



Article

# Effects of Ambient Fine Particles PM<sub>2.5</sub> on Human HaCaT Cells

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**Abstract:** The current study was conducted to observe the effects of fine particulate matter (PM<sub>2.5</sub>) on human keratinocyte cell line (HaCaT) cells. The potential mechanism linking PM<sub>2.5</sub> and skin was explored. HaCaT cells were cultured and then accessed in plate with PM<sub>2.5</sub>. Cell viability was tested by Cell Counting Kit-8. The mRNA and protein expression of Filaggrin, Loricrin, Involucrin, and Repetin were analyzed. The levels of Granulocyte-macrophage Colony Stimulating Factor, Thymic Stromal Lymphopoietin, Tumor Necrosis Factor- $\alpha$ , Interleukin-1 $\alpha$ , and Interleukin-8 were detected in the supernatant of the HaCaT cell with enzyme-linked immunosorbent assay kits. Cell viability decreased with the increase in PM<sub>2.5</sub>. Compared with the control group, the protein expression of Filaggrin, Repetin, Involucrin, and Loricrin showed different expression patterns in PM<sub>2.5</sub> treatment groups. The level of Tumor Necrosis Factor- $\alpha$ , Thymic Stromal Lymphopoietin, Interleukin-1 $\alpha$ , and Interleukin-8 significantly increased in the cells treated with PM<sub>2.5</sub>. Ambient PM<sub>2.5</sub> may increase the risk of eczema and other skin diseases. The relative mechanism may be associated with the impairment of the skin barrier and the elevation of inflammatory responses.

**Keywords:** PM<sub>2.5</sub>; HaCaT cell; skin damage

## 1. Introduction

Airborne particulate matter (PM) pollution is receiving an increasing amount of attention in China. Particles with an aerodynamic diameter under 2.5  $\mu\text{m}$  (fine particles, PM<sub>2.5</sub>) have become the primary pollutant, which is the most important pollutant when hazes form. In most cities in China, 24 h average concentrations of PM<sub>2.5</sub> show an obviously high level especially in fall and winter seasons according to the data from Ministry of Environmental Protection of the People's Republic of China. The Chinese government has established the air quality standard for PM<sub>2.5</sub> in 2012 [1]. Several experimental and epidemiological studies have demonstrated that PM<sub>2.5</sub> mainly affect the respiratory system [2] and the cardiovascular system [3], via exerting complex biological effects in these tissues. Recently, the effect of PM<sub>2.5</sub> on the skin has also been noticed by clinical dermatologists [4,5].

Ambient air pollution was recognized as a crucial risk factor in skin diseases. Previous studies have shown that air pollution may induce or aggravate atopic dermatitis [6,7], which is also associated with an increase in oxidative stress of the skin tissue, the elevation of skin aging, and the impairment

of immune function. In addition, one study has found that exposure to ambient PM<sub>2.5</sub> can aggravate symptoms in children with allergic dermatitis and eczema deterioration [8]. Exposure to high concentrations of PM<sub>2.5</sub> lead to more pigmented spots in the face and increase the nasolabial folds [9]. However, until now, mechanisms linking ambient PM<sub>2.5</sub> and skin damage have not been elucidated, and the relevant research is limited.

- The mechanism linking skin damage and environmental factors may have the following aspects.
- Oxidative damage: PM may damage skin through oxidative stress production, which is one of the important causes of skin aging [10].
- Skin barrier function impairment: In the process of keeping skin barrier integrity, several important structural proteins play an important role in the process of skin cell differentiation, such as Loricrin (LOR), Involucrin (IVL), and Filaggrin (FLG) [11].
- Immune injury: Airborne particulate matter may increase keratinocyte inflammatory mediators [12] and cause cutaneous injury in cellular or humoral through immunity and innate immunity pathways. Increasing evidence has shown that the immune injury may play a role in the pathogenesis of various cutaneous inflammatory diseases by releasing cytokines or by expressing membrane-associated adhesion molecules [13,14].

The present study aims to observe the effects of ambient PM<sub>2.5</sub> on human keratinocyte cell line cells (HaCaT) and explore the potential mechanisms linking PM<sub>2.5</sub> and skin damage.

## 2. Materials and Methods

### 2.1. PM<sub>2.5</sub> Sampling

The PM<sub>2.5</sub> particles were collected using a Thermo Anderson G-2.5 air sampler (Thermo Scientific, Franklin, MA, USA) from May 2014 to December 2014 in a non-industry district in Shanghai, China. Glass microfiber filters (Glass Microfiber Company, Shanghai, China) were used to collect PM<sub>2.5</sub>. After the sampling ended, the filters were cut into small pieces, immersed in three-fold-distilled water, and sonicated for 4 × 30 min with a sonicator (Jeken, Shenzhen, China) for sterilization. The suspension was treated by vacuum-freeze dry, and concentrated fractions were weighted and stored at −20 °C. Before the stimulation, the PM<sub>2.5</sub> was diluted to 1000 µg/mL and stored at 4 °C.

### 2.2. Cell Culture

HaCaT cells, a spontaneously transformed aneuploid immortal keratinocyte cell line from adult human skin, were purchased from Cells Center of Shanghai Institutes for Biological Sciences (Chinese Academy of Science, Shanghai, China), cultured in defined keratinocyte serum-free medium (K-SFM) (Gibico, Grand Island, NY, USA), and grown at 37 °C in a humidified incubator in a 5% CO<sub>2</sub> atmosphere. The medium was refreshed every two days, and cells were sub-cultured every four days. Cell culture was performed according to the manufacturer's manual.

### 2.3. PM<sub>2.5</sub> Treatment

HaCaT cells were cultured and then accessed in plate with concentrations of 5 × 10<sup>3</sup> cells/100 µL. After two days of culture, the cells were treated with a series of concentrations (0, 5, 10, 25, 50, 100, 200, 300, 400, 500, and 800 µg/mL) of PM<sub>2.5</sub> for 24 h to evaluate the concentration-dependent effect. For observing the time-response induced by PM<sub>2.5</sub>, the effects were determined at different exposure times in cells after being treated with PM<sub>2.5</sub>. The morphology of HaCaT cells was observed with a microscope (Nikon, Tokyo, Japan).

### 2.4. Cell Viability Determination

Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Tokyo, Japan) is widely used to test cell proliferation and cytotoxicity with high accuracy. In order to observe the cell viability of different

concentrations of PM<sub>2.5</sub>, cell viability was determined in HaCaT cells after being treated with 0–800 µg/mL PM<sub>2.5</sub>. Then, the time-response was determined at different exposure times. After treatment with 50 µg/mL PM<sub>2.5</sub>, the HaCaT cells were incubated for 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, 16 h and 24 h, respectively. Enzyme ferment was used to test the cell viability by reading absorbance at 450 nm. The inhibition ratio was calculated and a growth curve was printed. The calculation formula is as follows:

$$\text{Viability (\%)} = (\text{Optical Density (OD) control group} \times \text{OD treatment group}) / \text{OD control group} \times 100\%$$
$$\text{Relative activity (\%)} = (1 - (\text{test-background}) / (\text{control-background})) \times 100\%.$$

### 2.5. Western Blot

After treatment with different concentrations (0, 10, 25, 50, and 100 µg/mL) of PM<sub>2.5</sub> for 24 h, the HaCaT cells were rinsed twice in phosphate buffered saline (PBS). Then, the protein extracts were obtained by cell lysis buffer (Beyotime, Haimen, China) and spun at 14,000× g for 10 min at 4 °C. Total proteins for each sample were loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (Beyotime). After electrophoresis, proteins were transferred onto a nitrocellulose membrane. Blots were rinsed twice in Tris-buffered saline–Tween (TBST). After being blocked for 2 h at room temperature in TBST plus 5% skim milk powder, the nitrocellulose membrane was incubated with different dilutions of primary antibodies—FLG, LOR, IVL, Repetin (RPTN), or β-actin (Abcam, Cambridge, UK)—over 12 h at 4 °C. Then, the membrane was rinsed three times in TBST (10 min each at room temperature) and incubated for 2 h at room temperature with a secondary antibody (Beyotime). Blots were finally rinsed clearly and detected by Immobilon Western (Millipore, Boston, MA, USA). The protein bands were scanned with a LAS3000 imaging system (Fujifilm, Tokyo, Japan), and band density was calculated by Quantity One software (Bio-Rad, Hercules, CA, USA). β-actin was used as a control.

### 2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

The cell supernatant samples were collected from the HaCat cells after treatment with different concentrations (0, 10, 25, 50, and 100 µg/mL) of PM<sub>2.5</sub> for 24 h. The cytokines Granulocyte-macrophage Colony Stimulating Factor (GM-CSF), Thymic Stromal Lymphopietin (TSLP), Tumor Necrosis Factor-α (TNF-α), Interleukin-1α (IL-1α), and Interleukin-8 (IL-8) were determined using ELISA kits (AMEKO, Shanghai, China). The tests were performed strictly according to the manufacturer's instructions.

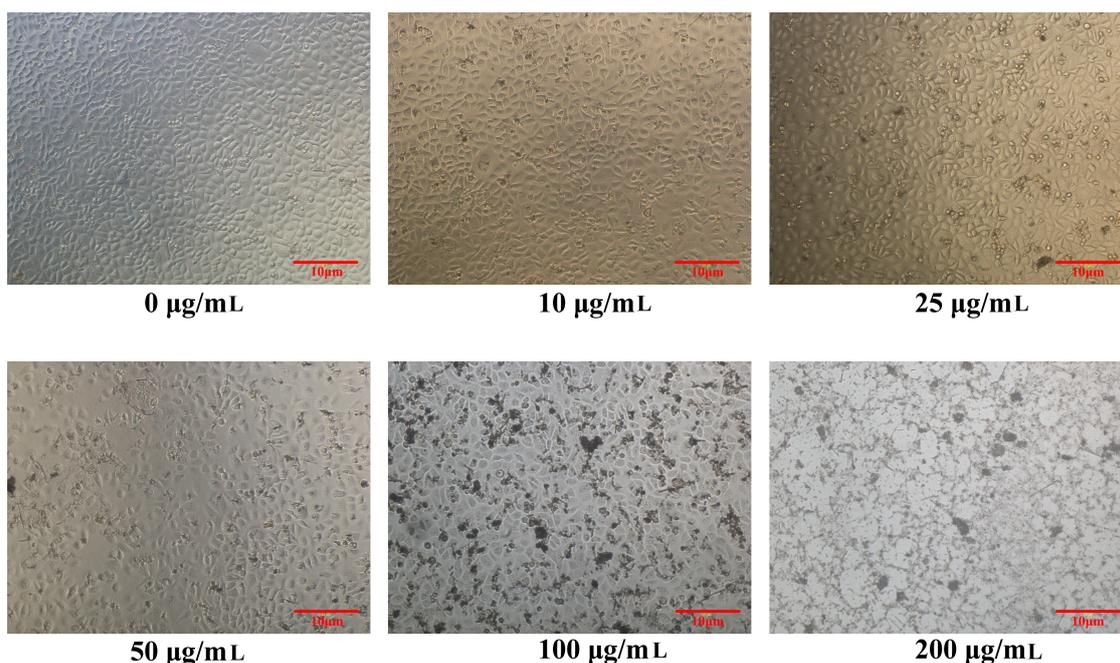
### 2.7. Statistical Analyses

All analyses were carried out three times independently. The results were presented as means ± standard deviation (SD). The Graphprism 5.0 software (GraphPad Software, San Diego, CA, USA) was used for statistical analysis and graph plotting. The differences among the exposure groups were analyzed using one-way analysis of variance.

## 3. Results

### 3.1. The Morphology of HaCaT Cells

The morphology of HaCaT cells was observed after being stimulated by different concentrations of PM<sub>2.5</sub> (Figure 1). When treated with 0 µg/mL PM<sub>2.5</sub>, the HaCaT cells showed a normal shape. However, with the increase in PM<sub>2.5</sub> concentration, the cell membrane was impaired and the dead cells increased.

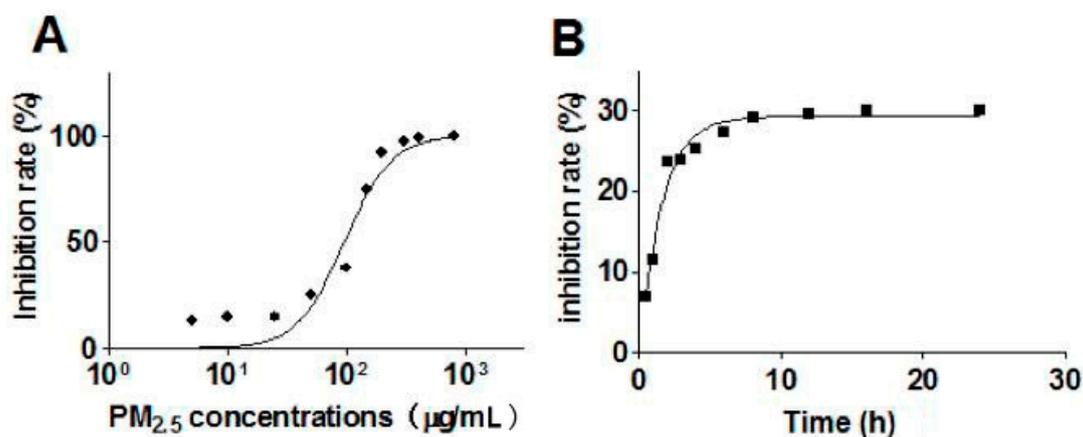


**Figure 1.** Morphology of human keratinocyte cell line (HaCaT) cells after being exposed to 0, 10, 25, 50, 100, and 200  $\mu\text{g}/\text{mL}$   $\text{PM}_{2.5}$ , respectively. Scale bar: 10  $\mu\text{m}$ .

### 3.2. Cell Viability Determination

#### 3.2.1. Relationship between $\text{PM}_{2.5}$ Concentration and Cell Viability

The CCK-8 assay was used to detect the cell viability of HaCaT cells after being treated with  $\text{PM}_{2.5}$ . As Figure 2A shows, with the rise in  $\text{PM}_{2.5}$  concentration, cell viability decreased. At a lower dose of  $\text{PM}_{2.5}$ , the cell inhibition rate maintained a low level. When the concentration of  $\text{PM}_{2.5}$  exceeded 50  $\mu\text{g}/\text{mL}$ , the inhibition rate elevated rapidly. The results indicate that the cytotoxicity of  $\text{PM}_{2.5}$  significantly increases when the concentration exceeds 50  $\mu\text{g}/\text{mL}$ .



**Figure 2.** Effects of  $\text{PM}_{2.5}$  concentrations and exposure times on HaCaT cells viability. (A) HaCaT cells were treated with different concentrations of fine particles ( $\text{PM}_{2.5}$ ) (from 0 to 800  $\mu\text{g}/\text{mL}$ ) for 24 h; HaCaT cell viability maintained a low level with lower doses of  $\text{PM}_{2.5}$ , and, when the concentrations of  $\text{PM}_{2.5}$  exceeded 50  $\mu\text{g}/\text{mL}$ , the damage of the cells increased significantly; (B) HaCaT cells were treated with 50  $\mu\text{g}/\text{mL}$   $\text{PM}_{2.5}$ , and the CCK-8 assay was used to detect cell viability at 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, 16 h, and 24 h respectively. (Each analysis was carried out three times independently).

### 3.2.2. Relationship between Exposure Time and Cell Viability

According to the above result, the exposure concentration of PM<sub>2.5</sub> was set as 50 µg/mL to evaluate the relationship between PM<sub>2.5</sub> exposure time and HaCaT cells viability. The CCK-8 assay was used to detect cell viability at 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, 16 h, and 24 h, respectively (Figure 2B). When compared with 0 h, a significant reduction of cell viability was observed at 2 h. Nevertheless, from 6 h to 24 h, the cell inhibition rate did not show a significant alteration, which means that the cell viability maintains a stable level (70%) starting from 6 h (Figure 2B).

### 3.3. Western Blot

The proteins expression of FLG, IVL, LOR, and RPTN were detected in HaCaT cells by western blot (Figure 3). Increases in IVL and RPTN protein expression in a dose-dependent manner occurred with rises in PM<sub>2.5</sub> concentration. PM<sub>2.5</sub> induced a high expression of FLG in cells only in the high PM<sub>2.5</sub> treatment groups but did not cause significant changes in LOR.

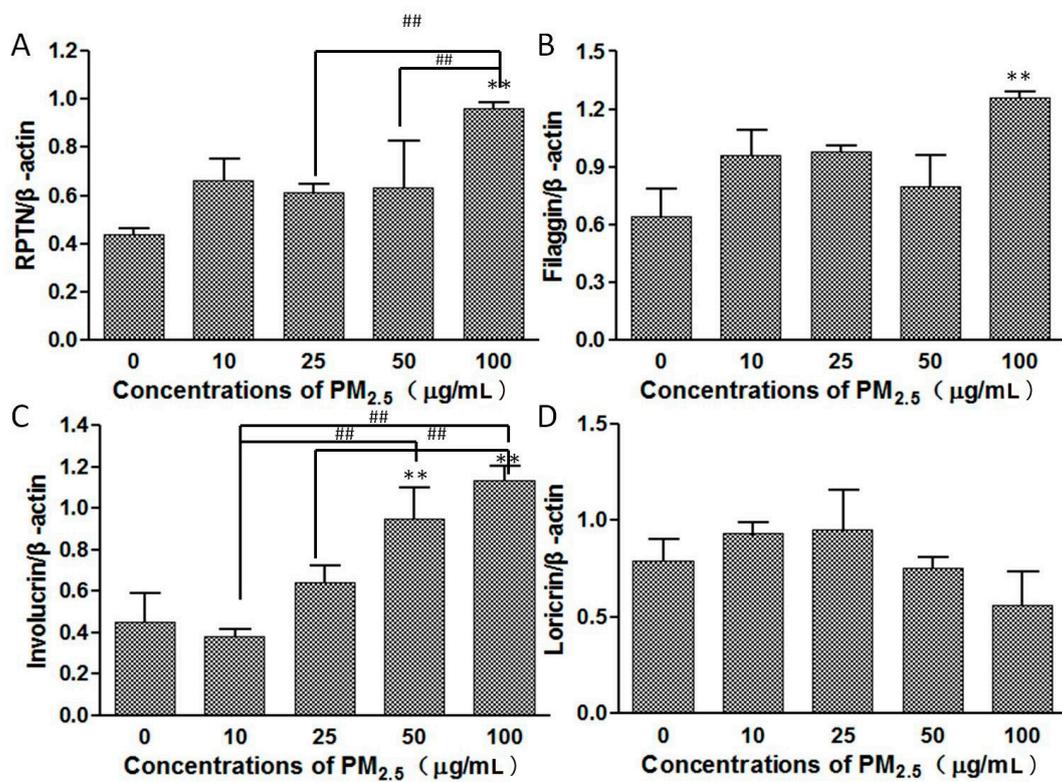
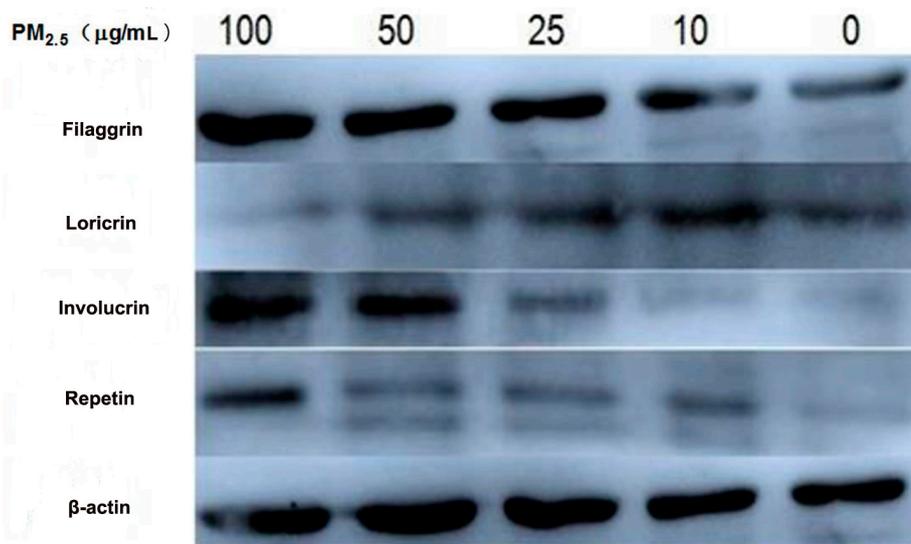


Figure 3. Cont.



**Figure 3.** Proteins expression of Repetin (A); Filaggrin (B); Involucrin (C); and Loricrin (D) in HaCaT cells after being exposed to different concentrations of PM<sub>2.5</sub> (0, 10, 25, 50, and 100 µg/mL) for 24 h. \*\* There were significant differences ( $p < 0.01$ ) between the exposed group and the control. ## There were significant differences ( $p < 0.01$ ) between the exposure groups. (Analysis of variance (ANOVA) statistics was used to calculate the statistical difference between the exposed group and the control and between the four exposed groups. Each analysis was carried out three times independently).

### 3.4. ELISA

The secretion of GM-CSF, TSLP, TNF- $\alpha$ , IL-1 $\alpha$ , and IL-8 were detected in the supernatant of the HaCaT cells (Figure 4). The rise in PM<sub>2.5</sub> was associated with increased production of the above cytokines, except GM-CSF. The level of TNF- $\alpha$  sharply increased when the concentration of PM<sub>2.5</sub> exceeded 25 µg/mL. The secretion of IL-8 by HaCaT cells was elevated significantly at low concentrations of PM<sub>2.5</sub> and maintained a high level with increased concentrations of PM<sub>2.5</sub>. The production of TNF- $\alpha$ , TSLP, and IL-1 $\alpha$  increased in a dose-dependent manner, but GM-CSF did not.

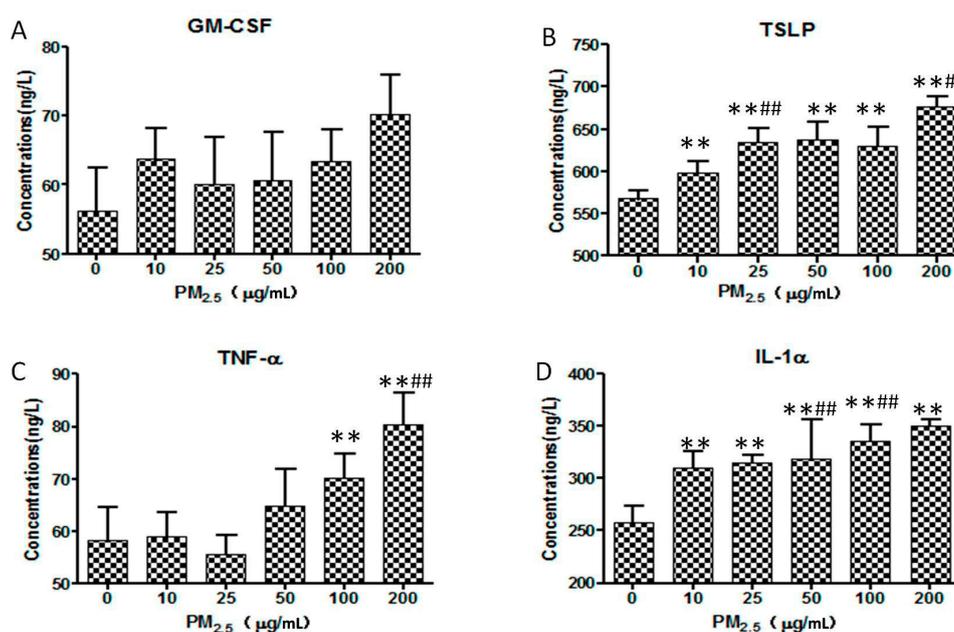
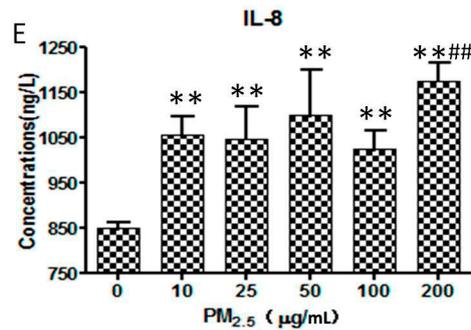


Figure 4. Cont.



**Figure 4.** Levels of Granulocyte-macrophage Colony Stimulating Factor (GM-CSF) (A); Thymic Stromal Lymphopoietin (TSLP) (B); Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) (C); Interleukin-1 $\alpha$  (IL-1 $\alpha$ ) (D); and Interleukin-8 (IL-8) (E) in HaCaT cells after being exposed to fine particles for 24 h. In each figure, the exposed doses from left to right are 0, 10, 25, 50, 100, and 200  $\mu\text{g}/\text{mL}$ . \*\* There were significant differences ( $p < 0.01$ ) between the exposed group and the control. ## There were significant differences ( $p < 0.01$ ) between the group and the former group. (ANOVA statistics was used to calculate the statistical difference between the exposed group and the control and between the exposed group and its adjacent former group. Each analysis was carried out three times independently).

#### 4. Discussion

Keratinocytes in the skin are the first carrier to be exposed to environmental stresses. The keratinocyte exposed to Asian dust storm particles could upregulate the pro-inflammatory mediators and increase the arylhydrocarbon receptor (AhR) expression, and the latter activation may lead to an increased production of reactive oxygen species [12]. Thus, the current study was aimed to observe the influence of ambient PM<sub>2.5</sub> on HaCaT cells.

A previous study has reported that organic compounds presenting on the surface of PM<sub>2.5</sub> may penetrate into skin and have direct effects on viable skin cells including keratinocytes [7]. In our study, ambient PM<sub>2.5</sub> could affect not only the viability of HaCaT cells, but also the expression of skin barrier-related proteins and the synthetic immune-related indicators. Meanwhile, different concentrations of PM<sub>2.5</sub> induced different responses. With the use of microscope detection and the test of cell activity, the rate of cell damage gradually rose with the rise in PM<sub>2.5</sub> concentration. When the concentration of PM<sub>2.5</sub> rose to 50  $\mu\text{g}/\text{mL}$ , the cell viability reached a stable level (around 70%). When the concentration of PM<sub>2.5</sub> exceeded 100  $\mu\text{g}/\text{mL}$ , the majority of cells were dead.

The onset of dermatitis is closely related to the reduction of the skin barrier function. Recent studies found that the FLG gene often showed a reduced expression in patients with atopic dermatitis and eczema. An increase in LOR expression, induced by the skin barrier dysfunction was indicated by the immunohistochemistry and Western blot results [15]. IVL is considered to be a human keratinocyte differentiation marker [16]. Previous literature reported that mRNA and protein expression of IVL significantly reduced when the skin barrier function was impaired [15,17,18], yet some studies showed that IVL was upregulated in patients with atopic dermatitis [19,20]. In our study, the results showed that the expression of FLG, IVL, and RPTN elevated significantly after exposure to PM<sub>2.5</sub>, while the expression of LOR showed no change in cells after being stimulated by PM<sub>2.5</sub>. This indicates that the acute exposure of PM<sub>2.5</sub> might impair the skin barrier by reducing the cell viability directly instead of downregulating the expression of skin barrier-related proteins. On the other hand, the increased levels of FLG, IVL, and RPTN might present a reaction of the skin cells to acute PM<sub>2.5</sub> exposure. The response of human keratinocytes to chronic and low-concentration PM<sub>2.5</sub> exposure needs further study.

It has been reported that the immunotoxicity of PM plays an important role in the innate immune system and in cellular or humoral immunity [21]. Ambient PM<sub>2.5</sub>-related skin damage might be caused by activating the innate immune system. TSLP, a member of the cytokine superfamily, plays an important role in the regulation of immune responses by inducing the maturation and activation of

multiple immunocytes [22]. TSLP has a high expression in patients with asthma, allergic rhinitis, and atopic dermatitis, which is associated with the relative signal pathway in the pathogenesis of these diseases [14,23,24]. The activation of TSLP could affect innate immunity or the immune response of Th2 cells and activate a nuclear transcription factor (NF- $\kappa$ B) to negatively regulate FLG expression on skin cells [25–27]. This study also supports the increased expression of TSLP in HaCaT cells after exposure to PM<sub>2.5</sub>. The results indicate that PM<sub>2.5</sub> can affect non-specific immunity and cause skin damage.

Additionally, PM<sub>2.5</sub> can also stimulate human keratinocytes to release a series of cytokines and pro-inflammatory cytokines. Our data showed the levels of TNF- $\alpha$ , IL-1 $\alpha$ , and IL-8 significantly increased with the increase in PM<sub>2.5</sub> concentration, excluding GM-CSF. Pro-inflammatory cytokines further stimulate epithelial cells, fibroblasts, and endothelial cells to secrete cytokines and adhesion molecules (such as IL-8, IL-2, and IL-1). Then, these adhesion molecules and cytokines can accumulate inflammatory cells (such as neutrophils, macrophages, monocytes, and polymorphonuclear leukocytes), resulting in inflammatory response [28,29]. IL-1 $\alpha$  was expressed in keratinocytes and was released when the skin was stimulated by external stimulation, which is regarded as an early indicator of skin irritation [30]. However, the mechanisms linking PM<sub>2.5</sub> or their organic compounds and the expression of inflammation-related cytokines in skin are still limited. Further studies using a suitable experimental system to clarify the mechanisms involved in the modulation of cytokines from keratinocytes by PM<sub>2.5</sub> pollution are necessary.

## 5. Conclusions

In conclusion, the skin, being an important barrier of the body, can display a series of responses when exposed to external harmful substances. The current study indicates that acute PM<sub>2.5</sub> exposure can reduce the viability of HaCaT cells and induce the release of inflammatory factors. These results suggest that ambient PM<sub>2.5</sub> may increase the risk of eczema and other skin disease occurrences dependent on inflammatory activity.

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**Author Contributions:** Feng Xu and Jinhua Xu proposed the research question and designed the research. Qiao Li, Zhihua Kang, Shuo Jiang, Jinzhao Zhao and Shuxian Yan collected and analyzed the data; Qiao Li, Zhihua Kang and Shuo Jiang wrote the paper.

**Conflicts of Interest:** The authors declare no conflict of interest.

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