between two groups were evaluated using the two-tailed unpaired Student's t-test. A value of \( p < 0.05 \) was considered significant; n.s. = not significant.

Data Availability

The data generated in this study are available within the article and its supplementary data files.

Acknowledgements

We thank Professor Ke Zeng in Nanjing University for his kindly providing the OT-1 TCR transgenic mice and OVA-LLC cells. This study was funded by the National Natural Science Foundation of China (31971309, 32001069, 81973273), the Natural Science Foundation of Jiangsu Province (BK20200318), and the Fundamental Research Funds for the Central Universities (020814380115). C.W. acknowledges the financial support from the Science and Technology Development Fund, Macao SAR (FDCT 0018/2019/AFJ, 0060/2020/AGJ) and the University of Macau Research Committee (MYRG2020-00084-ICMS). This study also supported by the funds for the International Cooperation and Exchange of the Natural Science Foundation of China and the Science and Technology Development Fund (31961160701).

Conflict of Interest
The authors declare no potential conflicts of interest.

Reference


1353 504-526.


46. Soudi, M., Paumann-Page, M., Delporte, C., Pirker, K.F., Bellei, M., Edenhof, E.,


OVA-LLC Model in OT-1 Mice

PBS: 16.1%

PM1: 3.40%

TSP: 4.48%
A

Injection of FPM

PM1

TSP

7 d

Intratracheal Injection of IP-10

IP-10

Tissue Extraction

B

IFN-γ^+CD8^+/CD45^+CD3^+

PBS

PM1

TSP

p < 0.0001
Normal or FPM-exposed Lung Tissue

- Seed CTLs on one side
- Drop IP-10 on the other side

50 μm Slide of Lung Tissue

- Capture CTLs’ movement
- Analysis CTLs’ trajectories

Resting CTLs
Moving CTLs
Chemokine IP-10
Interstitial ECM
Neutral Salt and Acetic Acid Treatment
Extraction: Soluble Collagen

Pepsin Treatment
Extraction: Moderately Cross-link Collagen

Remaining Insoluble Fraction
Extraction: Highly Cross-link Collagen
Hydroxy-lysine 211

Sulfilimine bond

Methionine 93
Incubation → Centrifugation → Washing

LH Fluid → 0.3 M Sucrose → LH-FPM Complex

Elution → Protein Corona

Western Blotting → LC-MS
The graph illustrates HOCl production (mM/µg) across different conditions: PBS, PM1, and TSP. The data points show a statistically significant difference between the conditions, as indicated by the asterisks and p-values:

- PM1 vs. PBS: p = 0.0028
- TSP vs. PBS: p = 0.0001
A. M2-10B4 Cells

Relative level of PXDN mRNA expression

shNC  shPXD N

B. M2-10B4 Cells

PXDN  GAPDH

shNC  shPXD N

C. Lung Tissue

Relative level of PXDN mRNA expression

shNC  shPXD N

D. Lung Tissue

PXDN  GAPDH

shNC  shPXD N

p = 0.0039

p = 0.0091