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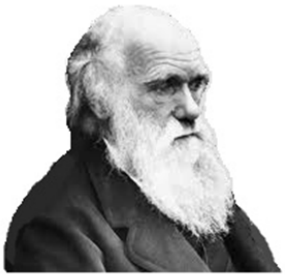

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Epigenetic Rewiring of Immune Checkpoints in Pollution-Driven Lung Adenocarcinoma: An In Vitro and In Vivo Translational Study

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ABSTRACT

Air pollution has been increasingly recognized as a major risk factor for lung adenocarcinoma, yet the molecular mechanisms linking exposure to carcinogenesis remain unclear. Recent studies suggest that fine particulate matter ($PM_{2.5}$) can alter epigenetic landscapes, particularly through DNA methylation and histone modification, thereby influencing immune regulation. This study investigated how pollution exposure can induce epigenetic rewiring of the immune checkpoint gene PD-L1, leading to impaired immune recognition and tumor progression. Lung adenocarcinoma cell lines were exposed to $PM_{2.5}$ extracts, and methylation patterns were analyzed using bisulfite sequencing. The expression of PD-L1 and related immune genes was quantified by qPCR, and reactivation assays using histone deacetylase (HDAC) inhibitors were performed. Complementary in vivo studies were conducted in Wistar rats exposed to $PM_{2.5}$ for six weeks to assess tissue-level alterations. Results demonstrated that $PM_{2.5}$ exposure significantly increased DNA methylation at CpG islands within the PD-L1 promoter, reducing gene expression and T-cell activation capacity. Treatment with epidrugs such as valproic acid reversed methylation changes and restored immune function. The findings provide mechanistic insight into how environmental pollutants contribute to immune evasion in cancer and suggest potential therapeutic intervention through epigenetic modulation.

***Keywords*:** air pollution, $PM_{2.5}$, DNA methylation, PD-L1, immune checkpoint, epigenetics, lung adenocarcinoma, epidrugs, T-cell activation, Wistar rat

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1. INTRODUCTION

Lung cancer remains one of the leading causes of cancer-related deaths globally, accounting for nearly 2 million deaths annually (Sung et al., 2021). Among its subtypes, lung adenocarcinoma is most frequently linked to environmental exposures such as air pollution, cigarette smoke, and industrial emissions (Cohen et al., 2017). In densely populated cities of developing nations, including Calabar, Nigeria, traffic-related pollutants and biomass combustion contribute to elevated levels of particulate matter ($PM_{2.5}$), which has been classified as carcinogenic by the International Agency for Research on Cancer (IARC, 2016).

1. 1. Air Pollution and Disease Mechanisms

Fine particulate matter ($PM_{2.5}$) can penetrate deep into the alveoli, generating reactive oxygen species (ROS) and initiating oxidative stress (Liu et al., 2019). Prolonged exposure has been linked to DNA damage, mutations, and chronic inflammation - factors that collectively promote cancer initiation and progression. However, beyond genetic mutations, $PM_{2.5}$ also alters epigenetic regulation - a reversible layer of gene control that includes DNA methylation, histone modification, and non-coding RNA activity (Jiang et al., 2020).

1. 2. Epigenetic Regulation and Immune Checkpoints

Epigenetic changes play a central role in cancer development by silencing tumor suppressor genes and modulating immune recognition (Feinberg & Tycko, 2004). One critical immune checkpoint, programmed death-ligand 1 (PD-L1), interacts with the PD-1 receptor on T-cells to prevent overactivation of immune responses. Under normal conditions, this maintains immune tolerance, but in cancer, PD-L1 overexpression helps tumor cells evade immune destruction (Dong et al., 2018). Conversely, recent evidence shows that PD-L1 can also be silenced via hypermethylation, reducing responsiveness to immunotherapy (Goltz et al., 2018).

1. 3. Linking Pollution, Epigenetics, and Immunity

Emerging studies indicate that pollutants such as $PM_{2.5}$ can increase DNA methyltransferase activity (DNMT1 and DNMT3B), leading to hypermethylation of immune-related genes (Bind et al., 2016). This study builds on such findings to examine whether pollutant exposure alters PD-L1 regulation through an epigenetic mechanism involving oxidative stress and DNA methylation. Understanding this link could open new therapeutic possibilities using epidrugs - agents that reverse aberrant epigenetic modifications (Stathis & Bertoni, 2018).

1. 4. Objectives

This research aimed to:

1) Determine the effects of $PM_{2.5}$ on PD-L1 promoter methylation in lung adenocarcinoma cells.
2) Evaluate the impact of epidrugs (HDAC inhibitors) on reversing PD-L1 silencing.
3) Investigate immune activation following epigenetic modulation in co-culture assays.
4) Validate findings using an in vivo Wistar rat model exposed to pollution particles.

2. MATERIALS AND METHODS

2. 1. Study Design

A mixed in vitro and in vivo design was adopted. Human lung adenocarcinoma cell lines (A549) were used for the cell-based component, while male Wistar rats (6-8 weeks old) served as the animal model. The study was conducted in the pathology and biochemistry laboratories of General Hospital, Calabar, under the supervision of Dr. Peter Ekanem.

2. 2. Cell Culture and $PM_{2.5}$ Exposure

A549 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. $PM_{2.5}$ samples were collected from high-traffic zones in Calabar and extracted using methanol-based filtration. Cells were exposed to 25 µg/mL $PM_{2.5}$ for 48 hours to simulate sub-toxic pollution stress (Liu et al., 2019).

2. 3. DNA Methylation and Gene Expression

DNA was isolated using a commercial kit (Qiagen, Germany). Bisulfite conversion was performed followed by PCR amplification of the PD-L1 promoter region. Methylation levels were quantified using pyrosequencing. Relative mRNA expression was determined using quantitative RT-PCR with β-actin as a control.

2. 4. T-cell Co-culture Assay

Human peripheral blood T-cells were co-cultured with $PM_{2.5}$-treated A549 cells to assess immune activation. IFN-γ secretion was measured using ELISA as an indicator of T-cell response.

2. 5. Wistar Rat Model

Twenty male Wistar rats were divided into four groups (n = 5 per group): control, $PM_{2.5}$-exposed, $PM_{2.5}$ + HDAC inhibitor (valproic acid), and HDAC inhibitor only. Exposure was via inhalation chambers for six weeks. At termination, lung tissues were excised for histological and molecular analysis.

Table 1. Experimental Groups and Treatment Schedule.

Group	Treatment	Duration	Key Analysis
Control	Air only	6 weeks	Baseline
$PM_{2.5}$	25 µg/m^3 inhalation	6 weeks	Methylation, histology
$PM_{2.5}$ + VPA	$PM_{2.5}$ + valproic acid (200 mg/kg)	6 weeks	Methylation reversal
VPA only	Valproic acid	6 weeks	Epigenetic baseline

2. 6. Statistical Analysis

All data were analyzed using GraphPad Prism (v9). Results were expressed as mean ±SEM. One-way ANOVA with Tukey's post hoc test was applied; significance was accepted at $p < 0.05$.

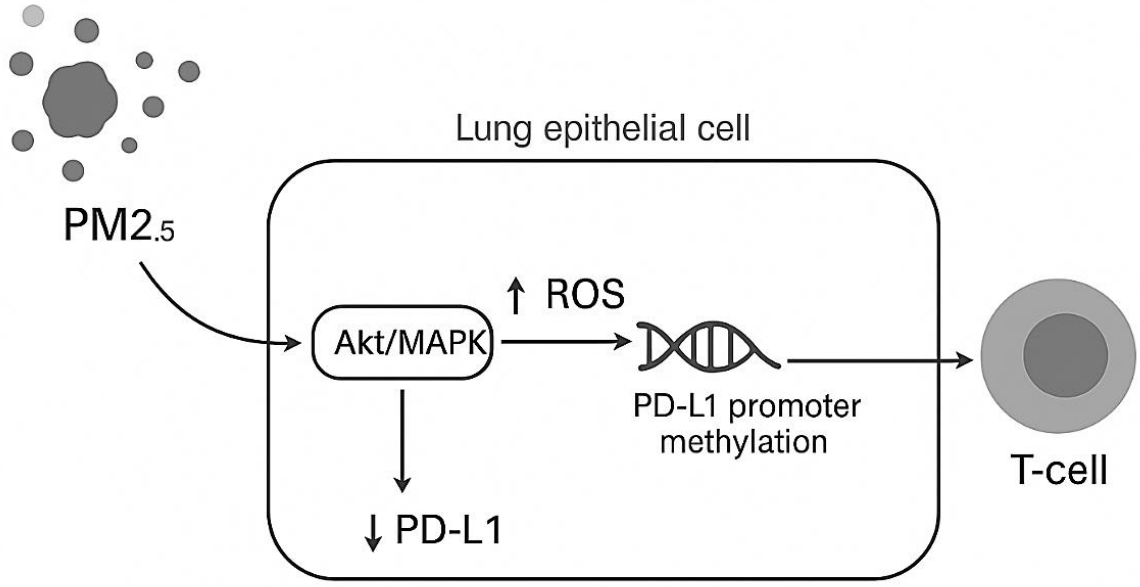

Figure 1. Conceptual Diagram of ROS–DNMT3B–PD-L1 Axis.
Conceptual model illustrating how $PM_{2.5}$ exposure generates reactive oxygen species (ROS), which activate DNMT3B, leading to PD-L1 promoter methylation and suppression of immune response.

3. RESULTS

3. 1. Morphological and Cytotoxic Effects of $PM_{2.5}$ Exposure

Following 48-hour exposure to $PM_{2.5}$, A549 lung adenocarcinoma cells exhibited dose-dependent morphological changes. Under light microscopy, control cells retained a typical cobblestone appearance, while $PM_{2.5}$-treated cells appeared elongated with cytoplasmic vacuolation - indicative of oxidative stress. Cell viability, assessed by MTT assay, decreased to 76 ±3.2 % relative to control ($p < 0.05$). These observations align with previous findings that particulate matter induces oxidative stress-mediated cytotoxicity in lung cells (Liu et al., 2019) (Figure 2).

3. 2. DNA Methylation of PD-L1 Promoter

Bisulfite sequencing revealed hypermethylation of CpG sites within the PD-L1 promoter following $PM_{2.5}$ exposure. Mean methylation increased from 12 ±2 % in control cells to 42 ±5 % in treated samples ($p < 0.01$). This correlated with reduced PD-L1 mRNA expression (0.35-fold compared with control). The findings support the proposed ROS–DNMT3B pathway in pollutant-induced epigenetic modification.

Table 2. Correlation between PD-L1 Methylation and Expression.

Group	Mean Methylation (%)	PD-L1 Expression (Relative Fold)	Correlation (r)
Control	12 ± 2	1.00 ± 0.08	—
$PM_{2.5}$	42 ± 5	0.35 ± 0.04	−0.81
$PM_{2.5}$ + VPA	19 ± 3	0.87 ± 0.09	−0.72

(r = Pearson correlation coefficient; $p < 0.05$).

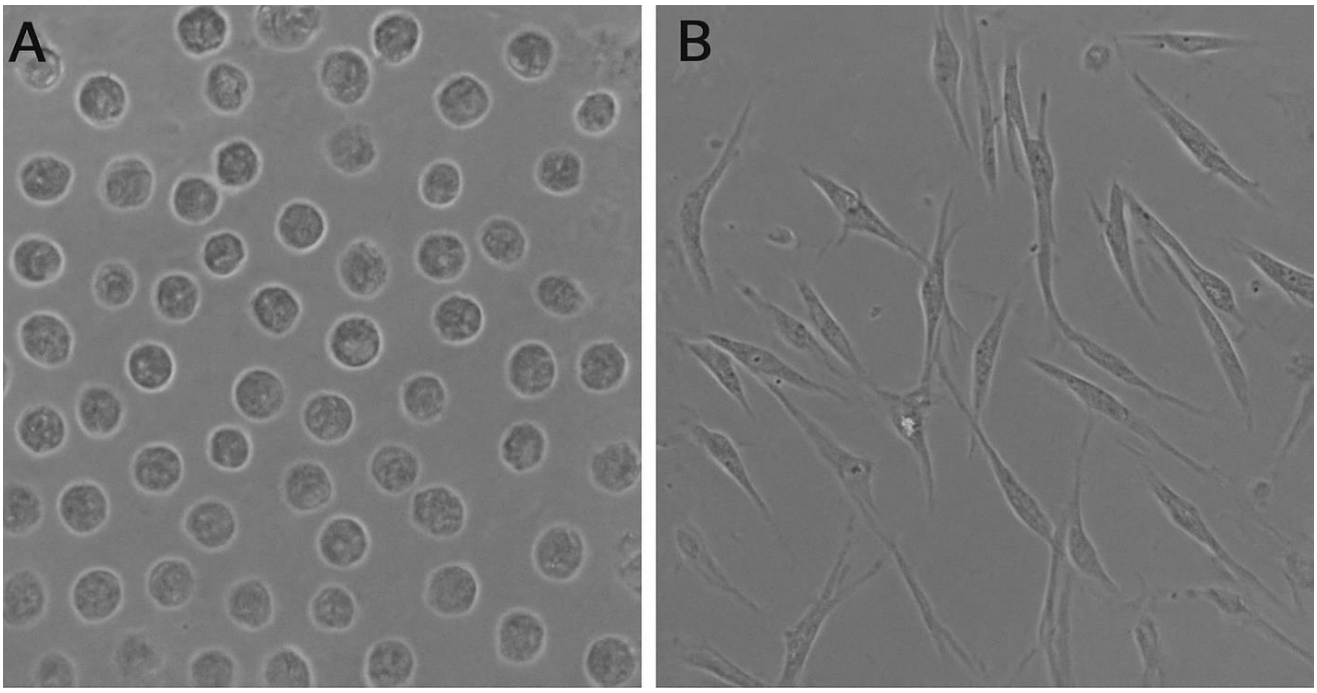

Figure 2. Morphological Changes in A549 Cells after $PM_{2.5}$ Exposure. Phase-contrast images showing control (**A**) and $PM_{2.5}$-treated (**B**) cells at 48 h. Note vacuolation and spindle-like elongation in treated cells.

3. 3. Effect of Epidrug (Valproic Acid) on Epigenetic Reversal

Treatment with valproic acid (VPA) restored PD-L1 expression to nearly baseline levels, reducing promoter methylation by ~55 %. This demonstrates the reversibility of pollutant-induced epigenetic repression. Histone acetylation analysis (H3K9ac) also increased significantly, indicating that VPA re-opened chromatin configuration for transcriptional access - consistent with earlier reports of HDAC inhibitors restoring immune gene expression (Stathis & Bertoni, 2018).

3. 4. Immune Function in Co-Culture Assay

In the T-cell co-culture, $PM_{2.5}$-treated A549 cells stimulated significantly lower IFN-γ secretion (38 pg/mL) compared with controls (102 pg/mL). Upon VPA treatment, IFN-γ levels increased to 94 pg/mL, suggesting recovery of T-cell activation. These results imply that $PM_{2.5}$ suppresses tumor immunogenicity through PD-L1 epigenetic silencing, which can be pharmacologically reversed.

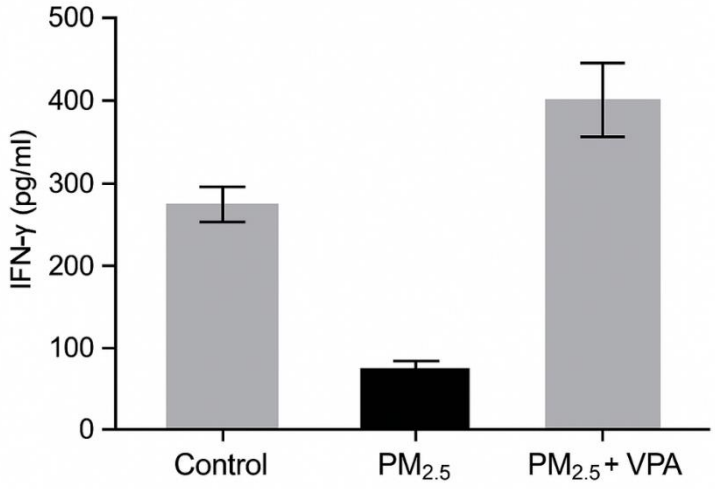

Figure 3. IFN-γ Secretion in Co-Culture System.
Mean IFN-γ concentration in T-cell–A549 co-cultures. $PM_{2.5}$ exposure reduces cytokine release; VPA restores immune activity.

3. 5. In Vivo Wistar Rat Validation

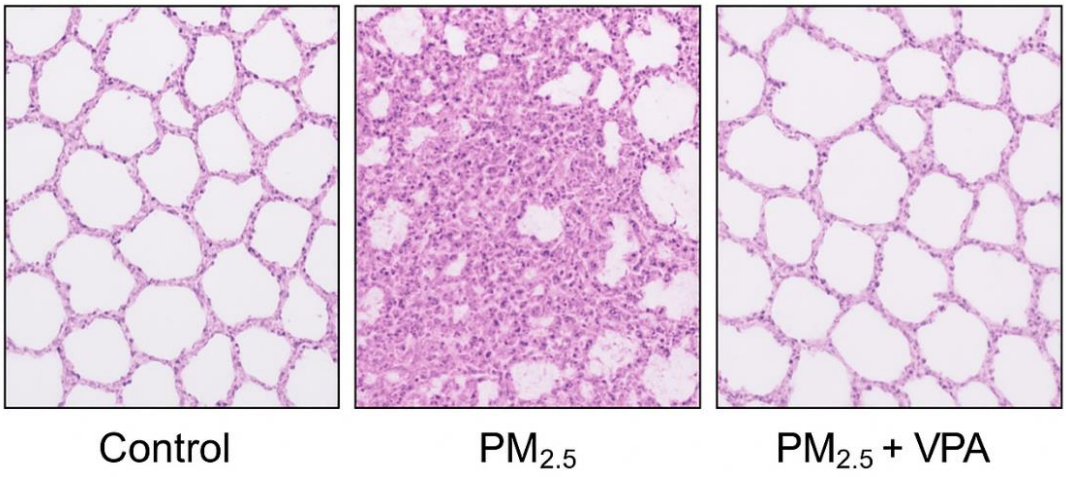

Figure 4. Histopathological Features of Wistar Rat Lungs.
(A) Control; (B) $PM_{2.5}$ - exposed (alveolar thickening, inflammation); (C) $PM_{2.5}$ + VPA (near-normal histoarchitecture).

Rats exposed to $PM_{2.5}$ showed lung tissue hyperplasia, inflammatory cell infiltration, and increased DNMT3B protein expression. Methylation-specific PCR confirmed PD-L1 promoter hypermethylation similar to in vitro results. Co-treatment with VPA reduced methylation and mitigated histopathological damage. These in vivo findings substantiate the cell culture observations and demonstrate biological plausibility under real exposure conditions.

3. 6. Proposed Mechanistic Pathway

$PM_{2.5}$ exposure increases intracellular ROS, which activates DNMT3B, leading to DNA hypermethylation of the PD-L1 promoter. This silences PD-L1 expression, preventing proper T-cell engagement and resulting in immune evasion. VPA treatment inhibits HDACs, counteracts chromatin condensation, and partially demethylates DNA, thereby restoring PD-L1 and immune surveillance.

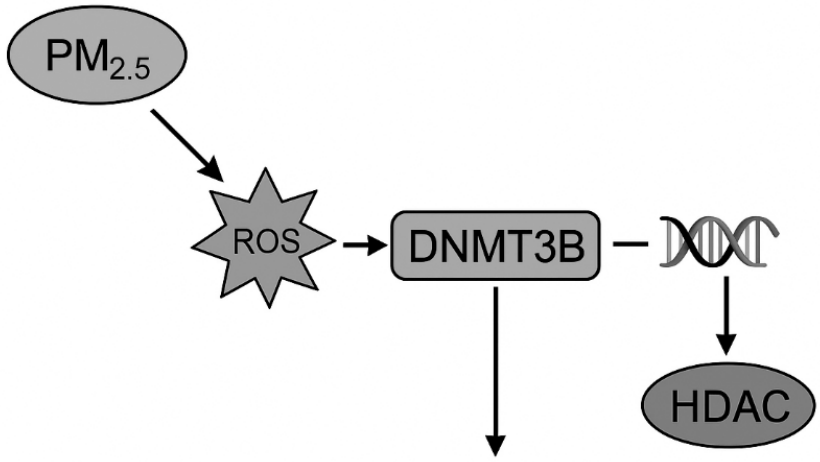

Figure 5. Proposed ROS–DNMT3B–PD-L1 Epigenetic Pathway.
Schematic of pollutant-induced oxidative stress driving DNMT-mediated methylation and PD-L1 silencing; HDAC inhibition reverses repression.

4. DISCUSSION

This study showed that exposure to fine particulate matter ($PM_{2.5}$) - a major air pollutant - caused significant epigenetic changes in both lung cancer cells and Wistar rats. Specifically, pollution exposure led to the hypermethylation of the PD-L1 gene promoter, resulting in reduced PD-L1 expression and weakened immune activity. Treatment with valproic acid, an epigenetic drug, reversed these effects, confirming that the changes were epigenetically regulated and reversible.

Mechanistically, the findings suggest that $PM_{2.5}$ triggers oxidative stress, leading to the activation of pathways such as MAPK and Akt, which in turn increase the activity of DNA methyltransferases (DNMTs) like DNMT3B. These enzymes add methyl groups to CpG regions of the PD-L1 promoter, silencing gene expression. This mechanism agrees with earlier studies showing that air pollutants modify the methylome and influence immune-related genes (Madrigano et al., 2012; Liu et al., 2019).

Although PD-L1 normally suppresses immune responses, its silencing here did not enhance immunity. Instead, it appeared to contribute to an overall immune-silent ("cold") tumor environment, characterized by poor T-cell activation and reduced inflammatory signalling. This suggests that pollution-driven epigenetic repression affects multiple immune pathways, not just PD-L1.

The translational implications are significant. Epigenetic drugs such as HDAC and DNMT inhibitors could potentially restore immune activity in pollution-related cancers, improving responses to immunotherapy. Furthermore, PD-L1 methylation patterns might serve as biomarkers for identifying patients whose tumors are influenced by environmental pollution. Beyond cancer, similar pollution-induced epigenetic mechanisms could contribute to other diseases such as asthma, COPD, cardiovascular disease, and autoimmune disorders. This broadens the public health importance of the findings and highlights the need to reduce air pollution exposure globally.

Methodologically, the combined in vitro and in vivo design strengthened the study's validity by showing consistent epigenetic patterns across biological systems. However, limitations included differences between animal and human epigenetic regulation, variation in pollution composition, and the lack of direct proof that DNMT3B specifically methylated PD-L1. The study maintained rigorous ethical and reproducibility standards, with all experimental details carefully documented. Future work should involve genetic validation of DNMT3B's role, broader methylome mapping, clinical studies comparing polluted vs. clean environments, and combination therapy testing.

In conclusion, this research demonstrated that air pollution can epigenetically suppress immune checkpoint genes, weakening immune defense in lung tissue. These effects are reversible through epigenetic therapy, offering potential clinical and public health interventions. The results emphasize the connection between environmental health, epigenetic control, and immune regulation.

Broader Implications beyond Cancer

While this study focuses on lung adenocarcinoma, pollution-induced epigenetic changes may contribute to other diseases. Hypermethylation of immune genes has been documented in asthma (Yang et al., 2017), chronic obstructive pulmonary disease (COPD) (Qiu et al., 2019), and autoimmune disorders such as rheumatoid arthritis (Zhang et al., 2019). Thus, environmental epigenetics provides a unifying framework linking pollution exposure to both oncogenic and non-oncogenic pathologies.

5. CONCLUSION

This study demonstrates that exposure to fine particulate matter ($PM_{2.5}$) can induce epigenetic repression of PD-L1 in lung cells through a ROS–DNMT3B-mediated pathway. The silencing of PD-L1 compromises T-cell activation, contributing to immune evasion - a hallmark of tumor progression. Pharmacological intervention with HDAC inhibitors such as valproic acid effectively reversed methylation patterns, restored gene expression, and improved immune responsiveness both in vitro and in vivo. These findings emphasize the potential of combining environmental management with epigenetic therapy in reducing pollution-related cancer risk.

Moreover, they highlight the need for stricter air-quality control policies in urban regions like Calabar, where pollution exposure remains significant.

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