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Presentación

Uno de los contaminantes atmosféricos más relevantes por sus efectos adversos para la salud, es el material particulado (PM) con un diámetro menor a 10 micras, ya que puede penetrar profundamente en los pulmones e inducir respuestas inflamatorias con la sobreactivación de neutrófilos, principal población celular que migra a los sitios de inflamación del pulmón, llevando al desarrollo de diversas enfermedades del sistema respiratorio. La mayoría de estos contaminantes son el producto de la quema de combustibles fósiles, pero su composición puede variar según sus fuentes.

En el desarrollo de esta tesis se muestran los resultados de la evaluación de los efectos del material particulado PM_{10} de la ciudad de Medellín en la respuesta inflamatoria mediada por neutrófilos.

En el Capítulo 1, se presentan los resultados de un trabajo publicado en la revista Heliyon, en el cual se revisó sistemáticamente las investigaciones disponibles que han evaluado el efecto de los contaminantes atmosféricos en la respuesta mediada por neutrófilos en modelos *in vivo* e *in vitro*, en el período 1980-2020. Una de las principales conclusiones sugirió que los contaminantes ambientales inducen el reclutamiento de neutrófilos como primer signo de inflamación, y que posteriormente estas células liberan citoquinas proinflamatorias y radicales libres de oxígeno (ROS) que dependen del tipo de contaminante.

En el Capítulo 2, se presentan los resultados de un trabajo publicado en la revista Scientific Reports, en el cual se muestra la evaluación experimental *in vitro* del efecto del PM_{10} sobre neutrófilos obtenidos de individuos humanos sanos. Las células fueron estimuladas con diferentes concentraciones de PM_{10}, se evaluó la citotoxicidad y viabilidad celular, y se cuantificó los siguientes mediadores inflamatorios, i) radicales libres de oxígeno mediante

citometría de flujo ii) producción de interleucina 8 (IL-8) por RT-PCR en tiempo real y ELISA iii) la expresión de peptidil arginina deiminasa 4 (PAD_4), mieloperoxidasa (MPO) y elastasa de neutrófilos (NE) por RT-PCR en tiempo real, y iv) la inducción de netosis por microscopía de fluorescencia.

Adicionalmente, se presentan los resultados del análisis experimental del efecto de la exposición de PM_{10} en un modelo murino. En este caso, ratones BALB/c fueron expuestos a PM_{10} y posteriormente en el lavado broncoalevolar (BALF) se realizó recuentos diferenciales para neutrófilos y en los tejidos pulmonares, análisis histopatológico y expresión de la quimiocina (CXCL1) con el fin de evaluar la infiltración de neutrófilos.

Finalmente se presentan las conclusiones generales y con base en los resultados experimentales obtenidos, más los resultados obtenidos en la revisión sistemática de literatura, proponemos que los neutrófilos se infiltran rápidamente en el pulmón e inician respuestas inflamatorias mediante varios mecanismos que incluyen, producción de ROS, producción de IL-8, liberación de trampas extracelulares de neutrófilos (NETs) y la liberación de múltiples enzimas proteolíticas, como MPO y NE.

Resumen

La contaminación del aire representa una gran amenaza para la salud humana. Según la organización mundial de la salud (OMS), la contaminación del aire, causa alrededor de unas 4.2 millones de muertes prematuras por año; esta mortalidad está relacionada con los efectos adversos en la salud respiratoria y cardiovascular causados por la exposición al PM. La exposición a este contaminate ambiental puede inducir daño pulmonar por estrés oxidativo e inflamación de las vías respiratorias, pérdida de las funciones inmunes frente a microorganismos, trombosis, coagulación y disfunción vascular que puede conducir al desarrollo de diferentes enfermedades. Investigaciones previas han demostrado que el PM estimula la liberación de citoquinas y quimiocinas que atraen neutrófilos a las vías respiratorias. Estas células responden rápidamente a las señales inflamatorias que provienen de áreas dañadas, migran y mediante un arsenal de defensa a su disposición, que incluyen fagocitosis, degranulación, ROS y NETs pueden degradar los agentes invasores. Sin embargo, el papel de estas células en la respuesta inflamatoria inducida por el PM_{10} aún no está completamente establecido.

Para este trabajo, la heterogeneidad en la evidencia sobre la respuesta inmune mediada por neutrófilos a la exposición de contaminantes, nos llevo en primer lugar a analizar sistemáticamente los estudios preclínicos que han evaluado la respuesta inmune mediada por neutrófilos a contaminantes atmosféricos en el período 1980-2020, siguiendo las recomendaciones de la Colaboración Cochrane y la guía PRISMA, a través de 54 estrategias de búsqueda en nueve bases de datos.

En total para la revisión sistemática, se incluyeron 234 estudios (*in vitro* e *in vivo*), y el análisis determinó el uso más frecuente de cultivos primarios, ratones BALB/C y C57BL6/J, y ratas Sprague-Dawley y Wistar. Además, las lecturas más frecuentes fueron recuentos celulares, citocinas e histopatología y el análisis temporal mostró que en la última década

ha predominado el uso de ratones con medición histopatológica y de citoquinas. Igualmente, los estudios incluidos sugieren que los contaminantes ambientales inducen el reclutamiento de neutrófilos a los sitios de inflamación, y que estas células liberan citoquinas proinflamatorias y ROS que dañan el tejido pulmonar y contribuyen a la respuesta inflamatoria sistémica.

También se determinó *in vitro* el efecto del PM_{10} presente en el aire de Medellín sobre la respuesta inflamatoria mediada por neutrófilos. Las muestras de PM_{10} fueron colectadas en las unidades de monitoreo de la ciudad de Medellín (SIATA, Área Metropolitana) y para los ensayos *in vitro*, los neutrófilos aislados de voluntarios humanos sanos, se expusieron a diferentes concentraciones de PM_{10}. A continuación, se evalúo la viabilidad celular mediante el ensayo de metil tetrazolio (MTT) y la liberación de lactato deshidrogenasa (LDH), se cuantificó la producción ROS por citometría de flujo, la expresión de IL-8 mediante RT-PCR y ELISA, la expresión de PAD_4, MPO y NE por RT-PCR y se determinaron las NETs mediante microscopia de fluorescencia. Para los ensayos *in vivo* ratones BALB/c fueron expuestos a de PM_{10} durante cinco días. Luego, se tomaron muestras de lavado broncoalveolar para recuentos diferenciales y muestras de pulmón para análisis histopatológico y expresión de CXCL1 por RT-PCR.

Mediante el análisis experimental, se determinó que la exposición de neutrófilos humanos al PM_{10} tuvo un efecto citotóxico que se evidencia con la liberación de LDH. Además, se observó que el PM_{10} induce la producción de ROS, la expresión y producción de IL-8, la expresión de PAD_4, MPO y NE y la liberación de NETs. A demás, observamos una infiltración moderada de neutrófilos en BALF y cambios histopatológicos en los tejidos pulmonares con infiltración de células inflamatorias y aumento en la expresión de CXCL1 en los pulmones de ratones BALB/c expuestos a PM_{10}.

En conjunto nuestros resultados demuestran el papel principal de los neutrófilos en la respuesta inflamatoria mediada por la exposición a contaminantes ambientales como el PM_{10}. A través de mecanismos que incluyen su reclutamiento en el tejido pulmonar, aumento de ROS, producción de IL-8, MPO, NE y liberación de NETs pueden contribuir a la exacerbación de patologías respiratorias, como las alergias y las enfermedades infecciosas y obstructivas.

Hipotesis

Las partículas (PM_{10}) de la ciudad de Medellín inducen la activación *in vitro* de los neutrófilos humanos y promueven alteraciones histopatológicas en los pulmones de un modelo de ratón, favoreciendo la respuesta inflamatoria evidenciada por la infiltración de neutrófilos, la producción de ROS, liberación de NETs y la expresión de MPO, NE e IL-8.

Pregunta de investigación

¿Cuál es el efecto del material particulado PM_{10} del aire de Medellín en la respuesta inflamatoria mediada por neutrófilos?

Objetivos

Objetivo general

Determinar el efecto del PM_{10} presente en el aire de Medellín sobre la respuesta inflamatoria mediada por neutrófilos

Objetivos específicos

1. Evaluar el efecto citotóxico *in vitro* del PM_{10} sobre neutrófilos, mediante los métodos de MTT y liberación de LDH.

2. Evaluar el efecto pro-inflamatorio del PM_{10} en neutrófilos, cuantificando la producción de ROS, la expresión de citoquinas proinflamatorias y la liberación de NETs.

3. Determinar el efecto del PM_{10} en la inducción de infiltrado pulmonar de neutrófilos en un modelo animal.

Capítulo 1

Revisión sistemática:

Systematic review of preclinical studies on the neutrophil-mediated immune response to air pollutants, 1980–2020

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Review article

Systematic review of preclinical studies on the neutrophil-mediated immune response to air pollutants, 1980–2020

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ABSTRACT

Preclinical evidence about the neutrophil-mediated response in exposure to air pollutants is scattered and heterogeneous. This has prevented the consolidation of this research field around relevant models that could advance towards clinical research. The purpose of this study was to systematic review the studies of the neutrophils response to air pollutants, following the recommendations of the Cochrane Collaboration and the PRISMA guide, through 54 search strategies in nine databases. We include 234 studies (*in vitro*, and *in vivo*), being more frequent using primary neutrophils, Balb/C and C57BL6/J mice, and Sprague-Dawley and Wistar rats. The most frequent readouts were cell counts, cytokines and histopathology. The temporal analysis showed that in the last decade, the use of mice with histopathological and cytokine measurement have predominated. This systematic review has shown that study of the neutrophils response to air pollutants started 40 years ago, and composed of 100 different preclinical models, 10 pollutants, and 11 immunological outcomes. Mechanisms of neutrophils-mediated immunopathology include cellular activation, ROS production, and proinflammatory effects, leading to cell-death, oxidative stress, and inflammatory infiltrates in lungs. This research will allow consolidating the research efforts in this field, optimizing the study of causal processes, and facilitating the advance to clinical studies.

1. Introduction

Exposure to air pollutants is associated with various health problems, including asthma, increased incidence of respiratory infections, reduced lung function, and heart conditions [1, 2]. Among air pollutants, particulate matter (PM) shows a strong association with adverse respiratory (including infectious diseases [3]) and cardiovascular health effects.

PM is a mixture of particles that vary in quantity, surface area, chemical composition, origin, and size; based on the latter property, it is classified as PM_{10}, $PM_{2.5}$ (fine particles), and $PM_{0.1}$ or UFP (ultrafine particles) [4]. Exposure to these environmental pollutants can cause inflammation of the respiratory tract [5], lung damage due to oxidative stress, increased mucus secretion, and immune alterations [6]. Besides, air pollution exposure has been associated with congenital and newborn alterations [7], cardiovascular and neurological disorders, as a consequence of inflammation and oxidative stress [8, 9]. The main immunological defects that have been reported, are the humoral response [10] and its association with asthma, and the production of reactive oxygen species in the development of chronic obstructive pulmonary disease (COPD) [11]. However, assessments of the acute inflammatory response and the role of one of its main mediators, neutrophils, still require research to clarify their involvement and thus design appropriate (therapeutic) control strategies.

Recently, multiple studies have focused on the effects of PM on immune cells in the lung, including neutrophils, the main mediators of acute inflammatory reactions, and infiltrating tissue since the early hours of injury. Thanks to their high production of cytokines, lipid mediators of inflammation, oxidative radicals, and multiple hydrolytic enzymes, including metalloproteases, the neutrophils modulate the progression of lung pathologies and the exacerbation of pre-existing diseases. For instance, *in vivo* studies have shown that exposure to these contaminants promotes the infiltration of neutrophils into the lungs to eliminate PM. However, aberrant accumulation of neutrophils in the lung causes tissue damage, as seen in the triggering of asthma or COPD, related to air

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pollution [12, 13]. In this sense, studies have reported variations in cell counts, cytokine production, reactive oxygen species, immunoglobulins, and other metabolic, immunological, and even tissue damage biomarkers [14, 15, 16, 17, 18]. Also, there is a high diversity of preclinical models (laboratory based-experiments) [13, 19, 20, 21], which include multiple cell lines, primary cultures, and diverse animal models, evidencing the heterogeneity of measurements of the immune response, especially the neutrophil-mediated one.

This heterogeneity presents several implications as contradictory results when using different sources of air pollutants, evaluating pleiotropic factors such as IL-6 (with pro- and anti-inflammatory functions), or murine models with contrasting immune responses such as C57BL6/J and Balb/C mouse strains, which predominantly present Th1 and Th2 responses (antagonistic immune profiles), respectively [22].

The above shows that the preclinical evidence on the neutrophil-mediated immune response to exposure to airborne contaminants is scattered and heterogeneous, which prevents the consolidation of this research field since there are no recommendations on the cellular or animal designs, the type of exposure, or the most relevant outcomes to advance to subsequent phases of clinical research [23, 24].

Therefore, the objective of this research was to analyze the methodological characteristics of the preclinical studies that have evaluated the neutrophil-mediated immune response to air pollutants in the period 1980–2020. The specific objectives included: i) to describe the studies according to the year and place of origin; ii) to identify the different *in vitro* and *in vivo* models that have been used; iii) to describe the main pollutants and immunological outcomes used in the studies; iv) to analyze the temporal changes in the design of the selected preclinical studies; and v) to identify the immunological effects reported in the most frequently used preclinical models in this field.

This work systematically reviewed the available research that has evaluated the effect of air pollutants in the neutrophil-mediated response in *in vivo* and *in vitro* models, with a broad approach according to the Cochrane Collaboration [25], where an empirical approach of synthesis of scientific evidence in a field is implemented. Besides, the current recommendations can be updated, and allow to analyze the potential for generalization of published studies, to define hypotheses with substantial or insufficient evidence; to identify areas for further research, to locate countries in need of further research, among other relevant uses for those interested in environmental health issues, immunology, preclinical research methodologies, among others. Besides, defining the role of neutrophils in the pulmonary inflammatory response to airborne contaminants can help understand their implications in respiratory and cardiovascular disease and provides information to support decision making regarding unified methodologies for immunological assessments with airborne contaminants.

2. Materials and methods

2.1. Type of study

A systematic review of preclinical studies, following the recommendations of the Cochrane Collaboration and the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guide.

2.2. PICO (Population intervention comparator outcome) question - modified

2.2.1. Experimental unit (population)

The experimental unit depended of the type of preclinical study carried out. For *in vitro* studies, it corresponded to the type of cell line or primary culture, and in the *in vivo* studies, it was the type of animal (rats, mice, hamsters, or rabbits) model.

2.2.2. Pollutant (intervention)

The atmospheric pollutants evaluated were particulate matter, Ozone, sulfur dioxide, aerosols, and others included in the preclinical studies, in different concentrations.

2.2.3. Control (comparator)

For *in vitro* studies, the control was the negative control, while, for *in vivo* studies, it was the initial or pre-exposure measurement.

2.2.4. Outcome

The outcomes were the type of neutrophil-mediated immune response, including cell count, cytokine measurement, cytokine mRNA, oxidative activity, immunoglobulins, histopathological changes.

2.3. Search and selection protocol according to the PRISMA guide

2.3.1. Identification

A search of scientific articles was performed in four multidisciplinary databases MedLine-PubMed, Science-Direct, Scielo, LILACS. Also open searches in Google Scholar (web search engine), and four field-specific databases: Infection and Immunity (American Society for Microbiology), EPA (Environmental Protection Agency, United States), OECD i Library and HEI (Health Effects Institute), were included. A query of terms was carried out in the thesauri MeSH (Medical Subject Headings) and DeCS (Health Sciences Descriptors), and it was complemented with a process of "pearl harvest". Two terms were identified for the topic of environmental pollution: "particulate matter" and " air pollution", which were combined with the terms found for the neutrophil-mediated immune response, i.e., "neutrophils", "granulocytes" and "white blood cells". According to the above, three search strategies were applied in each database (Table 1).

2.3.2. Screening

In this phase, three inclusion criteria were applied. The first criterion was that the articles had the search terms in title or abstract; the second consisted of original investigations, with which reviews, book chapters, and abstracts were eliminated; the third inclusion criterion was that the study was classified as preclinical, thus eliminating the clinical-epidemiological studies carried out in people from different populations and the mathematical modeling studies. In this phase, the identified studies were saved in a common source in Zotero to eliminate duplicates.

2.3.3. Eligibility

At this stage, six exclusion criteria were applied, *i)* publications in other languages such as Russian or Chinese, *ii)* studies in animals, but that did not classify as preclinical, for example, studies that had dogs, fish, or horses as units of analysis but that were not *in vivo* models (animal health studies), *iii)* studies about plant species, *iv)* studies not related to air pollutant or studies that did not specify the type air pollutant, *v)* studies that did not specify the immunological outcome and *vi)* studies not available in full text or eliminated from the database.

2.3.4. Inclusion

The articles that fulfilled the two previous stages were included in this review. The variables title, authors, journal, year of publication, country, type of preclinical model (this was grouped into *in vitro* and *in vivo* models), type of *in vitro* model, type of *in vivo* model, pollutant studied, and type of neutrophil-mediated immune response (outcome) were extracted.

2.4. Evaluation of the reproducibility

The reproducibility of the search and selection protocol was validated through a review performed by two researchers independently. It was determined *a priori* that disagreements would be resolved by referral to a

Table 1. Search syntax applied in each database.

	Search 1	Search 2	Search 3
PubMed	(particulate matter [Title/Abstract] OR air pollution [Title/Abstract]) AND (neutrophils [Title/Abstract])	(particulate matter [Title/Abstract] OR air pollution [Title/Abstract]) AND (granulocyte [Title/Abstract])	(particulate matter [Title/Abstract] OR air pollution [Title/Abstract]) AND (white blood cell [Title/Abstract])
Science-Direct	Title, abstract, keywords: (particulate matter OR air pollution) AND neutrophils	Title, abstract, keywords: (particulate matter OR air pollution) AND granulocyte	Title, abstract, keywords: (particulate matter OR air pollution) AND white blood cell
Scielo	(ab:(particulate matter OR air pollution)) AND (ab:(neutrophils))	(ab:(particulate matter OR air pollution)) AND (ab:(granulocyte))	(ab:(particulate matter OR air pollution)) AND (ab:(white blood cell))
LILACS	(tw:(particulate matter OR air pollution)) AND (tw:(neutrophils))	(tw:(particulate matter OR air pollution)) AND (tw:(granulocyte))	(tw:(particulate matter OR air pollution)) AND (tw:(white blood cell))
Google Scholar	allintitle: ((particulate matter OR air pollution) AND neutrophils)	allintitle: ((particulate matter OR air pollution) AND granulocyte)	allintitle: ((particulate matter OR air pollution) AND white blood cell)
Infection and Inmunity	For abstract or title " (particulate matter OR air pollution) AND neutrophils" (match all words)	For abstract or title " (particulate matter OR air pollution) AND granulocyte" (match all words)	For abstract or title " (particulate matter OR air pollution) AND white blood cell" (match all words)
EPA	DC.title:(particulate AND DC.title:matter AND DC.title:OR AND DC.title:air AND DC.title:pollution) AND DC.title:AND ANDDC.title:neutrophils	DC.title:(particulate AND DC.title:matter AND DC.title:OR AND DC.title:air AND DC.title:pollution) AND DC.title:AND ANDDC.title: granulocyte	DC.title:(particulate AND DC.title:matter AND DC.title:OR AND DC.title:air AND DC.title:pollution) AND DC.title:AND ANDDC.title: white blood cell
OECD i Library	From (Abstract contains '(particulate matter OR air pollution)') AND from (Abstract contains 'neutrophils') AND from (IGO collection contains 'OECD')	From (Abstract contains '(particulate matter OR air pollution)') AND from (Abstract contains 'granulocyte') AND from (IGO collection contains 'OECD')	From (Abstract contains '(particulate matter OR air pollution)') AND from (Abstract contains 'white blood cell') AND from (IGO collection contains 'OECD')
HEI	(particulate matter OR air pollution) AND neutrophils	(particulate matter OR air pollution) AND granulocyte	(particulate matter OR air pollution) AND white blood cell

Note 1: The searches were carried out in Spanish without finding additional results. A total of 54 search strategies were applied (3 in English +3 in Spanish x 9 databases). Note 2: Combining the terms particulate matter OR air pollution in a unique search yielded the same studies as separating them into two searches (one for each term). This was not the case for the terms "neutrophils", "granulocytes" and "white blood cells", for which separate searches (one for each term) returned more results than a single search (with all three terms linked with "OR").

third researcher. Disagreements were dealt with in two phases: referral to a third party, then consensus among the three (the two initial parties and the third party consulted in case of disagreement). The information was extracted, filling a database in SPSS 25.0 with the study variables, independently, by two reviewers.

2.5. Analysis of the information

The included studies were analyzed by a qualitative synthesis of the extracted variables were described with absolute (n) and relative (%) frequencies. A bivariate analysis was performed using Pearson's Chi-square to compare the preclinical study type by time period. The database and the analyzes were processed in SPSS 25.0, with 95 % confidence interval.

3. Results

With the application of the search strategies in the 9 databases, 129,013 results were found. After applying the title or abstract filter and eliminating duplicates, 471 abstracts were read to apply the other selection criteria; at the end, 234 studies met the search and selection protocol (Figure 1).

The higher frequency of studies came from the United States (42.3%), China (14.3%), Canada (7.3%), Brazil (4.7%), United Kingdom (4.7%), and Japan (4.7%), with a lower frequency of studies in Latin America, Asia, Oceania and without any studies from Africa (Supplementary Figure 1).

In the systematized studies, 17.5% (n = 41) used *in vitro* models, 65.8% (n = 154) *in vivo* and 16.7% (n = 39) both. Of the 80 studies that implemented *in vitro* model, 42.5% (n = 34) corresponded to primary culture, 36.3% (n = 29) cell lines and in 21.3% (n = 17) both were applied. In turn, in the 193 studies that implemented *in vivo* models, 59.6% (n = 115) were mice, 32.6% (n = 63) rats, 1.6% (n = 3) both rats and mice, and in less than 3% other models were used such as rabbits, hamsters and guinea pigs.

In the *in vitro* studies, 20 cell lines were identified, being more frequent the use of respiratory tract epithelial cells, representing 52.2% of the total number of studies with cell lines and 30% of the total number of *in vitro* studies. Likewise, 25 different primary cultures were identified, with a highest proportion of respiratory tract epithelial cells, representing 37.3% of total culture and 23.8% of the total *in vitro* studies, followed by neutrophil culture representing 19.6% and 12.5%, respectively (Table 2).

In the *in vivo* models, 34 strains of mice were identified, of which the Balb/C and C57BL6/J subtypes presented the highest proportion with 48.3% and 33.9% of the total studies in mice, respectively. Likewise, studies in rats included 11 different subtypes, more frequent Sprague-Dawley with 50%, Wistar with 25.8%, and Fischer-344 with 13.6% 11 of rats (Table 3).

Concerning the type of contaminant, 91% studied PM, with 45.7% that included the evaluation of the three types (PM_{10}; $PM_{2.5}$, and $PM_{0.1}$), and the primary immunological outcomes analyzed were cell count in 85%, measurement of cytokines (mainly proteins) in 71.4% and histopathological effects in 53.4% (Table 4).

In the most frequently used *in vitro* model (cell line or respiratory tract epithelium culture), a similar proportion of publications was found between 1980-2010 and 2011–2020; somewhat different from what was found for the most used rat models (Sprague-Dawley and Wistar), which were more used until 2010 (around 60%), while studies in mice have increased their frequency in the last decade since the period 2011–2020 was recorded 83% of studies with C57BL6/J and 66% with Balb/C; That is, the temporal analysis shows that in the last decade the study of this

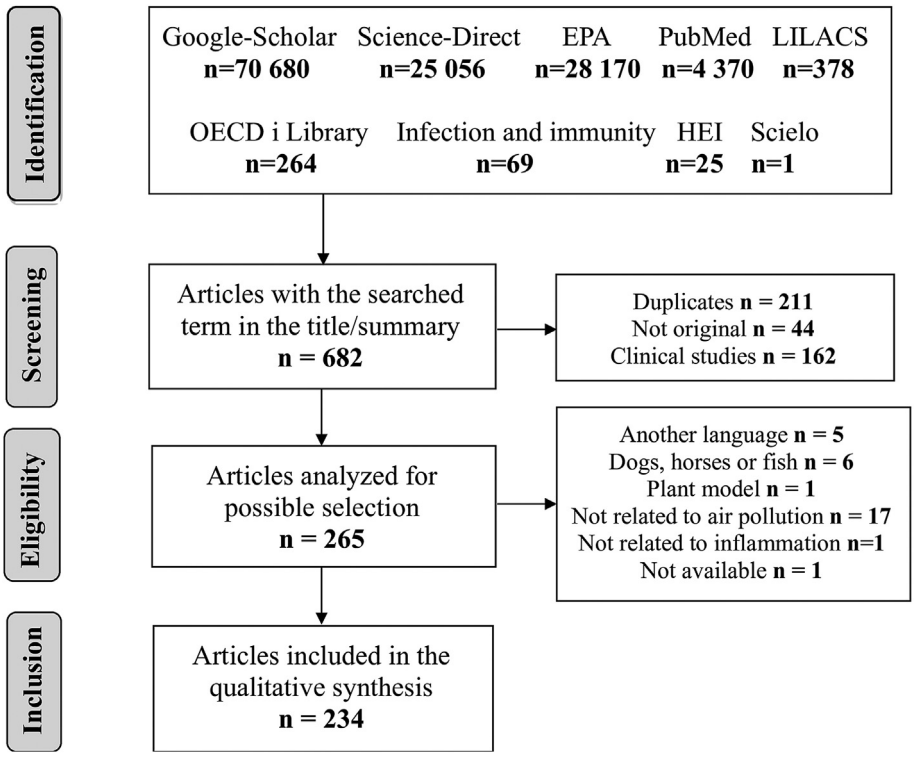

Figure 1. Study search and selection flowchart.

Table 2. Percentage distribution of in vitro studies (N = 80).

Subtype	Model	n	% between the subtype	Total % *in vitro*
Cell lines[a]	Respiratory tract epithelial cells	24	52.2	30
	RAW 264.7	5	10.9	6.3
	Human aortic endothelial cells (HAEC)	2	4.3	2.5
	Murine lung epithelial cells (MLE-12)	2	4.3	2.5
	Human monocytic cells (MM6)	2	4.3	2.5
	Subtotal cell lines	**46**	**100**	**57.5**
Primary culture[b]	Respiratory tract epithelial cells	19	37.3	23.8
	Neutrophils (unspecified)	10	19.6	12.5
	Rat/mouse/rabbit macrophages	8	15.7	10.0
	Human alveolar macrophages	8	15.7	10.0
	Dendritic cells	6	11.8	7.5
	PBMC	3	5.9	3.8
	Differentiated human monocytes in macrophages	2	3.9	2.5
	Subtotal primary culture	**51**	**100**	**63.8**

[a] **The following cell lines presented n = 1:** murine macrophages J774, THP-1, HaCaT, 3LL, HTB54, HL60, L929, 4T1, ATII, RLE-6TN, BW5147, R1, EL4, P815, and TG180.

[b] **The following primary culture presented n = 1:** human monocytes, mouse bone marrow monocytes, human neutrophils, rat neutrophils, primary tracheal epithelial cells of mouse, dendritic cells, endothelial cells, fibroblasts, rat fibroblasts Primary human keratinocytes, rat macrophages, rat alveolar macrophages, mouse bone marrow-derived macrophages, virgin CD4+ T-cells, human CD8+ T-cells, rat leukocytes, rat neonatal cardiomyocytes, and mast cells.

field with C57BL6/J and Balb/C mice has increased, with statistically significant differences ($p < 0.05$) in the three periods analyzed (Figure 2A). Likewise, it is observed that in the last decade, the proportion of studies on immunoglobulins, immunohistochemistry, histopathology, and cytokines has been statistically higher ($p < 0.05$), with a tendency to decrease the use of cell counts and other biomarkers (Figure 2B).

In general, the evidence available in the most frequently used preclinical models shows an alteration of the neutrophil-mediated immune response to PM, characterized by an increase in neutrophils in blood and lung tissue (accompanied by macrophages, monocytes, eosinophils, and lymphocytes), expression of biomarkers such as PAI-1, Saa3, PCR, LDH, alkaline phosphatase, GGT, NAG, ROS, chemotaxis and eicosanoids; as well as the reduction in the expression of iNOS and NADPH oxidase components, and decrease in transcriptional factors like as STAT1. There is divergent evidence in those referring to the increase or decrease of pro-inflammatory cytokines (mRNA and protein) and oxidative stress. Despite this profile's heterogeneity, in histopathological terms,

Table 3. Percentage distribution of *in vivo* studies (N = 193).

Subtype	Model	n	% between the subtype	Total % *in vivo*
Mice[a]	Balb/C	57	48.3	29.5
	C57BL6/J	40	33.9	20.7
	ICR	4	3.4	2.1
	TO	3	2.5	1.6
	Kunming	3	2.5	1.6
	CD-1	3	2.5	1.6
	C3HeB/FEJ	2	1.7	1.0
	DO11.10	2	1.7	1.0
	Subtotal mice	**118**	**100**	**61.1**
Rats[b]	Sprague-Dawley	33	50.0	17.1
	Wistar	17	25.8	8.8
	Fischer-344	9	13.6	4.7
	SH (Spontaneously hypertensive)	3	4.5	1.6
	SHR/NCrIBR	3	4.5	1.6
	WKY (Wistar Kyoto)	3	4.5	1.6
	Subtotal rats	**66**	**100**	**34.2**
Rabbits	New Zealand white rabbits	5	–	2.6
Hamster	Syrian Hamsters (*Mesocricetus auratus*)	4	–	2.1
Guinea pigs	Hartley Guinea pigs	3	–	1.6

[a] **The following mice strains presented n = 1:** Albins (*Mus musculus*), Ahr knockout, Deficient in apolipoprotein E (ApoE-/-), Deficient in MyD88 (MyD88−/−), Homozygotes with mutations in the TNFp55 (Tnfrsf1a) and p75 (Tnfrsf1b) receptor genes, Endogamic (A, AKR, C3, B6, CBA, DBA/2, FVB/N, and F1 specific crosses and backcrosses), Null mutants in EC-SOD, Transgenics (CD2-LacZ80/HazfBR), Transgenics that overexpress EC-SOD, AKR/J, B6C3F1, CB6F1, Hgu CftrTgH, IL-13−/− and IL-4-/IL-13−/−, IL-4−/− and MHC II−/−, IL-17−/−, IL17Ra−/− and IL23p19−/−, Jα18−/−, NC/Nga, NIH, Nlrp3 (−/−), Thy1. 1, TRPC6−/− and TRPC6+/-, Stat1 --, VAChT-KD and 4Get.

[b] **The following rats strains presented n = 1:** SHHF (Spontaneously Hypertensive Heart Failure), SH/NHsd, SPF HsdCpb: WU, SD, and Long-Evans.

preclinical evidence is consistent in the reporting of inflammatory infiltrates and lung tissue damage (Table 5).

4. Discussion

Environmental pollution is a major social, economic, and health problem worldwide. According to the World Health Organization (WHO), about 90% of people living in urban areas are exposed to air quality levels that exceed the accepted limits for $PM_{2.5}$ and PM_{10} (10 or 20 μg/m^3, respectively) [68]. This exposure has been associated with an increase in cardiovascular and respiratory adverse effects [69] and has contributed to decreased cognitive functions, depressive symptoms, and neurodegenerative pathologies [70]. Also, it has generated an economic impact for the countries, represented by an increase in mortality and morbidity, cost overruns for the care of related health events, loss of working hours, school and work absenteeism due to illness or the need to care for a sick person, a decrease in family income and a reduction in productivity [69, 71].

Against this background, in recent years, awareness of the impact of air pollution has increased, leading to changes in public policies. In addition, an increase in research focused on elucidating the mechanisms by which exposure to PM affects human health has been performed, focused on this field, in which preclinical studies play a determining role, given the limitations of human research to establish physiopathological and organic causality [72]. In this work, *in vitro* (primary cell culture and cell lines) and *in vivo* (rat, mouse, rabbit and hamster) models were analyzed to evaluated the effect of air pollutants on the neutrophil-mediated immune response. A total of 324 studies were systematically reviewed, in which were used 90 different preclinical models, with high heterogeneity of study sites, type of contaminant, and immune outcomes.

Most of the studies were carried out in the United States and China. In the case of the United States, the findings of this review demonstrate a greater interest in having substantial preclinical evidence, which in turn is related to previous studies that have reported the association between $PM_{2.5}$ exposure and death from cardiovascular and cerebrovascular disease, COPD, type 2 diabetes, lung cancer, pneumonia, chronic renal disease, hypertension, and dementia [73]. Although the average annual concentration of $PM_{2.5}$ has decreased by 42% between 2000 and 2015 in

Table 4. Percentage distribution of the types of contaminant and immunological outcome studied.

	n	%
Type of contaminant		
PM_{10}, $PM_{2.5}$, $PM_{0.1}$	107	45.7
$PM_{2.5}$	45	19.2
PM_{10}	35	15.0
$PM_{0.1}$	26	11.1
Ozone (O_3)	16	6.8
Sulfur dioxide (SO_2)	1	0.4
Secondary Organic Aerosols (SOA)	1	0.4
Zinc Sulfate ($ZnSO_4$) Aerosol	1	0.4
Concentrated environmental particles (CAP) and ozone (O_3)	1	0.4
Nicotine	1	0.4
Immunological outcome		
Differential counts[a]	164	85.0
Histopathology[a]	103	53.4
Immunohistochemistry[a]	35	18.1
Immunoglobulins[a]	22	11.4
Cytokines	167	71.4
Cytokine mRNA[b]	18	10.8
Cytokine proteins[b]	109	65.3
RNA and cytokine proteins[b]	40	24.0
Biomarkers	92	39.3
Oxidative Activity	67	28.6

[a] N = 193 since it only applies to *in vivo* studies.

[b] N = 167 since it only applies to studies that measured cytokines.

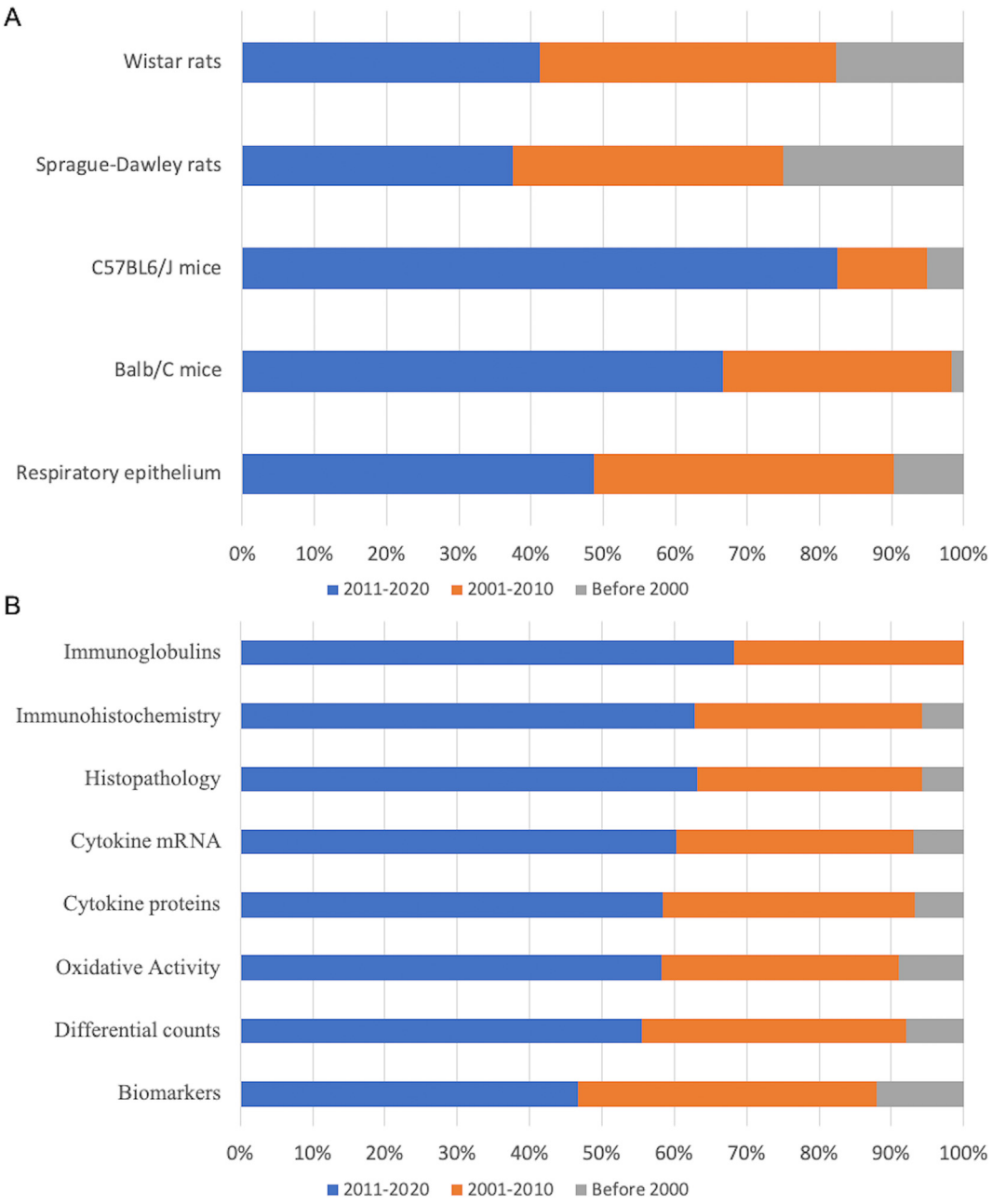

Figure 2. Percentage distribution of the leading preclinical models and immunological outcomes identified according to the publication period. A. Preclinical models, according to the study period. B. Immunological outcomes, according to the study period.

this country, as well as the mortality associated with $PM_{2.5}$ exposure [74], there is evidence that long-term exposure to fine PM contributes to the risk of mortality, especially from lung and cardiopulmonary diseases, including cancer [75].

In addition, China's air pollution problem has become the fourth largest health threat [76]. A study reported that exposure to polycyclic aromatic hydrocarbons (PAH) by inhalation resulted in a higher incidence of lung cancer [77], the most common cancer in the Chinese population [78]. PM_{10} exposure has resulted in about 1.5 million premature deaths in China [79]. Besides, during 2014–2015, the economic cost of health impact due to PM_{10} exposure represented 2.94% of China's gross domestic product (GDP) [79].

In this review, the effect of air pollutants on the neutrophil-mediated response was addressed. Most of the studies included used PM (including mixtures of PM_{10}, $PM_{2.5}$, and $PM_{0.1}$), the main air pollutant [80]. Although considering the availability of monitoring stations in the world, especially those that can collect $PM_{0.1}$, the experimental approaches must be focused on PM_{10} and $PM_{2.5}$, independently, to allow precise comparisons among the studies.

Regarding air pollutants, an increase in macrophages, neutrophils, eosinophils and lymphocytes was observed in the alveolar lavage fluid of mice exposed to PM [18, 26, 29, 30]. Furthermore, it has previously been reported that the inflammatory response may differ according to the type of PM [30, 80, 81, 82]. For instance, $PM_{0.1}$ induces grater eosinophilic

Table 5. Main immunological results in murine, rat, and neutrophil culture models exposed to air pollutants.

Murine models		
C57BL6/J mice	BALB/c mice	Type of pollutant
Increased neutrophils in blood and lung tissue, accompanied by other cell types such as macrophages, monocytes, eosinophils, and lymphocytes [26, 27, 28, 29]	Increased neutrophils in blood and lung tissue, accompanied by other cell types such as macrophages, monocytes, eosinophils, and lymphocytes [30, 31, 32, 33]	Particulate matter (PM_{10}, $PM_{2.5}$, $PM_{0.1}$), and DEP.
Increased pro-inflammatory cytokines (mRNA and protein)* [29, 34, 35]	Increased pro-inflammatory cytokines (mRNA and protein)* [5, 31, 36]	Cigarette smoke and Particulate matter (PM_{10}, $PM_{2.5}$).
Increased oxidative stress** [37, 38]	Increased oxidative stress** [39, 40]	ROFA, Diesel, DEP, and PM_{10}
Increased expression of plasminogen-1 activator inhibitor (PAI-1), Saa3, and CRP [5, 41]	Increased release of LDH (lactate dehydrogenase), alkaline phosphatase, and Saa3 [42] [5] [43].	Particulate matter ($PM_{2.5}$, PM_{10}), DEP, O_3 and tobacco smoke.
Inflammatory infiltration and lung tissue damage [34, 37]	Inflammatory infiltration and lung tissue damage [27, 44, 45]	$PM_{2.5}$, ROFA, and DEP.
Rat Models		
Sprague-Dawley Rats	**Wistar Rats**	
Increased neutrophils in blood and lung tissue, accompanied by other cell types such as macrophages, monocytes, eosinophils, and lymphocytes [21, 46, 47, 48]	Increased neutrophils in blood and lung tissue, accompanied by other cell types such as macrophages, monocytes, eosinophils, and lymphocytes [49, 50, 51, 52]	Particulate matter (PM_{10}, $PM_{2.5}$, $PM_{0.1}$) DEP, CFA, and O_3
Increase in pro-inflammatory cytokines (mRNA and protein) [46, 53, 54]	Increase in pro-inflammatory cytokines (mRNA and protein) [50, 55, 56]	DEP, CAP, ROFA, O_3, and PM_{10}.
Increased oxidative stress [57, 58, 59]	Increased oxidative stress [50, 51, 60]	Particulate matter (PM_{10}, $PM_{2.5}$, $PM_{0.1}$), DEP, and secondary organic aerosols (SOA).
Increased expression of CRP [61]. Increased release of LDH (lactate dehydrogenase) [47, 48, 53]	Increased release of LDH (lactate dehydrogenase), γ-glutamyl transferase (GGT), and n-acetyl glucosaminidase (NAG) [50, 51]	Particulate matter (PM_{10}, $PM_{2.5}$, $PM_{0.1}$), and CAP.
Inflammatory infiltration and lung tissue damage [21, 48, 54]	Inflammatory infiltration and lung tissue damage [56, 62, 63]	CFA, Particulate matter (PM_{10}, $PM_{2.5}$), ROFA, and DEP.
Primary neutrophils culture		
Human primary neutrophils	**Murine primary neutrophils**	
Increase in pro-inflammatory cytokines [64]	Increase in pro-inflammatory cytokines [19] Reduction of the TNFα production [65]	Particulate matter (PM_{10}, $PM_{2.5}$, $PM_{0.1}$)
Production of free oxygen radicals (ROS) [13, 64, 66, 67]	Decreased expression of iNOS and NADPH oxidase components [65]	Particulate matter (PM_{10}, $PM_{2.5}$, $PM_{0.1}$).
Eicosanoid enhancement (LTB_4, LTC_4, and PGE_2) [64]	Reduction in STAT1 activation [65]	Particulate matter (PM_{10}, $PM_{2.5}$, $PM_{0.1}$).

* Some reports indicate a decrease in pro-inflammatory cytokines.
** Some reports indicate a decrease in oxidative stress.

inflammatory effect than $PM_{2.5}$ [83], and also show increased potential for vascular dysfunction and heart damage [81]. Further, Cho et al. [81] observed an increased number of neutrophils in CD-1 mice exposed to PM_{10} compared to those exposed to $PM_{2.5}$ and $PM_{0.1}$ [81]. These results could be associated with chemical agents present in PM_{10} such as Al, Fe, Si, Cu and Ti [84, 85], and higher levels of endotoxin [80, 81, 86] in comparison with other PM [80].

On the other hand, it has been reported that PM stimulates ROS production and release in resting neutrophils, causing tissue damage, thus initiating, or exacerbating the inflammatory response [64, 87] (Figure 3). By the way, Miyake et al. found that endocytosis of neutrophils was involved in the triggering of ROS production. However, these cells endocytosed $PM_{2.5}$, but not PM_{10} [88], suggesting that oxidative response differed according to the type of PM [84, 88]. In this regard, it was identified that PM_{10} was more potent in inducing cytokines but not ROS than $PM_{2.5}$ and $PM_{0.1}$ [84]. In contrast, Li et al. identified the oxidative properties of PM_{10} by increased lactate dehydrogenase concentrations in BALF of syngeneic Wistar-derived rats [89]. These apparently contradictory results indicate that the oxidative stress in response to exposure to PM_{10} was different from that induced by $PM_{2.5}$, the latter being dependent on neutrophil endocytosis.

With respect to cytokine profiling, *in vitro* and *in vivo* studies suggest that IL-6, TNF-α and keratinocyte chemoattractant (KC) may represent key mediators of the inflammatory response induced by PM exposure [20, 25, 90, 91]. Particularly, Vieira et al. found an association between the cytokines release and the recruitment and activation of neutrophils in the BALF of BALB/c mice exposed to PM [90]. Van der Toorn et al. [92] reported that PM induced an increase in cytokines such as MIP-1α (macrophage inflammatory protein-1α), MCP-1 (monocyte chemotactic protein-1), KC, IL-5, and GM-CSF (granulocyte-macrophage colony stimulating factor) in the lungs of BALB/c mice [92]. Besides, Hitzfeld et al. [67] demonstrated that exposure to PM caused the release of IL-8 by neutrophils, inducing the accumulation of these cells at the sites of inflammation due to an autocrine mechanism [67](Figure 3). This cytokine was also released in the human bronchial epithelial cells [25] and the human nasal septum cells [91], indicating its important role in recruiting neutrophils in tissues affected by PM. Additional to the above, exposure to SO_2 in C57BL/6J mice induced infiltration of neutrophils in the alveolar space and release of cytokines such as TNF-α, IL-1β and IL-6. This stimulates the bone marrow stromal cells (mainly adipocytes) and regulates hematopoiesis. As result, alterations of the blood cell counts are observed in leukocytes, erythrocytes and platelets [93].

Besides, previous studies have shown a negative correlation between PM_{10} and O_3 [94], reflecting the importance of research studying the effect of contaminants other than PM. The exposure to O_3 caused the infiltration of neutrophils, macrophages, lymphocytes and eosinophils into the lungs of rabbits [95], mice [96] and rats [97, 98]. Also, Bosson et al. [88] found that O_3 exposure enhanced MPO (Myeloperoxidase)

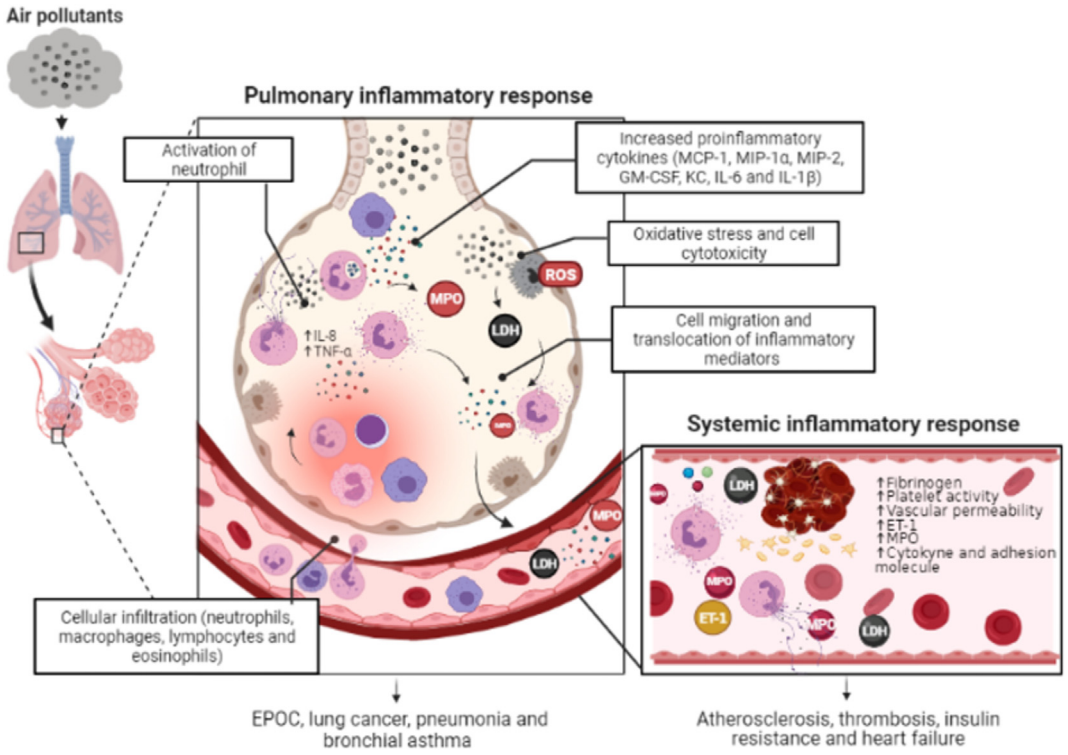

Figure 3. Immunological effects of PM, on neutrophil activation, and pulmonary and systemic homeostasis. Inhalation and deposition of PM in the lungs trigger inflammatory responses leading to the release of inflammatory cytokines (MCP-1, MIP-1α, MIP-2, GM-CSF, KC, IL-6 and IL-1β); and recruitment of innate immune cells (neutrophils, macrophages, lymphocytes and eosinophils). Neutrophil activation (degranulation, phagocytosis and NETs) induces the production of ROS and the release of inflammatory mediators. These cellular mechanisms also promote changes at the pulmonary level, including increased oxidative stress, and cellular cytotoxicity with LDH release. These events are associated with increased circulating levels of adhesion molecules, inflammatory mediators (MPO and ET-1), cytokines, increased platelet activity and vascular permeability. Together, these responses favor the development of pulmonary diseases such as COPD, lung cancer, pneumonia and bronchial asthma; and systemic inflammatory responses that are related to pathologies such as atherosclerosis, thrombosis, insulin resistance and heart failure.

release in induced sputum [88]. These findings suggested that the activated neutrophils induced MPO release, and ROS production, causing cell damage [88, 99].

Regarding aerosol and volatiles, there was a limitation in the number of studies. However, the *in vivo* toxicity resulting from secondary organic aerosols exposure evidenced ROS generation and lung inflammation [59]. Likewise, prolonged exposure to SO_2 caused chronic lung inflammation and pulmonary fibrosis in mice [93]. Moreover, a study evaluating the effect of nicotine present in electronic cigarettes showed higher levels of neutrophils and alveolar macrophages in bronchoalveolar lavage fluid of SR rats than in unexposed rats [100]. Contrary to what was observed for other pollutants, exposure to ZnSO4 was not associated with a change in the number of macrophages or neutrophils [101]. Therefore, although the evidence indicates that exposure to these pollutants affects the respiratory system, given that those pollutants can be generated from different sources of emission, complementary studies are still required.

Furthermore, the evidence indicates that PM exposure may contribute to morbidity and mortality from cardiac diseases. The proposed mechanisms include increased oxidant production, direct cardiac effects, and systemic mediators leading to cardiac responses [102]. Farina et al. [103] demonstrated that the inflammatory response in the lungs after exposure of BALB/c mice to PM_{10}, is also associated with translocation of inflammatory mediators like as MPO and ET-1, from the lungs to the bloodstream, thus triggering systemic and cardiac effects. On the other hand, ET-1, a marker of inflammation implicated in the progression of cardiovascular diseases, has been found to be increased in plasma and cardiac tissue of rats exposed to O_3 and diesel exhaust particles [104, 105, 106]. The increased oxidative stress, limited antioxidative compensation and cytotoxic potential of PM have been implicated in the pathogenesis of cardiovascular diseases in humans and animal models, including those with systemic hypertension and other complications such as atherosclerosis, stroke, and myocardial infarction [107, 108]. Previous studies have reported an increase in blood neutrophils and an attenuated antioxidant response in rats exposed to residual oil fly ash (ROFA) [109], showing PM-induced cardiophysiological changes such as acute depression in ST-segment area of electrocardiography [102]. It has been reported that neutrophil activation (with the expression of inflammatory cytokines, and adhesion molecules) induces endothelial dysfunction through ROS formation, and increased vascular permeability, thus resulting in structural damage of the arterial wall with smooth muscle cell proliferation, and atherosclerotic plaque formation [110, 111, 112]. Exacerbation of vascular oxidative stress in ApoE −/− mice exposed to PM has been reported, which enhanced vascular inflammation and atherosclerosis [113]. Further, a study by Haberzettl et al. [114] showed that exposition of mice to $PM_{2.5}$ suppressed endothelial insulin-stimulated nitric oxide synthase (eNOS) phosphorylation and suppresses vascular insulin signaling, thus suggesting that vascular insulin resistance could be one such "sensitive" target of $PM_{2.5}$ that in turn affects processes such as tissue perfusion, endothelial function, and atherogenesis [115, 116, 117]. The suppression of insulin-stimulated phosphorylation of eNOS results in alteration of the vascular tone, thrombosis, and atherogenesis [115, 117, 118]. Collectively, these changes could increase cardiovascular risk and mortality in humans exposed to PM.

In addition to oxidative stress and neutrophilic inflammation, PM exposure has been associated with activation of the coagulation and fibrinolytic system [119, 120]. Cascio et al [121] found, in a mouse

model, that after exposure to ultrafine particulate matter (UFP) there was an increase in the platelets, plasma fibrinogen, and soluble P-selectin levels, with reduced bleeding time, implying an increased thrombogenic potential. Increased expression of adhesion molecules by pulmonary capillary endothelium in hypertensive rats exposed to ROFA induces neutrophil migration and activation that consequently alter fibrin degradation and stimulate fibrinogen synthesis, ultimately leading to hyperfibrinogenemia [109]. Aberrant fibrinogen increase is a risk factor for cardiovascular disease in humans as by increasing blood viscosity, recruits platelets, and promotes thrombi [122].

While several studies have reported the link between cardiopulmonary mortality and exposure to air pollution, few reports have focused on the central nervous system (CNS). In BALB/c mice exposed to ultrafine particles, increased brain biomarkers of oxidative stress and tissue injury have been observed [123]. Similarly, the acute O_3 exposure in mice induces neurological issues and an increase in infiltrating neutrophils [124]. A study found that Alzheimer's and Parkinson's patients have significantly elevated levels of neutrophil-mediated oxidative stress compared with healthy donors [125]. As neutrophils are potent producers of ROS, including those in response to PM exposure, these cells could induce an oxidative imbalance and consequent oxidative injury to neighboring tissues, thus contributing to the pathogenesis of neurodegenerative disorders [124]. However, additional research should address the effects of neutrophil activation following exposure to air pollution and the related effects on neuroinflammation and neurotoxicity.

Recent studies link neutrophils as one of the most important players in the effects of pollutant exposure and cancer development. Rocks et al. [126] demonstrated that O_3 exposure greatly facilitates lung metastasis by inducing tissue injury and neutrophilic inflammation in a mouse model. Furthermore, O_3 exposure influences metastasis and activates the release of neutrophil extracellular traps (NETs) that favor tumor cell colonization in the lungs. However, there are limited studies that focus on evaluating this effect in the presence of PM, therefore, the cellular and molecular mechanisms linking neutrophils to cancer development and progression in relation to PM exposure should be further explored. This may be an important explanation for the epidemiological correlation between exposure to environmental pollutants and high rates of hospitalization and mortality from cardiovascular complications [110, 111].

Preclinical study models, also called basic medical research, include experiments on animals, cellular studies, among others that seek to clarify biochemical, genetic, physiological, or immunological mechanisms for various purposes such as improving analytical, diagnostic, or therapeutic processes, as well as consolidating (and in some cases discarding) scientific knowledge that is indispensable for the subsequent development of research on patients. Animal models make it possible to visualize the neutrophil-mediated immune response in a physiologically relevant environment that includes cellular interactions or specific signals that can be evaluated by *in vitro* models [127, 128]. In this review, it was evidenced that most of the studies included an *in vivo* model, in which mainly mice and rats were used. This trend has been maintained over time. It is worth mentioning that, since the 20th century, mice have represented a key element in the investigation of the immune response [129]. Moreover, given the metabolic and physiological similarities between humans and rats, the latter are widely used in immunological, pharmacological, and toxicological studies [130]. Nonetheless, primary research and characterization of leukocyte subpopulations in this species are less well known than for research in mouse models, limiting the development of complex immunological studies [131].

Although the use of animal models has been a critical element in biomedical research, it implies high costs and strict ethical considerations, which restrict their use [132]. With this limitation, the *in vitro* models represent a useful tool in the study of the effect of environmental contamination; although, in this model, in particular, there are different factors such as the concentration of the contaminants that can limit the conclusive and comparable information between the different studies [133]. Besides, few differences have been reported in the neutrophil's markers, depending on the mice or rat strain, because those different types of animals show differences in adaptive immunity, without affecting the innate immunity cells. In this sense, the PM concentrations that demonstrate a significant effect *in vitro* can be greater than the PM density that reaches the respiratory tract. Despite the above, these problems can also occur in animal models, so the use of *in vitro* models remains relevant [133].

In this work, primary cultures were the main *in vitro* models used in the studies. However, there were a limited number of *in vitro* studies that used neutrophils as a cellular model. Their selection instead of the cell line could be because the genetic manipulation of the cell lines can alter their phenotype, functionality, and capacity of response to stimuli, so it is possible that they do not adequately represent the primary cells and could provide deleterious results [134]. The general recommendations for *in vitro* experiments should be the use of primary human neutrophils, which can be easily obtained, compared with animal cells.

In general, this review showed high heterogeneity in the type of model used in the studies to evaluate the effect of air pollution, which may represent a limitation for the passage to clinical studies since it is difficult to determine the consistency of the results and the selection of the most significant exposures [135]. However, the identification of biological markers, that explain the effects of air pollutants is important itself, as provides information for experimental design looking for the understanding of molecular mechanisms. Besides, limited information was found on Ozone, SO_2 and $ZnSO_4$, evidencing the need to increase research focused on these pollutants.

Considering the studied outcomes, it is clear the natural change in the methodologies, promoted by the advances in molecular techniques, multiplex assays, and modern biochemical and immunological tools that allow, besides the classic measurements of cellular counts, functional aspects of them, including respiratory explosion and transcriptional expression of cytokines and other inflammatory factors, among others. These new measurements provide new information regarding the state of cell activation and the magnitude of the immune response in response to airborne contaminants, which could explain the alterations observed in the long term in individuals exposed to airborne contaminants, including increased susceptibility to infectious diseases of the respiratory tract.

Consistent with the characteristics of Cochrane's broad approach, we must declare some intrinsic limitations to this type of review, such as the impossibility of delving into the findings of some hypotheses on specific immunological outcomes or the mechanisms of damage of PM, especially those associated to the activity of the neutrophils.

5. Conclusions

The analysis of the studies and their heterogeneity allows us to recommend practical options that can be unified in future studies to obtain comparable data to move on to clinical studies, which will support the epidemiological observations widely reported in the area. These recommendations include the use of primary cultures of human neutrophils, considering that human donors' genetic variability allows a precise approximation to the expected results in the general population. Regarding the outcomes, the measurement of pro-inflammatory cytokines, either at the protein level in culture supernatants and BALF, by ELISA, or mRNA by RT-PCR, are widely used measures that allow building meta-analysis and understanding of global variations in response to contaminants found in different latitudes. Secondly, there is the use of BALBc or C57BL6/J murine models (understanding that it is not available in all research entities), and in this, the histopathological analyses (infiltrates counts and lung damage measures) that allow understanding of the expected immunopathological potential in the human population exposed to air pollutants.

Finally, the studies included in this review suggest that environmental pollutants induced neutrophil recruitment as the first sign of inflammation. The effect of neutrophils depended on the type, concentration and size of the PM particles. These cells release pro-inflammatory

cytokines and ROS that cause damage to lung tissue and contributes to the systemic inflammatory response related to cardiovascular diseases. The importance of future research to clearly establish the relationship between neutrophilic activation after exposure to air pollutants with the development of cancer and neurodegenerative diseases was also identified. Future studies with potential for clinical approaches include the use of primary cells, especially those obtained from pulmonary samples, such as bronchoalveolar lavages in human populations from different geographical areas with monitoring programs for air pollution. However, basic experimental studies are still incipient, indicating the need to improve and increase these investigations before the studies in human populations.

Declarations

Author contribution statement

All authors listed have significantly contributed to the development and the writing of this article.

Data availability statement

Data associated with this study has been deposited at https://doi.org/10.6084/m9.figshare.13140176.v1.

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Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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Capítulo 2

Artículo original:

Particulate matter (PM_{10}) induces *in vitro* activation of human neutrophils, and lung histopathological alterations in a mouse model.

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Particulate matter (PM_{10}) induces in vitro activation of human neutrophils, and lung histopathological alterations in a mouse model

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The epidemiological association between exposure to particulate matter (PM_{10}) and various respiratory and cardiovascular problems is well known, but the mechanisms driving these effects remain unclear. Neutrophils play an essential role in immune defense against foreign agents and also participate in the development of inflammatory responses. However, the role of these cells in the PM_{10} induced inflammatory response is not yet fully established. Thus, this study aims to evaluate the effect of PM_{10} on the neutrophil-mediated inflammatory response. For this, neutrophils from healthy adult human donors were in vitro exposed to different concentrations of PM_{10}. The cell viability and cytotoxic activity were evaluated by MTT. LDH, propidium iodide and reactive oxygen species (ROS) were quantified by flow cytometry. Interleukin 8 (IL-8) expression, peptidyl arginine deiminase 4 (PAD_4), myeloperoxidase (MPO), and neutrophil elastase (NE) expression were measured by RT-PCR. IL-8 was also quantified by ELISA. Fluorescence microscopy was used to evaluate neutrophil extracellular traps (NETs) release. The in vivo inflammatory responses were assessed in BALB/c mice exposed to PM_{10} by histopathology and RT-PCR. The analysis shows that PM_{10} exposure induced a cytotoxic effect on neutrophils, evidenced by necrosis and LDH release at high PM_{10} concentrations. ROS production, IL-8, MPO, NE expression, and NETs release were increased at all PM_{10} concentrations assessed. Neutrophil infiltration in bronchoalveolar lavage fluid (BALF), histopathological changes with inflammatory cell infiltration, and CXCL1 expression were observed in PM_{10}-treated mice. The results suggest that lung inflammation in response to PM_{10} could be mediated by neutrophils activation. In this case, these cells migrate to the lungs and release pro-inflamatory mediators, including ROS, IL-8, and NETs. Thus, contributing to the exacerbation of respiratory pathologies, such as allergies, infectious and obstructive diseases.

Air pollution represents a major threat to human health[1]. According to the World Health Organization (WHO), exposure to air pollutants is the cause of 4.2 million premature deaths per year[2]. This mortality is related to adverse respiratory and cardiovascular health effects caused by exposure to particulate matter (PM)[3,4].

Particulate pollutants are originated from the natural environment (volcanoes, forest fires and dust storms) and human activities (transportation, industry, power plants, combustion, and agriculture)[5]. According to their aerodynamic diameter, they are classified into particles including PM_{10} (less than 10 μm); $PM_{2.5}$ (less than 2.5 μm), and ultrafine particles (UFP, less than 0.1 μm)[6,7]. PM can penetrate the human respiratory tract and even reach circulation due to its small size, large surface area, penetration capacity, deposition, bioavailability, and long residence time in the air[8]. Exposure to PM can induce lung damage by oxidative stress and airway inflammation[9], loss of immune functions against microorganisms[10,11], thrombosis, coagulation, and vascular

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dysfunction that can lead to the development of different diseases[12–14]. Epidemiological and toxicological studies related to PM have shown positive correlations with the development of a wide range of diseases, including skin diseases[15], asthma[16], stroke, and ischemic heart disease[17], chronic obstructive pulmonary disease (COPD)[18] and lung cancer[19].

Although the pathogenesis of diseases produced by PM exposure is not completely clear, several studies have demonstrated mechanisms potentially associated with innate and adaptive immune alterations. These processes include cytotoxicity, production of cytokines and pro-inflammatory molecules, mutagenicity, oxidative DNA damage, and genotoxicity[20]. Inflammatory responses activated by PM exposure may underlie asthma, chronic obstructive pulmonary disease, and lung cancer[10,21].

Neutrophils constitute the most considerable fraction of leukocytes in the human body, with an essential role in lung immune response. These cells rapidly migrate to the lung, destroy the foreign agent and initiate an inflammatory response predominantly mediated by phagocytosis, ROS production, degranulation, and NETosis[22]. Neutrophil extracellular traps (NETs) are produced by the extracellular release of neutrophil nuclear material coated with antimicrobial peptides and enzymes, mainly activated by ROS production. NETosis activation is associated with exacerbated inflammatory response, tissue damage, and potentially, autoimmunity diseases[23–25]. Consequently, PM inhalation and deposition could induce ROS and oxidative stress molecules that induce neutrophil activation, NETs overproduction, and exacerbated inflammatory response.

Different in vivo studies have evidenced that PM exposure promotes neutrophil migration and lungs infiltration. Asthmatic BALB/c mice exposed to different concentrations of $PM_{2.5}$ exhibited a significant increase in the frequency of eosinophils and neutrophils and high production of TNF-α in bronchoalveolar lavage fluid (BALF). Aberrant accumulation and altered functions of neutrophils in the lung are related to increased inflammation and tissue damage in people exposed to PM[26]. In this regard, it has been shown that neutrophils activated by PM exposure are accumulated in the pulmonary vasculature and induce an elevated release of inflammatory mediators such as myeloperoxidase (MPO)[27] and leukotriene B4 (LTB_4)[28]. Similarly, an in vitro study showed that neutrophil exposure to PM increased the release of (LTB_4), leukotriene C4 (LTC_4), and IL-8[29]. These mediators may be responsible for oxidative and proteolytic tissue damage, leading to immune dysregulation and lung diseases.

Although different studies provide convincing evidence regarding PM exposure-related immune alterations, relatively few studies are available in the context of neutrophil-mediated inflammatory responses. Therefore, this study aimed to determine the in vivo and in vitro effect of PM_{10} on the neutrophil-mediated inflammatory response.

Materials and methods

Ethics statement. The study was performed according to the principles of the declaration of Helsinki and approved by the Ethical Committee of the Universidad Cooperativa de Colombia (certificate number 003/2018). The individuals enrolled provided signed informed consent forms.

Experiments in mice were approved by the corresponding Institutional Animal Care and Use Committees (CICUA), Universidad de Antioquia (certificate number 117), and performed following international guidelines and regulations.

The manuscript follows the recommendations in the ARRIVE guidelines.

Neutrophil isolation and culture. Neutrophilic polymorphonuclears (PMNs) were isolated from freshly drawn peripheral venous blood from healthy humans. Cells were separated by centrifugation at 3000 r.p.m for 40 min at room temperature in a double density gradient Ficoll/Histopaque 1119 and 1077 (Histopaque 1119 and 1077, Sigma—UK). The layer containing PMNs was then collected, washed twice with phosphate-buffered saline (PBS), and subsequently resuspended in RPMI medium (RPMI 1640, Gibco-USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin–streptomycin (GIBCO Invitrogen, Breda, The Netherlands). Cell viability after isolation and before the start of each experiment was > 95%, as measured by the trypan exclusion test.

PM_{10} collection. PM_{10} was collected in Valle de Aburrá, Colombia, between January-June of 2018, due to the most intense air pollution episode of the year, and using the local environmental authority of Valle de Aburrá (Sistema de Alerta Temprana—SIATA). The PM_{10} sample was obtained from 100 quartz filters at ten BAM-1020 monitoring stations (TISCH Environmental, BM2000H). The PM_{10} filters were cut into small pieces; high purity sterile water was added, mixed by immersion, and sonicated for 2 h and 15 min at 37 Hz. The mixture was then filtered using six layers of sterile gauze. After distributing the collected solution into sterile vials and freeze-drying under a vacuum, the tube was weighed to determine the mass of the extracted particles and stored at 80 °C before biological testing. A vacuum lyophilizer (ALPHAI-2LDplus, Martin Christ, Osterode, Germany) was used to dry the PM_{10} to particulate powder.

Cell culture and treatment. PMNs (2.5×10^5 cells/well) were seeded in 96-well plates (Greiner-Bio-One, Solingen, Germany). Increasing concentrations (0.1, to 100 µg/mL) of PM_{10} were prepared in RPMI 1640 medium. Subsequently, the freshly prepared PM_{10} solutions were added to the cells at a maximum volume of 200 µL per well. After exposure at 37 °C and 5% CO_2 for 5 h, the supernatant was removed and used for LDH analysis or stored at − 80 °C until further analysis (cytokine levels by ELISA). Cell pellets were used for total RNA extraction and MTT assay. As positive controls, we used cells stimulated with the following stimuli: MTT assays and LDH assay: 20% dimethyl sulfoxide (DMSO); RT-PCR and ELISA: 50 ng/mL lipopolysaccharide (LPS) (Invitrogen, San Diego, CA); flow cytometry: Zymosan; fluorescence microscopy: 50 nM Phorbol-12-myristate-

Gen	Primers 5'-3'	Annealing temperature
IL-8	Fw: 5′-ACTGAGAGTGATTGAGAGTGGAC-3′ Rv: 5′-AACCCTCTGCACCCAGTTTTC -3′	60 °C
PAD_4	FW: 5'-GGGGTGGTCGTGGATATTGC-3 ′ Rv: 5'-CCCGGTGAGGTAGAGTAGAGC-3 ′	64 °C
NE	FW: 5′-GTGGCGAATGTAAACGTC-3 ′ Rv: 5′-CCGTTGAGCTGGAGAATC-3′	58 °C
MPO	Fw: 5′-TGCTTCCTGGCAGGGGA-3′ Rv: 5′-CCACCTAG GGTTCAGGCTCT-3′	62 °C
PGK	Fw: 5′-GTTGACCGAATCACCGACC -3′ Rv: 5′-CGACTCTCATAACGACCCGC -3′	60 °C

Table 1. Primers.

13-acetate (PMA). In all assays, PM_{10} unexposed cells were used as a negative control. All reagents and materials used in the experiments were endotoxin-free.

Cytotoxicity assays. PM cytotoxic effect was evaluated using the colorimetric 3-(4,5-dimethylthiazol-2-yl) diphenyltetrazolium bromide (MTT) reduction, Lactate Dehydrogenase (LDH) release, and propidium iodide (PI) staining assays.

For MTT assay, PMNs were exposed to different concentrations of PM_{10} (0.1, 1, 10, 50, and 100 µg/mL) and 20% DMSO for 5 h. The supernatant was removed from each well, and the cells were washed with PBS. Then, the cells were incubated in a fresh medium containing 0.5 mg/mL MTT for 3 h at 37 °C. The formed crystalline formazan was dissolved in 100 µL DMSO. Finally, absorbance was measured at 570 nm using a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific). The data were normalized to untreated control cells.

LDH release in the PMNs culture medium was measured using the LDH toxicity assay kit (Roche, Germany) in supernatants of three different culture conditions: untreated PMNs (low control), treated with DMSO (high control) and exposed to different concentrations of PM_{10}. After 5 h of culture, supernatants were transferred to a 96-well plate in triplicate and incubated for 30 min at room temperature with the reaction mixture. The final absorbance was measured at 490 nm using a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific). Cytotoxicity was calculated as: % cytotoxicity = [(LDH activity with stimulus (PM_{10})-(LDH activity low control)/(LDH activity high control-LDH activity low control)] × 100%.

For Propidium iodide (PI) staining assays (for necrosis), PMNs were cultured at a density of 1×10^6 on round coverslips and treated with different concentrations of PM_{10} (10, 50, and 100 µg/mL) for 30 min at 37 °C. The cells were washed, stained for 1 h, and observed with a fluorescent microscope.

RNA extraction and quantitative real-time PCR. IL-8, PAD_4, NE, and MPO mRNA levels in PMNs exposed to different concentrations of PM_{10} (0.1, 1, 10, 50, and 100 µg/mL) and LPS (50 ng/ml) were determined by real-time PCR. Total RNA was isolated using the One-Step RNeasy Mini Kit extraction kit, according to the manufacturer's recommendations (Qiagen, Hilden, Germany). RNA concentration was quantified using a Nanodrop one spectrophotometer (Thermo Scientific). RNA was reverse transcribed into cDNA in a 20 µL reaction volume using the iScript High Capacity cDNA Reverse Transcription Kit, following the manufacturer's recommended protocol (Bio-Rad Laboratories, Hercules, CA). The qPCR was performed using SYBR Green Mastermix (Thermo Scientific, Waltham, MA) on a QuantStudio 3 real-time PCR detection system (Applied Biosystems). The list of primer sequences used to detect mRNAs is presented in Table 1. Relative gene expression levels obtained from reverse transcription qRT-PCR were calculated using the ΔΔCt method and normalized to phosphoglycerate kinase (PGK) gene expression.

Cytokine analysis. To investigate the effect of PM_{10} on IL-8 production, the concentration of this cytokine in cell culture supernatants was determined using a commercial ELISA assay (BioLegend, San Diego, CA) according to the manufacturer's instructions. All samples were performed in triplicate. Cytokine concentrations were calculated from a standard curve of the corresponding recombinant human cytokine.

NETs induction. Round coverslips were treated with ethanol and poly-L-lysine (Sigma) then placed in a 12-well plate with PBS. 5×10^5 PMNs were added per well with the following stimuli: (a) PMA as positive control; (b) 10 µg/ml of PM_{10}, (c) 50 µg/ml of PM_{10}, and (d) 100 µg/ml of PM_{10}, PBS was used as a negative control. The cells were incubated without FBS for 3 h at 37 °C in the presence of 5% CO_2, then fixed with 1 mL of 4% paraformaldehyde for 20 min at room temperature. Cell's DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI) and incubated at 4 °C overnight.

Afterward, cells were permeabilized with Triton X-100 and blocked with 1% BSA for 30 min at 37 °C. Then 1 mL of anti-neutrophil elastase (1:500, Abcam) and anti-myeloperoxidase (1:500, Abcam) primary antibodies were added to each well, and the plate was incubated for 1 h at 37 °C. 1 mL of anti-rabbit IgG (1:1000, Abcam) Alexa 488 and anti-rabbit IgG (1:500, Abcam) Alexa 647 secondary antibodies were then applied, the plate was incubated for 1 h at 37 °C. DNA, Neutrophil elastase (NE), and Myeloperoxidase (MPO) were identified by immunofluorescence. Images of random fields were taken through the 20 × and 40 × objectives. Each experiment was repeated at least three times.

Quantification of ROS production. Intracellular ROS production in PMNs exposed to PM_{10} was measured with the DHR-$_{123}$ ROS dihydrorhodamine assay kit (Thermo Fisher Scientific, USA), according to the manufacturer's protocol. Briefly, PMNs (5×10^5 cells) were cultured in flow cytometry tubes (BD Falcon polystyrene) and exposed to different concentrations of PM_{10} (0.1, 1, 10, and 50 µg/mL) and zymosan (10 µg/mL) for 30 min. An unstimulated control tube was included in each assay. Cells were then washed with PBS, and 100 µL PBS supplemented with DHR-123 at a concentration of 0.07 µg/mL was added and incubated at 37 °C for one hour. Cells were then washed and resuspended in 200 µL of PBS. The analysis was performed on a BD LSR Fortessa™, and the collected data were analyzed in FlowJo v10.4 software (FlowJo, LLC). The neutrophil population was classified by forward scatter (FSC), and side scatter (SSC), where at least 10,000 events were recorded. Doublets were excluded by forward scatter height (FSC-H) and forward scatter area (FSC-A). FL-1 channel mean fluorescence (FITC) was examined for an increase in Rho123 fluorescence representing ROS production in activated versus unstimulated cells (Fig. S1). ROS production was calculated by subtracting the percentage of ROS production of stimulated PMNs from the percentage of ROS of control PMNs.

Mice. 8-weeks-old male wild-type BALB/c mice weighing 18–20 g (Charles River, Portage, MI, USA) were bred and housed at 22 ± 1 °C under a 12 h light/dark cycle with food and water ad libitum and maintained under specific pathogen-free conditions at the animal facility of the Sede de Investigación Universitaria—Universidad de Antioquia (Medellín, Colombia). At the end of the experiments, euthanasia was performed using ketamine/xylazine 100/10 mg/kg intraperitoneally.

Mice PM_{10} model and experimental design. To establish a mouse model for PM-induced airway inflammation, mice were exposed to 100 µg PM_{10} (in 50 µl PBS) per day by intranasal instillation for six days. Meanwhile, control mice were treated with the same volume of PBS.

Lungs were perfused with 1 mL PBS sterile to obtain bronchoalveolar lavage fluid (BALF). The BALF cells were evaluated by Wright's stain, and neutrophil counts were performed. The mice's lung tissues were fixed with 4% buffered paraformaldehyde (Sigma-Aldrich, USA) for 48 h and processed using standard histological techniques. After embedding in paraffin, Sections (5-µm thick) were prepared and stained with HE (Sigma-Aldrich, USA) to evaluate neutrophils infiltration for histopathological analysis. A pathologist analyzed the histopathological sections. RT-PCR was performed as described above.

Statistical analysis. For data analysis, GraphPad® Prism 8.0.1 software. (San Diego, CA, USA) the software was used. Normality was determined using the Shapiro–Wilk test. Data were analyzed using the nonparametric Kruskal–Wallis test, followed by Dunn's multiple comparisons test. Values of $p < 0.05$ (*) were considered significant, and values of $p < 0.01$ (**) and $p < 0.001$ (***) were considered highly significant. Only statistically significant differences are stated with asterisks in each figure. All data are expressed as the mean ± standard error of the mean (SEM).

Results

PM_{10} did not reduce cell viability but increased plasma membrane damage and necrosis in PMNs. Cell viability was measured by MTT assay. PMNs exposed to 0.1, 1, 10, 50 and 100 µg/mL PM_{10} for 5 h did not show a significant concentration-dependent decrease in cell viability (Fig. 1a). However, 5 h after exposure of PMNs to the different PM_{10} concentrations, LDH release was increased in the two highest concentrations (50 µg/mL: 20.33 ± 2.77% and 100 µg/mL: 29.72 ± 3.18%) compared to control cells ($p < 0.05$) (Fig. 1b). As shown in Fig. 1c, stimulation of neutrophils with PM_{10} showed increased red fluorescence compared to control. Thus, PM_{10}-induced necrosis is confirmed.

Exposure to PM_{10} leads to ROS formation in PMNs. ROS production in response to increasing doses of PM_{10} was determined by flow cytometry to assess their oxidative properties. An increase in ROS production was observed at all doses evaluated. However, there was a statistically significant increase from 50 µg/mL PM_{10} to the negative control (Fig. 1d).

Exposure to PM_{10} induces IL-8 expression in PMNs. The IL-8 expression was quantified in PMNs after 5 h of PM_{10} exposure. IL-8 mRNA expression is significantly increased (7.49-fold) in PMNs after exposure to PM_{10} (50 µg/mL) ($p < 0.05$; vs. control group, Fig. 2a). Regarding IL-8 protein levels in PMNs supernatants, only a slight increase was observed at 50 µg/mL compared with the untreated control (Fig. 2b).

Exposure to PM_{10} induces the formation of NETs in PMNs. The formation of NETs in vitro in PMNs in response to PM_{10} was observed with 50 and 100 µg/mL of PM_{10} (35.76 and 23.90%, respectively) after 3 h, compared to unstimulated PMNs ($p < 0.05$; negative control) (Fig. 3a). Representative fluorescence photographs of NETs induction in PMNs, mediated by PM_{10}, negative (unstimulated cells) and positive (PMA) controls are shown in Fig. 3b.

Neutrophil exposure to PM_{10} causes altered expression of NETs-associated genes. Gene expression of NE, MPO, and PAD_4 was quantified in PMNs after PM_{10} exposure. MPO and NE mRNA expression increased in PMNs after PM_{10} exposure for 5 h (Fig. 4a,b). The PAD_4 mRNA expression exhibited a negative regulation in PMNs after PM_{10} exposure (Fig. 4c).

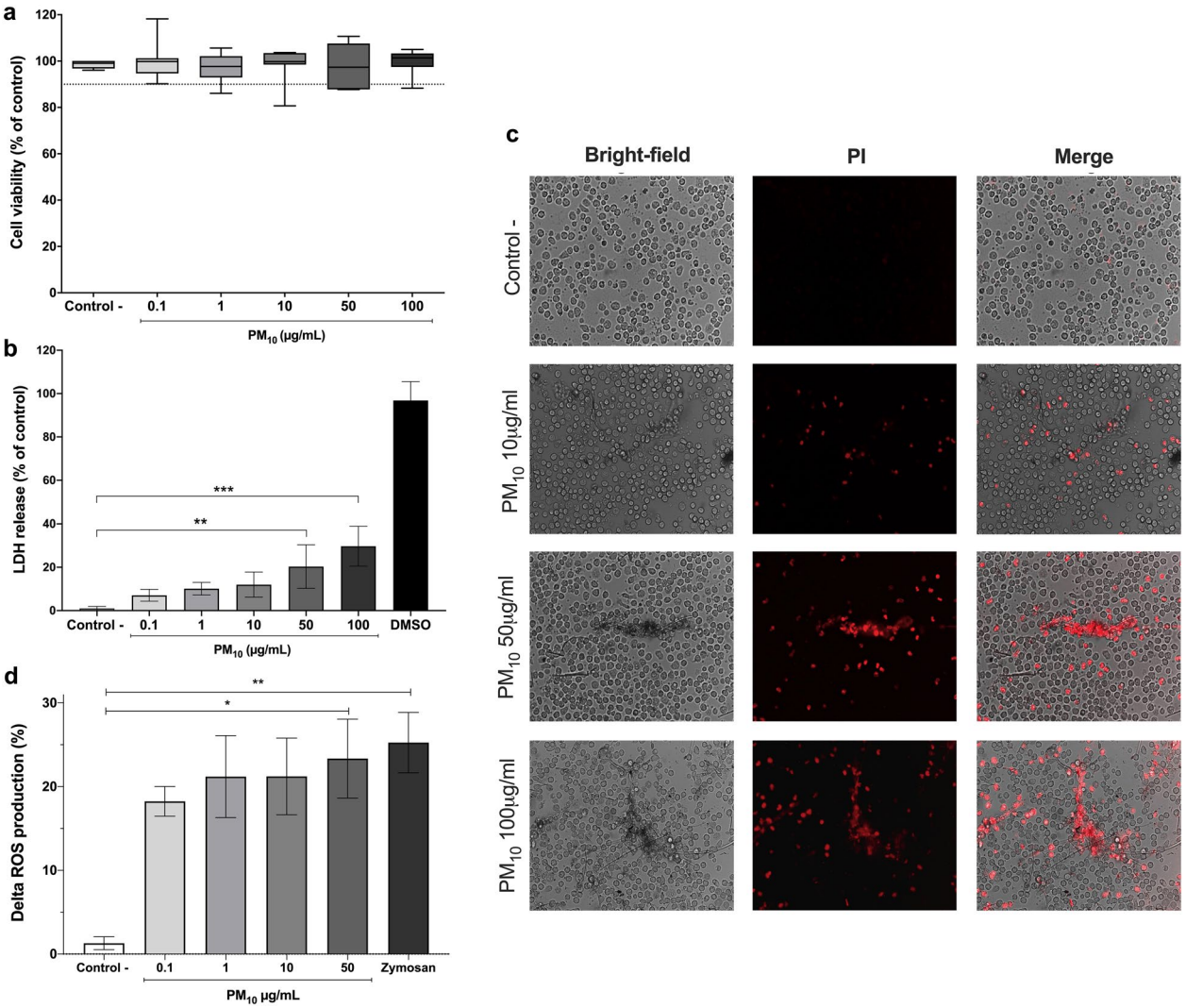

Figure 1. Cytotoxic effects of PM_{10} and ROS production in PMNs. (**a**) Cell viability assay by MTT and (**b**) LDH release in PMNs exposed to different concentrations of PM_{10} (0.1, 1, 10, 10, 50 and 100 µg/mL) for 5 h. Negative control (culture medium); positive control (DMSO). (**c**) Necrotic effects in PMNs exposed to different concentrations of PM_{10} (10, 50, and 100 µg/mL) for 30 min by PI staining. Photographs of each slide were observed at 200X magnification. (**d**) Intracellular ROS production in PMNs exposed to different concentrations of PM_{10} (0.1, 1, 10, and 50 µg/mL) for 30 min, determined by flow cytometry. Negative control (culture medium); positive control (zymosan). ROS levels are presented as the mean fluorescent intensity of DHR of treated cells relative to control. All data are represented as mean ± SEM of 8–14 healthy donors; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, by Kruskal–Wallis analysis with Dunn's post hoc test.

In vivo experiments with BALB/c Mice. The cellular infiltrate in the BALF of PM_{10}-exposed mice was analyzed to investigate the effects on lung inflammation induced by PM_{10} (Fig. 5a). PM_{10} induced an increase in the number of total inflammatory cells (Fig. 5b) and a statistically significant increase in the percentage of PMNs (Fig. 5c) in the BALF of exposed mice relative to controls. Histopathological changes in the lungs of BALB/c mice exposed to PM_{10} were also evaluated (Fig. 5d). We found pathological alterations with inflammatory cell infiltration and moderate PMNs infiltration score in the alveoli of PM_{10}-exposed mice (Fig. 5e). In addition, we observed that PM_{10} increased mouse CXCL1 mRNA expression in the lung lysate of exposed mice compared to PBS controls (Fig. 5f).

Discussion

Inflammation caused by exposure to PM is involved in the pathogenesis of numerous diseases, such as COPD, asthma, lung cancer, cardiovascular and neurodegenerative diseases[30]. In a systematic review, we recently summarized different in vivo studies demonstrating that PM_{10} reaches the bronchi with a predominant infiltration of neutrophils[31]. Therefore, in this study, we evaluate the effect of PM_{10} on the in vitro and in vivo neutrophil-mediated inflammatory response.

In the in vitro model, no differences were observed in cell viability/metabolism when human neutrophils were exposed to increasing concentrations of PM_{10} up to 100 µg/mL (Fig. 1a). Different studies have shown

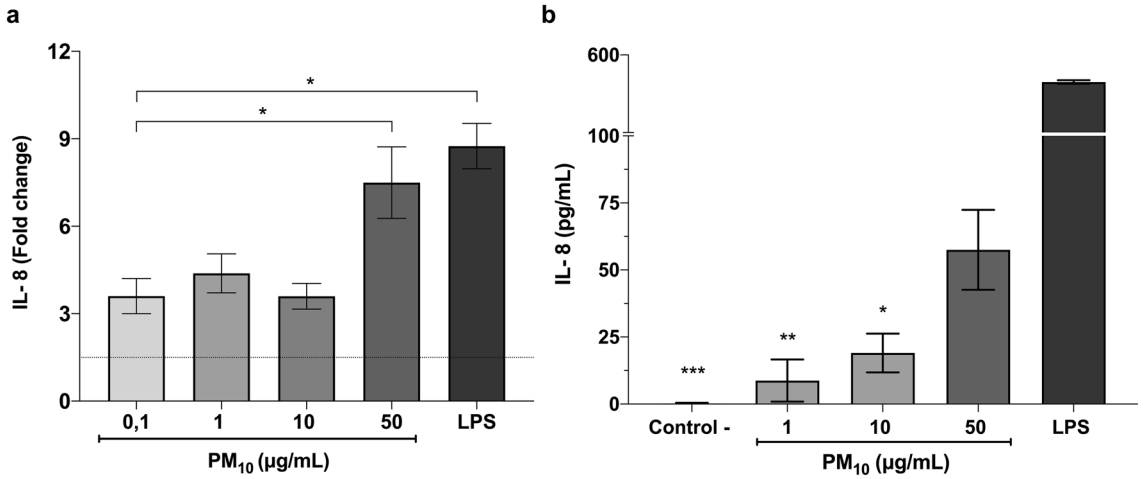

Figure 2. PM_{10} exposure induces IL-8 in PMNs. (**a**) IL-8 mRNA expression and (**b**) IL-8 release in PMNs exposed to different concentrations of PM_{10} (0.1, 1, 10 and 50 µg/mL) for 5 h. Negative control (culture medium); positive control (LPS). The dotted line illustrates the 1.5-fold increase over the untreated negative control. All data are represented by the mean ± SEM of 8 healthy donors. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, by Kruskal–Wallis analysis with post hoc Dunn's test. In (**b**), the asterisk represents differences compared with the LPS stimulated cells.

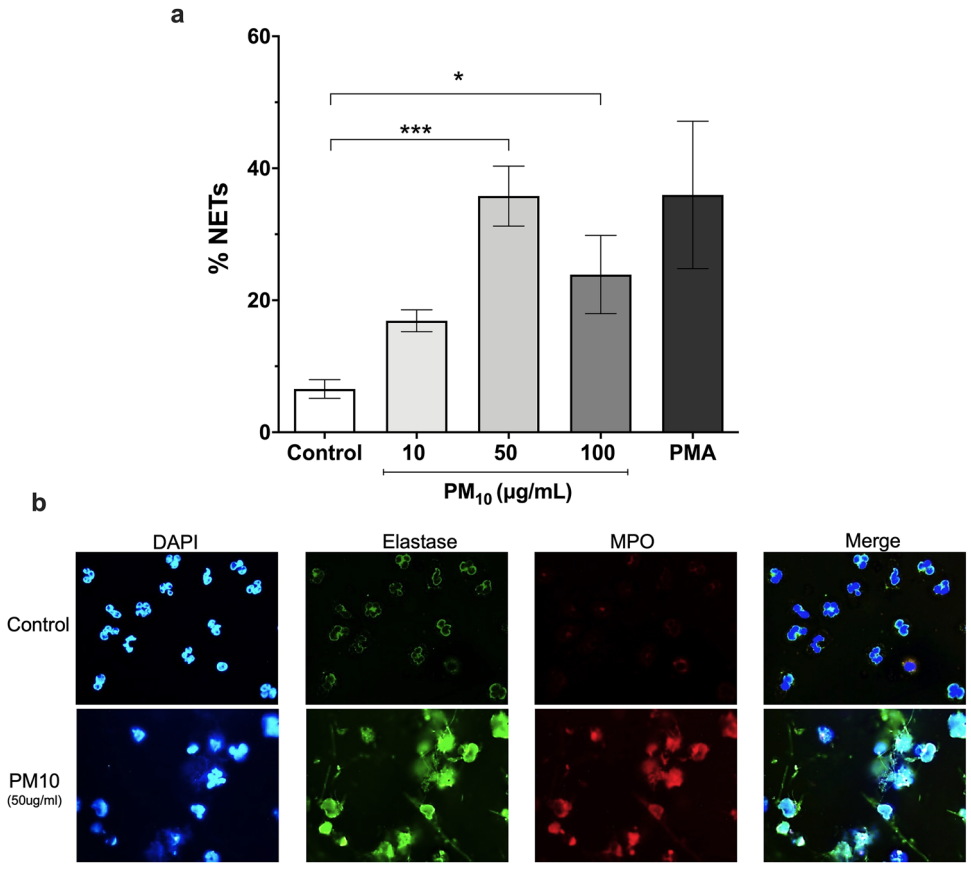

Figure 3. NETs formation in PMNs exposed to PM_{10}. PMNs were exposed to 10, 50, and 100 µg/mL PM_{10} for 3 h. Negative control (culture medium); positive control (PMA). (**a**) NETs release was analyzed microscopically, and MPO, NE, and DNA were quantified with ImageJ software. (**b**) Representative fluorescence microscopy images of NETs. Images show colocalization of MPO (red), NE (green) with DNA fibers released (blue) from released NETs. All data are represented as mean ± SEM of 9 healthy donors. * $p < 0.05$; *** $p < 0.001$ by Kruskal–Wallis analysis with Dunn's post hoc test.

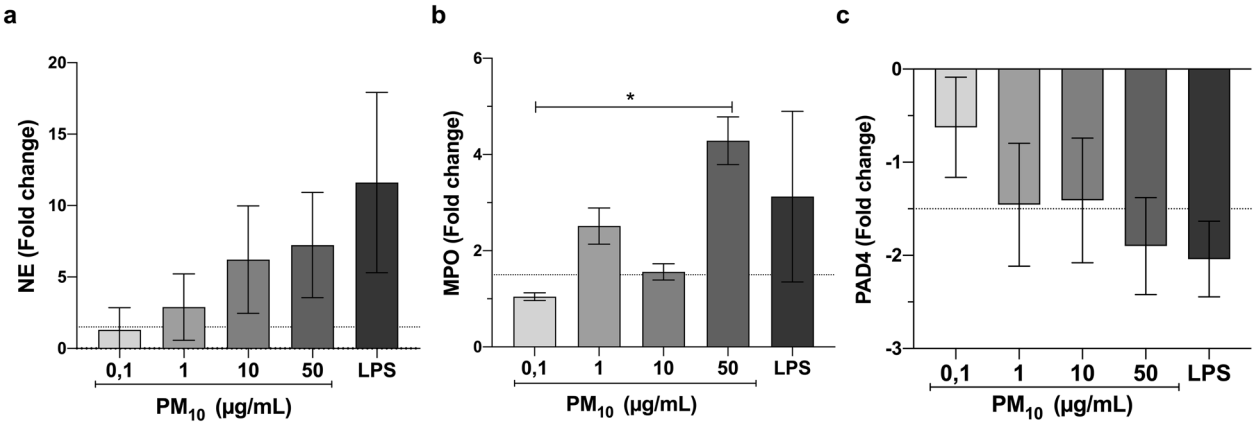

Figure 4. PM_{10} exposure alters NETS-associated gene expression in PMNs. mRNA expression of (**a**) NE (**b**) MPO and (**c**) PAD_4 in PMNs exposed to different concentrations of PM_{10} (0.1, 1, 10 and 50 µg/mL) for 5 h. Negative control (culture medium); positive control (50 ng/ml LPS). The dotted line illustrates the value of 1.5-fold change compared with the untreated negative control. All data are represented as mean ± SEM of 15–18 healthy donors. *$p < 0.05$ by Kruskal–Wallis analysis with Dunn's post hoc test.

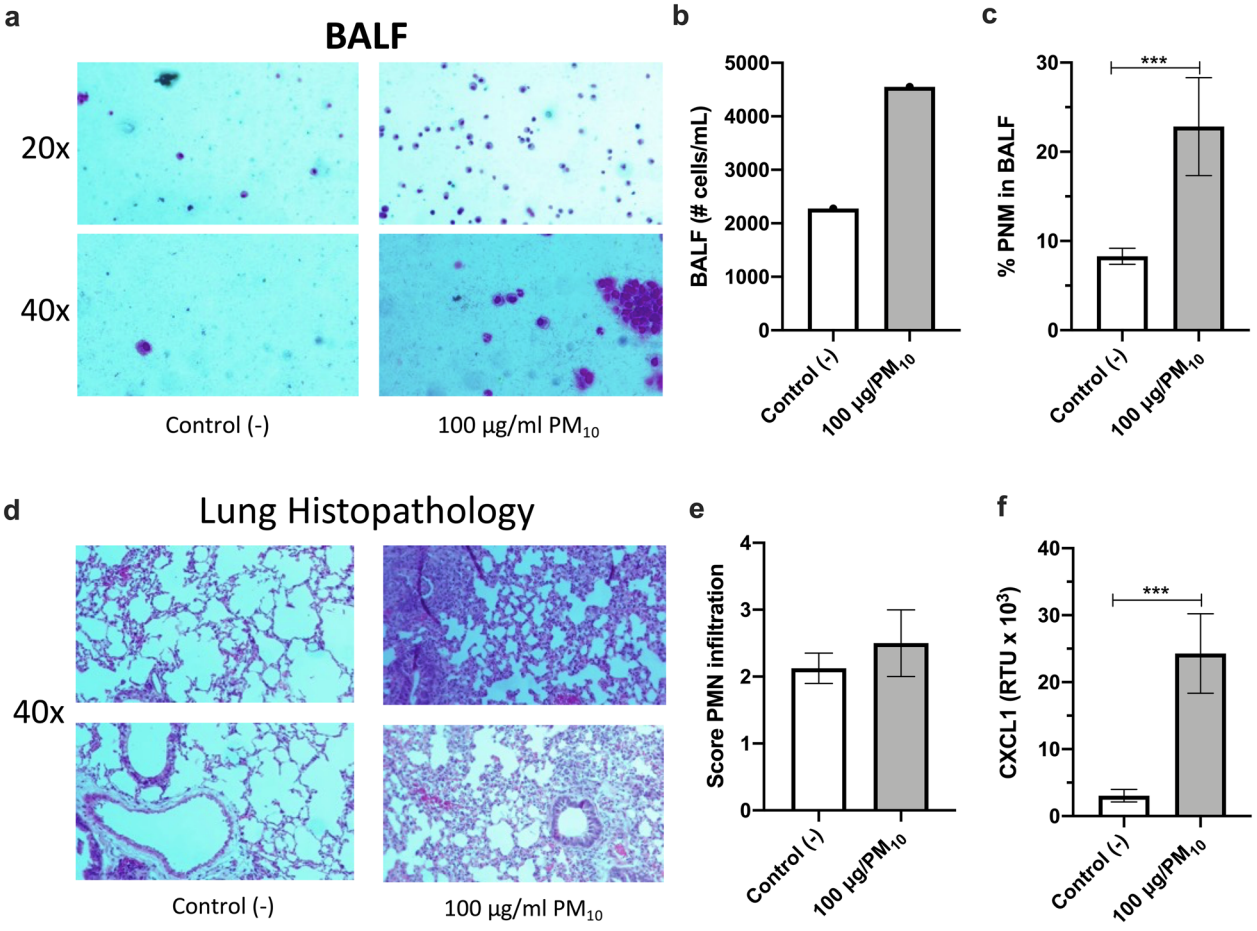

Figure 5. Intranasal treatment with PM_{10} increases cellularity and inflammatory infiltrate in the lung. BALB/c mice (n = 9 per group) were treated intranasally with 100 µg PM_{10} for five days. On day six post-treatment, the animals were euthanized, and bronchoalveolar lavage and lung tissue samples were collected. Wright's staining (**a**) quantification of total inflammatory cells (**b**) and quantification of PMNs in BALF (**c**) Representative images (**d**) and an inflammatory score of PMNs infiltrate (**e**) in hematoxylin–eosin stained lung sections, 1 = no infiltrate, 2 = mild infiltrate, 3 = moderate infiltrate, 4 = severe infiltrate. CXCL1 mRNA levels in lung lysate were measured by RT-PCR (**f**). All data are represented as mean ± SEM. ***$p < 0.001$ by Wilcoxon test.

that cellular exposure to PM_{10} decreased concentration- and time-dependent cell viability[32,33]. However, this is not consistent with the results of our investigation. On the other hand, two types of programmed cell death, pyroptosis, and necroptosis, are characterized by forming pores in the cell membrane and altering it, allowing the release of LDH[34,35]. In the present study, exposure to PM_{10} for 5 h increased LDH release at the highest concentrations (50 µg/mL: 20.33 ± 2.77% and 100 µg/mL: 29.72 ± 3.18%) in neutrophils (Fig. 1b). These results suggest that PM_{10} can alter the integrity of the neutrophil cell membrane, which can subsequently lead to cell death. Importantly, the assays used to analyze cytotoxicity in PMNs comprise respiratory chain activity (MTT) and membrane integrity (LDH). The latter is a much more sensitive assay, as it is based on the detection of LDH as an early event given by the loss of cell membrane integrity or damage. However, as mitochondrial metabolism is not yet affected, cytotoxicity is not detected by the MTT assay[36].

On the other hand, necrosis, a passive and disordered cell death generated in response to physical damage or severe toxic stress, causes detrimental consequences such as induction of inflammation[37]. In our study, exposure of PMNs to PM_{10} induced necrosis, as demonstrated by increased red fluorescence of PI compared to control (Fig. 1c). Previous studies have described the necrotic effects of PM_{10}[38]. In this study, PM_{10} exposure in the A549 cell line induced necrotic effects, probably due to the high metals content of PM. Therefore our data suggest that PM_{10} can generate different effects on PMNs, ultimately producing indirect toxicity.

Several factors are likely to contribute to the indirect toxic effects of PM, among which the most important may be the size and chemical composition[39]. However, there are no other studies on the cytotoxic effects of PM_{10} in neutrophils; some investigations have been carried out in vascular endothelial and epithelial cell lines of the respiratory tract, which corroborate the cytotoxic effects of PM_{10}[40]. Likewise, in the BALF of murine models exposed to PM_{10}, an increase in LDH concentrations has been demonstrated, evidencing epithelial injury[41]. Thus, exposure to these particles has been correlated with effects on human health[31,42]. Another study found that after a steel mill's closure, PM_{10} levels were decreased, and lower cases of respiratory admissions to hospitals were reported[42]. Interestingly, PM_{10} collected during this period produced less cytotoxicity and inflammatory response in human respiratory epithelial cells[43]. In addition, minor lung damage and neutrophilic inflammation in Sprague–Dawley rats were observed compared to PM_{10} collected while the steel mill was in operation[44].

An additional factor that may contribute to the indirect toxic effects of PM_{10} is its ability to cause the generation of ROS, which in turn may initiate or exacerbate an inflammatory response. ROS are unstable molecules formed that play a significant role in the degradation of PM_{10} within the phagolysosome[45]. ROS species include hydroxyl radical (OH^-), hypochlorous acid (HOCl), and hydrogen peroxide (H_2O_2)[45,46]. Their overabundance induces oxidative stress and reduces cell viability due to mitochondrial dysfunction, DNA damage, apoptosis, and genotoxicity[47–49]. In this study, we found that neutrophils exposed to PM_{10} presented an increase in ROS production (Fig. 1d). In this sense, our results agree with the study of Hitzfeld et al.[50], who demonstrated that PM_{10} extracts collected in two German cities stimulated the production of ROS in human neutrophils. A recent report suggested that PM_{10} caused significant toxicity in the cardiovascular development of zebrafish larvae, as it led to an increase in the level of ROS and the expression of genes involved in endoplasmic reticulum stress and Nrf2 signaling pathway factors, which are essential in the regulation of cellular oxidative stress[51].

In addition, to the indirect toxic effects of PM_{10} mediated by ROS, a recent study has shown that oxidative stress generated by PM exposure induces inflammatory responses by activating redox-sensitive transcription factors, which can lead to IL-8 expression in human bronchial epithelial cells[52]. Thus, scientific evidence has shown that IL-8 is a critical chemokine in airway inflammation induced by air pollutants[53,54]. In this study, we found up-regulation of IL-8 mRNA and IL-8 secretion in neutrophils exposed to PM_{10} (Fig. 2a,b). The secreted IL-8 mediates neutrophil recruitment into the lung and thus further amplifies inflammation in C57BL/6 mice exposed to cigarette smoke[55]. Oxidative stress and inflammation are essential factors in different respiratory and systemic diseases[56]. Dust particles can induce increased IL-8 transcriptional activity, leading to airway inflammation and exacerbation of asthma symptoms in human adults[57]. Concerning this, different studies have found that practices such as cooking with biomass are associated with increased IL-6, IL-8, and TNF-α, neutrophil infiltration, increased oxidative stress, hypertension, and tachycardia, increasing the risk of cardiovascular diseases[58,59].

NETosis is a process that is highly dependent on ROS production[60]. In the present study, we observed that neutrophils produce NETs in response to PM_{10} than control (Fig. 3a,b). The beneficial role of NETs in infections is clear; however, new evidence has revealed a massive presence of NETs and neutrophils in the sputum of patients with cystic fibrosis and COPD[61,62]. Furthermore, NETs have recently been linked to severe clinical manifestations of COVID-19 and dysregulated immune-thrombosis in response to SARS-CoV-2[63], strongly suggesting the role of NETs in chronic airway diseases. Likewise our findings align with a recent work that found a significant increase in the percentage of human neutrophils forming NETs measured by flow cytometry after stimulation with diesel exhaust particles (another air pollutant)[26]. Similarly, has been found that neutrophils isolated from male BALB/c mice exposed to cigarette smoke produced more NETs in response to PMA compared to control mice[64]. To note the induction of NETs seems to be decreased in neutrophils exposed to 100 µg/mL PM_{10} compared to those exposed to 50 µg/mL (without statistical differences), this may be explained because PM_{10} particles at this concentration form aggregates and thus are less accessible to cells or as shown in Fig. 1c PM_{10} is inducing another type of cell death.

Our results suggest that exposure of human neutrophils to PM_{10} resulted in altered expression of NETs-associated genes because high expression of the NE gene mRNA, MPO, was observed (Fig. 4a,b). NETs are released by a programmed form of cell death characterized by chromatin decondensation, nuclear membrane disassembly, and cell membrane rupture[65]. NE and MPO play a central role in coordinating these processes[66]. Likewise, PAD_4 is involved in NETosis through histone citrullination and chromatin decondensation[65,66]. However, our results show a negative regulation of the PAD_4 gene in neutrophils stimulated with PM_{10} (Fig. 4c).

In correlation to our results, scientific evidence demonstrates that various activating stimuli can mediate histone citrullination and PAD_4 requirement in NETs formation[67]. Therefore, there is a large discrepancy in the role of PAD_4 in NETs production, with several authors reporting that the involvement of PAD_4 depends on the type of stimulus used[68,69]. In our investigation, PM was used to induce NETs. It is well known that PM_{10} from urban areas could contain lipopolysaccharide and activate NF-κB through the MyD88 pathway, dependent on the bound LPS[70]. Thus, NF-κB has been reported to regulate PAD_4 in mouse and human cells differentially. For example, the human cell line HL-60 treated or not with TNF-α to activate the canonical NF-κB pathway showed a significant increase in IL-8 levels (a marker of NF-κB activation) and a significant decrease in PAD_4 levels[71].

Consistent with the in vitro results, our in vivo findings showed that five days after intranasal instillation with 100 μg/mL PM_{10}, BALB/c mice exhibited airway inflammation and increased cellularity and PMNs in BALF (Fig. 5). In lung tissue, an increase in CXCL1 mRNA expression was evident and histological analysis showed pathological alterations and neutrophil recruitment with a moderate infiltration in the lung airspaces of PM_{10}-exposed mice (Fig. 5). This inflammatory response could be partly due to the ability of PM_{10} to generate cellular cytotoxicity confirmed in vitro. Furthermore, although the composition of PM varies by region, our data are consistent with previous reports on neutrophilic inflammation. In the study by Yang et al.[72], BALF and immunohistochemical staining of lung tissues in mice 12 h after PM_{10} exposure showed visible signs of inflammation neutrophil infiltration in the airways. Likewise, CXCL1 (human IL-8 homolog) expression has been demonstrated in the BALF of C57BL/6 mice exposed to diesel exhaust particulate matter (DEP), a significant component of particulate matter that induces neutrophil-dominant inflammatory responses. In general, acute neutrophil-dominated inflammation is a mechanism of the immune system to eliminate pathological agents, including PM. However, IL-8 secretion, infiltration, and overactivation of neutrophils at sites of inflammation provide a feedback loop leading to uncontrolled inflammation that could promote the development or progression of asthma, COPD, and other diseases[73].

It is important to note that previous studies have suggested that PM_{10} exposure induces inflammatory cytokines production and immune cells recruitment, possibly due to their high content of transition metals[74]. On the other hand, recently published experimental studies evaluating the effects of air pollutant particles on inflammatory responses have focused mainly on $PM_{2.5}$, which includes most of the particles derived from fossil fuel combustion[75]. While these particles pose a risk to human health, coarser particles such as PM_{10} should not go unnoticed because, as our data show, they are potentially cytotoxic and inflammatory.

In conclusion, our results suggest that neutrophils are critical players in PM_{10}-induced inflammatory events. These molecules induce cytotoxicity, infiltration, and activation of neutrophils, leading to ROS production, NETs release, and MPO, NE, and IL-8 expression. Future studies should evaluate the effect of PM_{10} on other neutrophils responses, including their phagocytosis and tissue migration.

Data availability

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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Authors contributions

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Competing interests

The authors declare no competing interests.

Additional information

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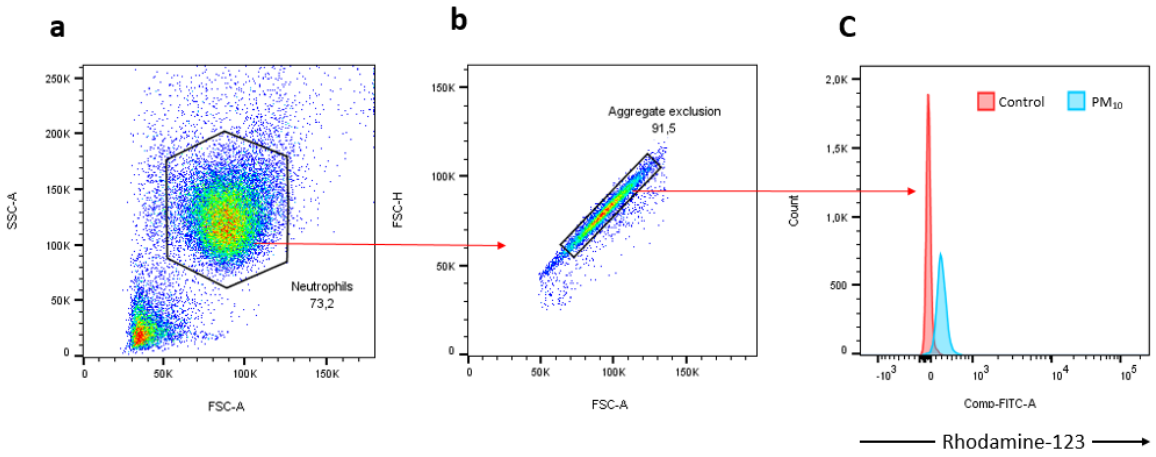

Fig. S1 Flow cytometry gating strategy for ROS quantification in neutrophils exposed to PM_{10}. (**A**) Neutrophils were separated by their higher FSC and SSC properties than other leukocyte/nucleate populations. (**B**) Aggregate exclusion was performed using a dot plot of FSC-A versus FSC-H. (**C**) Rhodamine-123 fluorescence was determined in neutrophils under control (unstimulated) and PM_{10}-stimulated conditions. The increase in fluorescence resulting from ROS production is indicated by a rightward shift on the x-axis. The graphs is representative of 7 independent experiments under the same conditions.

Table 2. ROS production (%)

	Control -	**Control +**	**PM_{10} (µg/mL)**			
	No stimulus	Zymosan	50	10	1	0.1
Mean	1,293	25,26	21,35	21,23	21,20	18,24
Std. Deviation	1,890	8,063	11,78	10,23	11,99	4,678

Conclusión general

En general, los resultados de este trabajo aumentan los conocimientos sobre la respuesta inmune relacionadas a la exposición de contaminantes ambientales como el PM, en primer lugar a través de la revisión sistemática de estudios preclínicos que evaluaron contaminantes ambientales, demostramos que esta evidencia preclínica es dispersa y heterogénea, sin embargo el análisis de estos estudios sugiere que la exposición a contaminantes ambientales induce el reclutamiento de neutrófilos, y que los mecanismos inmunopatologicos mediados por estas células incluyen la activación celular, la producción de ROS y liberación de citocinas/quimioquinas inflamatorias, que lleva a la muerte celular, estrés oxidativo e infiltrados inflamatorios en los pulmones. En segundo lugar a través la evaluación experimental *in vitro* e *in vivo* demostramos que la exposición a PM_{10}, disminuye la viabilidad celular, que se evidencia con la liberación de LDH, además provoca un aumento de ROS, liberación de IL-8 y aumenta la liberación de NETs y expresión de enzimas como MPO y NE. Mecanismos que pueden llevar al infiltrado inflamatorio de neutrófilos observado en las histopatologías y BALF de ratones BALB/c expuestos a PM_{10}, lo que pone de relieve los mecanismos a través de los cuales la exposición a PM puede ejercer sus efectos nocivos.

Perspectivas

A partir de la revisión sistemática y el análisis experimental, las perspectivas se orientan en, evaluar en neutrófilos el efecto *in vitro* de la exposición a PM_{10} sobre la expresión de otras citocinas pro-inflamatorias como IL-1α, IL-1β, IL-6, IL-16, IL-18, TNF y MIF; la expresión de citocinas antiinflamatorias como IL-1ra, IL-4,TGFβ1, TGFβ2; citoquinas inmunorreguladoras como IL-22, IL-23 y factores de crecimiento como G-CSF. Así mismo, sería interesante

analizar el efecto del PM_{10} sobre la expresión de quimioquinas (CXCL1, CXCL2, CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL3, CCL4, CCL17, CCL18, CCL19, CCL20, CCL22, CCL23) y moléculas de adhesión (L-selectin, PSGL1, ESL1, CD44) importantes en la cascada de reclutamiento de neutrófilos. Por otro lado también se podría evaluar el efecto del PM_{10} sobre la fagocitosis y destrucción intracelular de bacterias o virus mediado por neutrófilos. Además, es necesario explorar tipos de muerte celular como la piroptosis y necroptosis, y con ello evaluar mecanismos de regulación ya que pueden ser un tratamiento muy valioso y prometedor para enfermedades respiratorias relacionadas con la exposición a contaminantes ambientales. En este mismo sentido, otro punto a evaluar en un modelo animal seria los inhibidores de la formación de NETs e incluso inhibidores del reclutamiento de neutrófilos (ejemplo inhibidores de CXCR1 y CXCR2), ya que estos pueden ayudar a reducir la tasa general de mortalidad por enfermedades respiratorias y cardiovasculares relacionadas con el PM.