Original article

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The effects of prolonged exposure to cigarette smoke on phenotype and function of innate immune cells in the lungs

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Cigarette smoke contains various toxic compounds that can alter the phenotype and function of immune cells within the lungs. To investigate how extended exposure to cigarette smoke affects innate immune cells (including neutrophils, macrophages, dendritic cells (DCs), natural killer (NK) cells, and natural killer T (NKT) cells), we exposed BALB/c mice to cigarette smoke

for 4 or 8 weeks, with control animals being exposed to air. The cigarette smoke was generated using an LM1 Borgwaldt smoking machine. Flow cytometry analysis and intracellular staining of lung-infiltrated immune cells revealed that prolonged exposure to cigarette smoke notably increased the number of inflammatory CD14 receptor-expressing neutrophils, TNF-α-producing NK and NKT cells, and CD40-expressing macrophages. Additionally, significantly higher numbers of IL-12 and IL-23-producing DCs were observed in the lungs of mice exposed to cigarette smoke for 8 weeks compared to those exposed to air. Moreover, a greater number of TNF-α, IL-12, and IL-23-producing macrophages, along with TNF-α-producing neutrophils, NK, and NKT cells, were found in the lungs of mice exposed to cigarette smoke for 8 weeks compared to those exposed for 4 weeks. In conclusion, prolonged cigarette smoke exposure promotes an inflammatory phenotype in lung-infiltrating innate immune cells, contributing to the progression and worsening of inflammatory lung diseases.

Keywords: cigarette smoke; lungs; macrophages; neutrophils; dendritic cells

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Uticaj produženog izlaganja duvanskom dimu na fenotip i funkciju ćelija urodjene imunosti u plućima

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Dim cigareta sadrži razne štetne supstance koje mogu menjati fenotip i funkciju imunskih ćelija u plućima. Kako bismo bolje razumeli kako produženo izlaganje dimu cigareta utiče na ćelije urodjene imunosti (neutrofile, makrofage, dendritske ćelije, urodjenoubilačke (NK) i urodjenoubilačke T (NKT) ćelije) u plućima, koristili smo BALB/c miševe koji su bili izloženi dimu cigareta tokom 4 ili 8 nedelja. Dim cigareta generisan je pomoću LM1 Borgwaldt mašine.

Miševi iz kontrolne grupe bili su izloženi vazduhu. Korišćenjem protočne citometrije i intracelularnim bojenjem imunskih ćelija infiltriranih u plućima, uočeno je da produženo izlaganje dimu cigareta značajno povećava prisustvo inflamacijskih neutrofila koji eksprimiraju CD14 receptor, NK, NKT ćelija koje produkuju faktor nekroze tumora (TNF-α), kao i makrofaga koji eksprimiraju CD40 receptor. Takođe, značajno veći broj IL-12 i IL-23-produkujućih dendritskih ćelija primećen je u plućima miševa koji su bili izloženi dimu cigareta tokom 8 nedelja u poređenju sa životinjama izloženim vazduhu. Pored toga, značajno veći broj makrofaga koji proizvode TNF-α, IL-12, IL-23, kao i značajno veći broj neutrofila, NK i NKT ćelija koji proizvode TNF-α primećen je u plućima miševa koji su bili izloženi dimu cigareta tokom 8 nedelja u poređenju sa životinjama koje su bile izložene dimu cigareta tokom 4 nedelje. U zaključku, produženo izlaganje dimu cigareta pospešuje stvaranje inflamacijskog fenotipa u urođenim imunskim ćelijama u plućima, podstičući progresiju i pogoršanje inflamacijskih oboljenja pluća.

Ključne reči: dim cigareta; pluća; makrofagi; neutrofili; dendritske ćelije

Introduction

Cigarette smoke is composed of numerous harmful substances that severely impact the respiratory system and play a major role in the onset and advancement of lung-related diseases (1–5). Formaldehyde, acrolein, benzene and heavy metals provoke oxidative stress-induced damage of alveolar epithelial cells, causing structural changes in the lungs (1, 2). Continuous and long-term exposure to these harmful components can lead to the destruction of alveolar walls, resulting in the development of emphysema (3, 4). Additionally, alarmins released from injured alveolar epithelial cells can recruit fibroblasts and trigger excessive collagen deposition, leading to the development of pulmonary fibrosis (4). Moreover, as highly carcinogenic compounds, polycyclic aromatic hydrocarbons from cigarette smoke can cause mutations in lung parenchymal cells, paving the way for the development of lung cancer (5).

Cigarette smoke significantly influences the microbiome of the respiratory tract, leading to the development of dysbiosis, an imbalance in the microbial communities within the lungs (6, 7). Long-term exposure to the harmful chemicals of cigarette smoke creates a hostile environment for beneficial bacteria while promoting the growth of pathogenic microorganisms (6, 7). This dysbiotic state can impair the lungs' ability to defend against microbial pathogens and may induce the detrimental immune response in the lungs (6,

7). The inflammatory response triggered by the altered microbiome could create a vicious cycle, where the inflammation favors the persistence of harmful microbes, exacerbating lung damage and promoting the development and progression of chronic pulmonary diseases (8).

Importantly, harmful components of cigarette smoke may disrupt the normal homeostasis of the immune system in the lungs, enabling development of immune cell-driven inflammatory diseases (9). An altered expression of adhesion molecules (selectins and integrins) was observed on the endothelial cells that were continuously exposed to cigarette smoke (10). Accordingly, prolonged exposure to cigarette smoke affects migration of circulating immune cells in the lungs, impairing their capacity to elicit appropriate anti-microbial immune response (9). Additionally, harmful components of cigarette smoke could trigger a potent, detrimental immune response in the lungs, by inducing the massive release of pro-inflammatory cytokines from lung tissue-resident innate immune cells (natural killer (NK), natural killer T (NKT) cells, macrophages and dendritic cells (DCs)), setting off a cycle of chronic inflammation (11). Continuously activated innate immune cell orchestrate detrimental inflammatory response by promoting activation of adaptive immune cells, T and B lymphocytes (11, 12). Persistent activation of immune cells within the lungs over an extended period leads to significant tissue injury and structural changes in the airways, ultimately contributing to the

emergence of chronic inflammatory lung conditions such as asthma and chronic obstructive pulmonary disease (COPD) (9, 13).

In order to better understand how prolonged exposure to cigarette smoke affects lung neutrophils, macrophages, DCs, NK and NKT cells, we herewith used a well-established murine model of lung inflammation induced by cigarette smoke (3, 11) to examine the variations in phenotype and cytokine production of these innate immune cells following one and two months of cigarette smoke exposure.

Material and Methods

Experimental animals

This study involved the use of BALB/c mice aged between six and eight weeks. The animals were maintained under specific-pathogen-free conditions in a climate-regulated facility, featuring a 12-hour alternating light/dark cycle, at the Animal Facility of the Faculty of Medical Sciences, University of Kragujevac, Serbia. They were provided with unlimited access to clean water and a standard diet formulated for laboratory rodents. All procedures involving the animals complied with ethical standards and were approved by the Animal Ethics Committee of the same institution, ensuring the welfare of the animals throughout the study.

Design of the study

The mice were randomly assigned to three groups: a control group exposed only to ambient air, and two experimental groups subjected to cigarette smoke for either 4 weeks (Group 1) or 8 weeks (Group 2). Cigarette smoke was produced using Marlboro Red (MR) cigarettes and delivered via an LM1 Borgwaldt smoking apparatus. Exposure followed the Health Canada Intense (HCI) puffing protocol, which involves drawing nine puffs per cigarette with each puff lasting 2 seconds and delivering 55 mL of smoke in a bell-shaped flow profile (27.5 mL/s), taken every 30 seconds with blocked filter vents, down to a butt length of 36 ± 0.5 mm. Prior to use, MR cigarettes were conditioned for a minimum of 48 hours at 60 ± 3% relative humidity and 22 ± 1°C. Smoke was administered in an environment maintained at 60 ± 5% relative humidity and 22 ± 2°C, as per ISO 3402:1999 standards (11). The animals were euthanized after completing the exposure protocol, specifically 24 hours following their final exposure to cigarette smoke.

Isolation of immune cells from the mice lungs

Initially, the mice lungs underwent enzymatic digestion. Once removed, the lungs were placed in a petri dish, rinsed with phosphate-buffered saline (PBS), and finely minced using a scalpel or razor blade. The resulting tissue

fragments were transferred into 15 mL conical tubes containing a digestion mixture composed of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.5 mg/mL collagenase IV and 1 mg/mL DNase. The samples were incubated at 37°C in a water bath for 45 minutes with continuous agitation at 80 rpm. After digestion, the cell suspensions were passed through 100 µm nylon mesh filters into 50 mL tubes containing DMEM enriched with 10% fetal bovine serum (FBS), followed by centrifugation. The resulting cell pellets were separated from the supernatant and treated with 5 mL of lysis buffer for 5 minutes to remove red blood cells. A subsequent centrifugation step was performed, after which the cells were resuspended in fresh medium and prepared for intracellular staining and flow cytometric evaluation (11).

Flow cytometry analysis and intracellular staining of immune cells

Immune cells extracted from the lungs of mice exposed to either ambient air or cigarette smoke were assessed for a range of surface and intracellular markers using flow cytometry (11). To reduce potential cell loss or damage resulting from tissue dissociation processes, viable cells were selectively isolated using the MACS® Dead Cell Removal Kit (Miltenyi Biotec, Bergisch Gladbach, Germany; Cat. No. 130-090-101). For this process, single-cell suspensions were incubated with 100 µL of Dead Cell Removal MicroBeads per 10 million cells at room temperature for 15 minutes. The mixture was then

passed through MS columns pre-filled with MACS Binding Buffer, allowing live cells to pass through while dead cells were retained.

To prevent nonspecific interactions with Fc receptors, the isolated live cells were pre-treated with anti-mouse CD16/CD32 (BD Fc Block), using 1 µg per million cells in 100 µL of staining buffer composed of Dulbecco's PBS (DPBS) without calcium or magnesium, 1% heat-inactivated fetal bovine serum (FBS), and 0.09% sodium azide. This incubation was carried out for 15 minutes at 4°C. After blocking, cells were washed and stained with fluorescently labeled antibodies.

A total of 1 × 10^6 cells were incubated for 30 minutes in the dark at 4°C with antibodies targeting CD45, CD3, CD4, CD8, F4/80, CD11c, Ly6G/C, CD49, CD14, CD40, and TLR-2. These antibodies were conjugated with various fluorophores, including FITC, PE, PerCP, and APC (BD Biosciences, San Jose, CA, USA). The staining process concluded with two washing steps using staining buffer, followed by centrifugation to pellet the cells for subsequent analysis.

For intracellular marker detection, cells were activated using 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 500 ng/mL ionomycin for a duration of 5 hours. After this stimulation period, GolgiStop (BD Biosciences, San Jose, CA, USA; Cat. No. 554715) was introduced to inhibit protein transport. Cells were subsequently fixed and permeabilized using the BD Cytofix/Cytoperm™

Fixation/Permeabilization Kit (Cat. No. 554714), with incubation carried out at 4°C for 20 minutes. Following fixation, cells were rinsed twice with BD Perm/Wash™ buffer and centrifuged.

The permeabilized cells were then resuspended in 50 µL of BD Perm/Wash™ buffer containing pre-titrated fluorochrome-conjugated antibodies targeting inducible nitric oxide synthase (iNOS), NLRP3, TNF-α, IL-12, and IL-23. These antibodies, specific for mouse antigens, were labeled with FITC, PE, PerCP, or APC fluorophores (BD Biosciences, San Jose, CA, USA). Staining was carried out at 4°C in the dark for 30 minutes. Afterward, cells underwent two additional washes with Perm/Wash™ buffer and were finally resuspended in staining buffer for flow cytometric assessment. Data collection was conducted using a BD FACSCalibur system, and results were analyzed using Flowing software (Turku Bioscience Centre).

Statistical analysis

All statistical evaluations were performed using SPSS software version 21.0 for Windows (SPSS, Inc., Chicago, IL, USA). To determine variations in group means, a one-way analysis of variance (ANOVA) was employed. In cases where ANOVA revealed statistically significant differences, post hoc comparisons between specific groups were carried out using two-tailed Student's t-tests, applying the Tukey method to adjust for multiple testing. Results are expressed as mean values ± standard error of the mean (SEM). A

p-value less than 0.05 was regarded as indicating statistical significance, and all tests were two-tailed.

Results

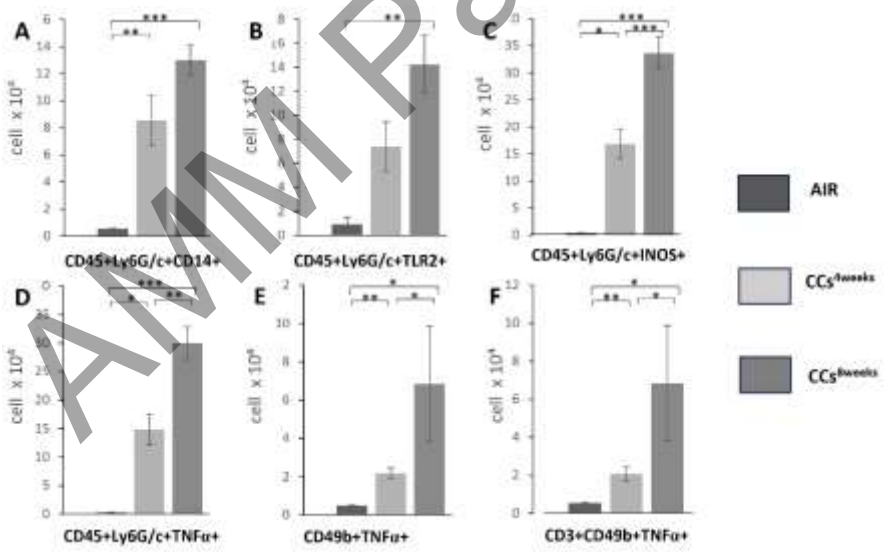
Extended cigarette smoke exposure promotes accumulation of inflammatory neutrophils, NK, and NKT cells in lung tissue

Flow cytometric evaluation of immune cells isolated from the lungs demonstrated that chronic exposure to cigarette smoke markedly elevated the levels of inflammatory neutrophils, natural killer (NK) cells, and natural killer T (NKT) cells in the lungs of treated mice (Figure 1). A substantial increase in $CD14^{+}CD45^{+}Ly6G^{+}$ neutrophil populations was observed in the lungs of mice exposed to smoke for 4 weeks (Figure 1A; $p<0.01$) and even more so after 8 weeks of exposure (Figure 1A; $p<0.001$), in comparison to the air-exposed control group. In addition, cigarette smoke inhalation for 8 weeks significantly enhanced the presence of neutrophils expressing TLR-2 ($Ly6G^{+}CD45^{+}$) in the lung tissue (Figure 1B; $p<0.01$).

Further intracellular staining revealed that ongoing smoke exposure drove these neutrophils toward a more pro-inflammatory profile (Figure 1C-D). Mice exposed to cigarette smoke for 8 weeks exhibited a markedly higher number of inducible nitric oxide synthase (iNOS)-positive neutrophils (Figure 1C; $p<0.001$) as well as TNF-α-secreting $CD45^{+}Ly6G^{+}$ neutrophils (Figure 1D; $p<0.01$), compared to those exposed for only 4 weeks.

In a similar manner, prolonged exposure to cigarette smoke led to an increased presence of inflammatory TNF-α-producing CD49b+ NK cells (Figure 1E) and CD49b+CD3+ NKT cells (Figure 1F) in the lungs of mice. Significantly higher numbers of TNF-α-producing NK and NKT cells were detected in the lung tissue of mice exposed to cigarette smoke for both 4 weeks (Figure 1E-F; $p<0.01$) and 8 weeks (Figure 1E-F; $p<0.05$), compared to the control group exposed only to air. Notably, extended cigarette smoke exposure notably increased TNF-α production by both NK and NKT cells in the lungs, with the 8-week exposure group showing a significantly higher proportion of TNF-α-producing CD49b+ and CD49b+CD3+ cells when compared to the 4-week exposure group (Figure 1E-F; $p<0.05$).

Figure 1. The impact of extended cigarette smoke exposure on the presence of neutrophils, NK, and NKT cells in the lungs of mice.



Flow cytometric analysis of immune cells in the lungs revealed that BALB/c mice exposed to cigarette smoke for 8 weeks had the highest levels of CD45+Ly6G/C+ neutrophils expressing CD14 (A), TLR-2 (B), iNOS (C), and TNF-α (D), as well as the highest numbers of TNF-α-producing NK (E) and NKT cells (F), compared to mice exposed to cigarette smoke for 4 weeks or air-exposed control mice. Data are shown as Mean ± SEM; n = 6 mice per group; *p < 0.05, **p < 0.01, *** p < 0.001.

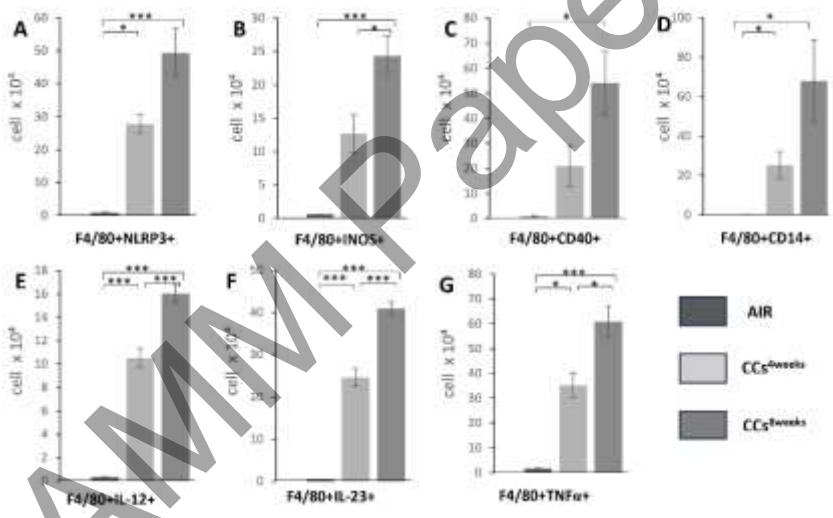
Chronic cigarette smoke exposure led to the development of an inflammatory profile in lung macrophages.

Analysis of lung cell populations revealed that extended exposure to cigarette smoke significantly increased the number of inflammatory macrophages (Figure 2). The presence of NLRP3 inflammasome-activated F4/80+ macrophages was notably higher in mice exposed to smoke for 4 weeks (Figure 2A; $p<0.05$) and 8 weeks (Figure 2A; $p<0.001$), compared to the control group exposed to air. Additionally, after 8 weeks of smoke exposure, there was a marked increase in the number of F4/80+ macrophages expressing iNOS (Figure 2B; $p<0.001$), CD40 (Figure 2C; $p<0.05$), and CD14 (Figure 2D; $p<0.05$) in the lungs of the experimental animals.

Intracellular staining further demonstrated that prolonged exposure to cigarette smoke prompted the production of pro-inflammatory cytokines such

as IL-12, IL-23, and TNF-α in lung macrophages (Figure 2D-F). Significantly elevated levels of IL-12- (Figure 2D; $p<0.001$), IL-23- (Figure 2E; $p<0.001$), and TNF-α-producing macrophages (Figure 2F; $p<0.05$) were detected in the lungs of mice exposed to cigarette smoke for 4 weeks, compared to those exposed to air. This effect was more pronounced in animals exposed to smoke for 8 weeks, with substantially higher numbers of IL-12- (Figure 2D; $p<0.001$), IL-23- (Figure 2E; $p<0.001$), and TNF-α-producing F4/80+ macrophages (Figure 2F; $p<0.05$) observed in the 8-week exposure group compared to the 4-week exposure group.

Figure 2. The lasting impact of cigarette smoke on the phenotype and functionality of lung macrophages



A notable increase in the total number of F4/80+ macrophages expressing NLRP3 inflammasome (A), iNOS (B), CD40 (C), CD14 (D), and producing IL-

12 (E), IL-23 (F), and TNF-α (G) was observed in BALB/c mice exposed to cigarette smoke for 4 or 8 weeks, compared to the air-exposed control group. Data are presented as Mean ± SEM; n = 6 mice per group; *p < 0.05, **p < 0.01, *** p < 0.001.

Chronic cigarette smoke exposure increased the number of inflammatory DCs in the lungs of mice

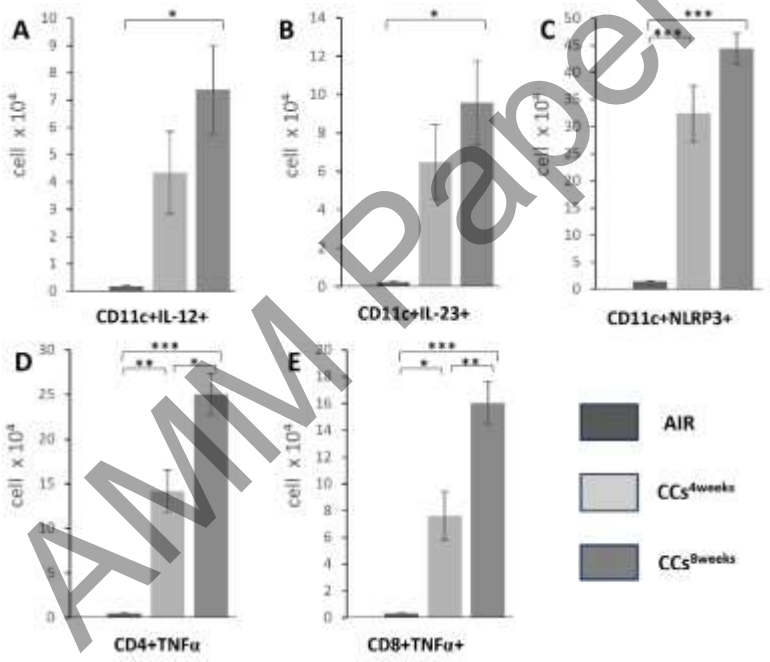
Similar to the effects seen in macrophages, sustained exposure to cigarette smoke promoted the development of an inflammatory phenotype in dendritic cells (DCs) in the lungs (Figure 3). A significant rise in the numbers of IL-12- and IL-23-producing CD11c+ DCs (Figure 3A-B; $p<0.05$) was observed in the lungs of mice exposed to smoke for 8 weeks, compared to the air-exposed control group. Additionally, the total count of NLRP3+CD11c+ DCs was significantly higher in the lungs of mice exposed to cigarette smoke for both 4 weeks and 8 weeks (Figure 3C; $p<0.001$), compared to the control group.

The substantial increase in inflammatory DCs in the lungs coincided with a significant rise in the number of inflammatory T cells infiltrating the lungs (Figure 3D-E). Notably, the number of TNF-α-producing helper CD4+ T cells was significantly higher in mice exposed to cigarette smoke for 4 weeks (Figure 3D; $p<0.01$) and 8 weeks (Figure 3D; $p<0.001$). Similarly, a marked

increase in TNF-α-producing cytotoxic CD8+ T cells was observed in the lungs of mice exposed to cigarette smoke for both 4 weeks (Figure 3E; $p<0.05$) and 8 weeks (Figure 3E; $p<0.001$).

Crucially, prolonged cigarette smoke exposure significantly enhanced TNF-α production in both helper and cytotoxic T lymphocytes, as evidenced by a higher number of TNF-α-producing CD4+ (Figure 3D; $p<0.05$) and CD8+ T cells (Figure 3E; $p<0.01$) in the lungs of mice exposed to smoke for 8 weeks, compared to those exposed for 4 weeks.

Figure 3. The effect of extended cigarette smoke exposure on the presence of DCs in the lungs.



Flow cytometry analysis of immune cells infiltrating the lungs showed that the highest numbers of IL-12 (A) and IL-23-producing (B) NLRP3 inflammasome-

activated (C) CD11c+DCs, as well as TNF-α-producing CD4+ T helper (D) and cytotoxic CD8+ T cells (E), were found in the lungs of BALB/c mice exposed to cigarette smoke for 8 weeks, compared to those exposed for 4 weeks or the air-exposed control group. Data are presented as Mean ± SEM; n = 6 mice per group; *p < 0.05, **p < 0.01, *** p < 0.001.

Discussion

Cigarette smoke exposure leads to an increased influx of neutrophils into the lungs, where they become activated and release various pro-inflammatory cytokines and chemokines (14, 15). These smoke-activated neutrophils generate reactive oxygen species (ROS) and proteolytic enzymes, which can damage lung tissue and worsen chronic inflammatory conditions such as COPD and asthma (14). Moreover, cigarette smoke alters neutrophil functions, hindering their ability to undergo apoptosis, thereby prolonging their lifespan in the lungs and contributing to ongoing inflammation (15). In our study, we observed that prolonged exposure to cigarette smoke enhanced the inflammatory response of neutrophils in the lungs. Specifically, long-term smoke exposure significantly increased the number of CD14 and TLR-2-expressing neutrophils, which play a crucial role in initiating and exacerbating lung inflammation. These receptors facilitate the detection of pathogen-

associated molecular patterns, leading to neutrophil activation and a surge in ROS and inflammatory cytokine production, which further damages lung tissue (16). Consistent with these findings, we noted a significant increase in the number of iNOS-expressing and TNF-α-producing neutrophils in the lungs of mice exposed to cigarette smoke for 8 weeks.

Furthermore, our data showed that prolonged cigarette smoke exposure led to an elevated presence of TNF-α-producing NK and NKT cells in the lungs. NK cells are essential for recognizing and eliminating infected or transformed cells through cytotoxic mechanisms (17), while NKT cells, with features of both T and NK cells, contribute significantly to the development and progression of lung inflammation (17). Upon activation, these cells release large amounts of TNF-α, which induces the expression of adhesion molecules such as E and P selectins on endothelial cells, thereby promoting the recruitment of circulating leukocytes into the lungs (8, 17). The increase in TNF-α-producing NK and NKT cells due to cigarette smoke exposure may play a critical role in driving tissue damage and worsening inflammatory lung diseases. Chronic inflammation caused by these cells can lead to the destruction of alveolar structures, which is a key factor in the development of emphysema in lungs exposed to cigarette smoke over time (9).

Macrophages play a crucial role in the immune defense of the lungs, involved in phagocytosing pathogens, presenting antigens, and modulating inflammatory responses (18). When exposed to cigarette smoke, a variety of

toxic chemicals are introduced that can alter their function and characteristics, leading to an imbalance in the macrophage-driven immune response (19). Macrophages exposed to cigarette smoke show reduced phagocytic activity, weakening their ability to eliminate inhaled microbial threats (19). This impairment is partly due to the oxidative stress caused by cigarette smoke, which interferes with the signaling pathways required for macrophage activation and phagocytosis (19, 20). In our study, we found that prolonged exposure to cigarette smoke led to a higher number of macrophages with an activated NLRP3 inflammasome and increased expression of iNOS.

Additionally, cigarette smoke affects the ability of lung macrophages to present antigens effectively (11, 19). The exposure to smoke can alter the expression of co-stimulatory molecules, which hampers their capacity to present antigens to T cells, potentially leading to ineffective T cell activation and contributing to T cell-mediated lung inflammation (8). Our results align with this, showing a significant increase in macrophages expressing the CD14 receptor and the co-stimulatory CD40 molecule in the lungs of mice exposed to cigarette smoke for 8 weeks.

Cigarette smoke significantly impacts professional antigen-presenting cells in the lungs, such as DCs and macrophages, by promoting a pro-inflammatory state marked by the elevated production of cytokines like TNF-α, IL-12, and IL-23 (8, 11, 19). These cytokines contribute to the sustained inflammatory environment in the lungs by driving Th1 and Th17 cell-mediated lung damage

(21, 22). In our study, we found that continuous exposure to cigarette smoke substantially increased the number of macrophages producing TNF-α, IL-12, and IL-23, as well as DCs expressing NLRP3 and producing IL-12 and IL-23, thereby aiding the development and progression of Th1 and Th17 cell-induced inflammation in the lungs. Notably, the increased presence of IL-12 and IL-23-producing DCs was associated with a higher count of TNF-α-producing CD4+ and CD8+ T cells in the lungs of mice exposed to cigarette smoke for 8 weeks. These T cells, in a TNF-α-dependent manner, trigger apoptosis in alveolar epithelial cells, resulting in alveolar damage and compromised gas exchange (23, 24). Moreover, TNF-α produced by T cells enhances the expression of E and P selectins on lung endothelial cells, facilitating the recruitment of circulating leukocytes into the lungs, which further intensifies the inflammatory response and worsens lung injury (24).

Conclusion

In summing up, extended exposure to cigarette smoke promoted the development of an inflammatory phenotype in innate immune cells within the lungs, contributing to the progression and worsening of immune cell-driven inflammatory lung diseases.

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