Effects of combustible cigarettes and electronic nicotine delivery systems on the development and progression of chronic lung inflammation in mice

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Abstract

Introduction: Although detrimental effects of combustible cigarettes (CCs) on the progression of lung inflammatory diseases are well known, changes in electronic nicotine delivery systems (ENDS)-exposed lung-infiltrated immune cells are still unrevealed.

Methods: The analysis of blood gas parameters, descriptive and quantitative histology of lung tissues, determination of serum cytokines, intracellular staining and flow cytometry analysis of lung-infiltrated immune cells were used to determine the differences in the extent of lung injury and inflammation between mice from experimental (CC and ENDS-exposed animals) and control group (Air-exposed mice).

Results: Continuous exposition to either CCs or ENDS induced severe systemic inflammatory response, increased activation of NLRP3 inflammasome in neutrophils and macrophages and enhanced dendritic cell-dependent activation of Th1 and Th17 cells in the lungs. ENDS induced less severe immune response than CCs. Serum concentrations of inflammatory cytokines were significantly lower in the samples of ENDS-exposed mice. Compared to CCs, ENDS recruited lower number of circulating leukocytes in injured lungs and had less capacity to induce CD14/TLR-2-dependent activation of NLRP3 inflammasome in lung-infiltrated neutrophils and macrophages. ENDS-primed dendritic cells had reduced capacity for the generation of Th1 and Th17 cell-driven lung inflammation. Accordingly, extensive immune cell-driven lung injury resulted in severe respiratory dysfunction in CCs-exposed mice, while ENDS caused moderate respiratory dysfunction in experimental animals.

Conclusions: Continuous exposition to either CCs or ENDS induced immune cell-driven lung damage in mice. ENDS triggered immune response which was less potent than inflammatory response elicited by CCs and, therefore, caused less severe lung injury and inflammation.

Keywords: combustible cigarettes; electronic nicotine delivery systems; immune cells; lung injury and inflammation

Implications: This is the first study that compared the effects of CCs and ENDS on lung-infiltrated immune cells. Although both CCs and ENDS elicited systemic inflammatory response, immune cell-driven lung injury and inflammation were less severe in ENDS-exposed than in CC-exposed animals. Continuous exposition to ENDS-sourced aerosols was less harmful for respiratory function of experimental animals than CC-derived smoke**.**

Introduction

The lungs are continuously exposed to the toxic air pollutants which induce oxidative stress in alveolar epithelial cells and trigger detrimental inflammatory response that cause irreversible damage of lung parenchyma [1]. Combustible cigarettes (CCs)-derived smoke contains more than seven thousand chemicals which negatively affect phenotype and function of lung-infiltrated immune cells, paving the way for the development of inflammatory and malignant lung diseases (chronic obstructive pulmonary disease (COPD), emphysema, chronic bronchitis, tuberculosis and lung cancer) [2-4].

Damage-associated molecular patters, massively released from CC-smoke-exposed alveolar epithelial cells, continuously activate immune cells in the lungs [5]. Smoke-primed immune cells secrete large amount of inflammatory cytokines which led to the development of persistent and chronic inflammation in the lungs of CCs users [5]. Continuous exposition to CCs-sourced smoke aggravates on-going inflammatory response in the lungs by modulating migratory properties, phenotype and function of monocytes/macrophages, neutrophils, dendritic cells (DCs), T lymphocytes, natural killer (NK) and natural killer T (NKT) cells [6]. By affecting expression of chemokine receptors, by modulating production of cytotoxic reactive oxygen species (ROS) and nitric oxide (NO) and by altering secretion of inflammatory cytokines (tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin (IL)-1 beta (IL-1β), IL-6, IL-8) in immune cells, CCs elicit immune cell-driven injury of alveolar epithelial cells which result in severe respiratory dysfunction that is frequently observed in long-term CCs users [6].

During the last few years, a large number of CCs users, in order to avoid detrimental effects of cigarette smoking, replace CCs with electronic nicotine delivery systems (ENDS), including e-cigarettes, tobacco heated products (THPs), vaping devices, etc. [7]. CC users switching to ENDS typically use ENDS in a way that maintains similar nicotine levels as during CC use [8-9]. By heating tobacco instead of burning it, ENDS generate fewer toxins than CCs [9]. After switching to ENDS, exposure to hazardous components of smoking is significantly reduced, with the exception of nicotine levels that were sufficient to satisfy consumer's needs [9-12].

Despite the fact that CC-dependent effects on the progression of lung inflammatory diseases are well known, changes in phenotype and function of ENDS-exposed lung-infiltrated immune cells are still un-revealed. In order to elucidate molecular and cellular mechanisms elicited by long-term use of CCs and ENDS, we herewith used mice model of chronic lung inflammation to compare the extent of lung injuries in CCs and ENDS-exposed mice and to analyze the differences in phenotype and function of immune cells which infiltrated lungs of these animals.

Material and Methods

Animals

Six–to-eight-week-old BALB/c mice were used in this study. Mice were maintained in animal facility of Faculty of Medical Sciences, University of Kragujevac, Serbia in a temperature-(22 ± 2 °C) controlled pathogen-free environment with 12/12 hour light–dark cycles. Fresh water and basic laboratory food were provided ad libitum. Human care was provided to all animals and all experimental procedures were approved by and performed in accordance with the ethical

guidelines of the Animal Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

Study design

Mice were randomly divided into two experimental (CC and ENDS-exposed mice) and one control group (Air-exposed mice). Both sex were included and divided evenly between groups. Mice from CC group were exposed to the smoke from 1R6F cigarettes (standardized experimental tobacco cigarette, University of Kentucky) generated by LM1 Borgwaldt smoking machine while animals from ENDS group were exposed to the IQOS-sourced aerosols generated by Borgwaldt LM4 machine.

Total number of used 1R6F cigarettes and IQOS sticks were determined according to nicotine dosimetry measurements. Increase in nicotine concentration in the blood of CCs and ENDS consumers depends on smoking parameters (inhaled volume, duration of smoke hold, frequency of puffing) and varies between 5-30 ng/ml (for single CC) and 9.31-30.55 ng/ml (for single IQOS stick) [13]. Blood nicotine levels decline rapidly within the 20 minutes after cigarette use due to rapid tissue distribution of nicotine to all body tissues [14]. Therefore, in order to mimic average nicotine levels in the bloodstream of CC and ENDS-exposed animals, nicotine concentrations were determined in 100% aqueous aerosol extracts (AqEs) of 1R6F cigarettes and IQOS sticks [15]. Amount of nicotine was measured by UPLC-ESI-TQD (Waters Acquity) with an Acquity UPLC® HSS T3 1.8 µm – 2.1 × 100 mm column, operating in Multiple Reaction Monitoring (MRM) and positive ion mode. Measurement on 100% diluted AqE samples showed nicotine concentrations of 12.8 µg/ml for 1R6F and 8.4 µg/ml for IQOS [15], indicating that CC and ENDS exposure regimens used in this study produced similar nicotine levels.

The smoke and aerosol generated by each 1R6F cigarette and IQOS stick were captured in line on a 44 mm diameter Cambridge filter pads (CFPs) which trap the same amount of mainstream smoke drawn through the cigarettes/IQOS filters by CC smokers, or ENDS-consumers [15]. CFPs collect the condensate of 1R6F cigarette and IQOS stick s smoked or vaped on smoking machines. Due to the fact that mice were exposed to CC-derived smoke or ENDS-derived aerosol in sealed chambers with no chance of smoke- or aerosol-constituent losses and that LM1 smoking and LM4 vaping machines released equal amounts of nicotine after every exposure, concentrations of nicotine in generated smoke/aerosol corresponded to the amount of nicotine in CFPs [15]. Accordingly, after each exposure, CFPs were collected and nicotine was measured in every 16RF and IQOS-exposed CFP. CFPs were weighed before and after smoking/vaping, and mean mass per number of smoked cigarettes represented the value of total particulate matter. Mean value of nicotine amount for 1R6F cigarettes was 1299 µg and for IQOS sticks was 1113 µg and these values are similar to nicotine amounts which were determined in the mainstream smoke of CC and ENDS consumers (1300 µg /cigarette and 1100 µg /IQOS stick) [15-17], indicating clinical relevance of exposure regimens that were used in this study.

CC and ENDS exposure regimens

In experimental studies which investigated the effects of CCs and ENDS on the development and progression of inflammatory diseases, the most frequently used regimes were International Organization for Standardization (ISO) and Health Canada Intense (HCI) smoking regime [15-19]. Considering that better insight into the smoking effects should be gained from higher amount of smoke that can be inhaled, more intense regimen (HCI) was chosen for this study.

To simulate typical average human behavior of CCs and ENDS users, experimental animals were exposed to 5 1R6F cigarettes or 5 IQOS sticks every day for four weeks. During the exposition mice were retained in closed chambers without source of clean air, breathing only the smoke generated by 1R6F cigarettes or aerosol generated by IQOS. 1R6F cigarettes were smoked according to HCI puffing regime (total puff volume of 55 mL and 2 s duration bell shape profile (27.5 mL/s), 1 puff every 30 s with filter vent blocked) for total number of nine puffs and to a butt length of 36 ± 0.5 mm. IQOS were vaped according to HCI puffing regime (1 puff every 30 s, for 6 min, with filter vent blocked) for total number of twelve (1 Heatstick) puffs. 1R6F cigarettes and IQOS sticks were conditioned for at least 48h (60 ±3% relative humidity, 22 ±1 ◦C) before smoke generation, and used in a test atmosphere of 60 ±5% relative humidity, 22 ±2 ◦C according to ISO 3402:1999.

When exposure is finished, mice were placed back into their room with fresh air. Chambers were carefully cleaned after every exposure in order to prevent formation of tar residues. All animals were sacrificed after 4 weeks of daily exposure to CCs or ENDS.

Blood gas analysis

In order to explore whether ENDS affected extracellular acid-base status and gas exchange, blood gas parameters (partial pressure of oxygen in arterial blood (PaO_2), partial pressure of carbon dioxide ($PaCO_2$) in arterial blood and pH) were analyzed. For this purpose, arterial blood samples were obtained from control and experimental animals and analyzed within a few minutes using a test cartridge blood analysis system (Premier GEM 3500, Instrumentation Laboratory, Bedford, Massachusetts, USA).

Histological analysis of lung tissue samples

After euthanasia, the lungs were excised from the chest cavity, rinsed with phosphate buffer solution (PBS), fixed in 10% formalin and embeded in paraffin. Fixed and embedded lung tissues were then sectioned at 5 μm for hematoxylin–eosin (H&E) staining. The degree of lung damage was determined based on pathohistological scores. Severity of damage was graded into four categories (0, 1, 2, and 3) with 0 being the absence of visible damage and 3 being the >50% lung parenchyma affected. For each mouse, total lung injury score was calculated by summing the field scores [18].

Immunohistochemistry

Paraffin-embedded sections of mouse lung tissue were deparaffinized and rehydrated. Heat-induced antigen retrieval was achieved by using citrate buffer (pH = 6.0). Thereafter, tissue sections were rinsed in PBS, incubated first with Hydrogen Peroxide Block for 10 minutes, rinsed again in PBS and then incubated with Protein Blocking buffer for 10 minutes. After blocking, glass slides were rinsed in PBS, stained with biotinylated primary F4/80 and Ly6G (ab15694, Abcam and **13-9668-82,** Invitrogen, respectively) antibodies and incubated overnight. Staining was visualized by using Streptavidin Peroxidase and DAB Substrate and sections were furthermore counterstained with Mayer's hematoxylin. Sections were photomicrographed with a digital camera mounted on light microscope (Olympus BX51, Japan).

Measurement of cytokines in serum samples of experimental animals

The levels of inflammatory cytokines (IL-1β, IL-6, IL-12 and IL-17) were determined in serum samples of mice from control and experimental groups. For this purpose, the blood samples were collected from abdominal aorta of sacrificed mice, centrifuged for 15 min and finally, serum was collected and stored at −80°C. Serum levels of selected cytokines were measured by commercial enzyme-linked immunosorbent assay (ELISA) sets (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Flow cytometry analysis and intracellular staining of immune cells

Immune cells isolated from the lungs of Air, CC and ENDS-exposed mice were screened for various cell surface and intracellular markers by flow cytometry, as described [20]. The MACS® Dead Cell Removal Kit (Miltenyi Biotec, Bergisch Gladbach, Germany; Cat. No. 130-090-101) was used for magnetic cell separation of viable cells. For intracellular cytokine staining, cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 500 ng/mL ionomycin for 5 h and GolgiStop (BD Biosciences, San Jose, CA, USA; Cat. No. 554715) [20]. Flow cytometric analysis was conducted on a BD Biosciences FACSCalibur. Acquired data was analyzed by using the Flowing software analysis program (Turku Bioscience Centre).

Statistics

Statistical analyses were performed using SPSS 21.0 for Windows software (SPSS, Inc., Chicago, IL). Differences between mean values within groups were determined using one-way ANOVA. In cases when ANOVA identified a significant difference, individual differences were examined using two tailed Student's *t* tests with a Tukey correction for multiple comparisons. Data were expressed as the mean ± SEM for each group. All reported p values were 2-sided and $p < 0.05$ was considered statistically significant.

Results

Less severe lung injury and inflammation were observed in ENDS-exposed than in CC-exposed mice

Severe respiratory dysfunction was observed in CC-exposed mice (Fig. 1A-C). Significant decrease in PaO2 (Fig. 1A, F=5,368, p<0.01) and pH (Fig.1B, F=11,186, p<0.05) and significant increase in PaCO2 (Fig., 1C, F=14,563, p<0.01) were observed in the arterial blood of CC-treated animals compared with control, Air-exposed mice, indicating strong disturbing effect of CCs on the gas exchange between the alveoli and lung capillaries. Interestingly, oxygen and carbon dioxide exchange was not notably altered by ENDS since there were no significant differences in arterial blood gas parameters between ENDS and Air-exposed mice (Fig.1A-C). Importantly, significantly higher levels of PaO2 (Fig. 1A, F=5,368, p<0.05) and pH (Fig. 1B, F=11,646, p<0.05) and lower levels of PaCo2 (Fig. 1C, F=14,563, p<0.01) were measured in arterial blood of ENDS-exposed compared to CC-treated mice, suggesting that ENDS which heated processed tobacco induced less severe detrimental effects on respiratory function of experimental animals than CCs.

The alveolar and blood vessel walls were intact and only several lymphocytes were observed in the lung parenchyma of Air-exposed mice (Fig.1Da). On the contrary, inflammation-induced pathological changes were seen in the lungs of CCs and ENDS-treated animals (Fig.1Db-c). Importantly, inflammatory cell infiltration and alveolar destruction were less severe in the lung parenchyma of animals that were exposed to ENDS (Fig.1Dc) than in CC-exposed mice (Fig.1Db). Precisely, partial alveolar wall destruction, widened alveolar septa, capillary dilation and congestion accompanied with *massive* leucocytes' infiltration were observed in the lung tissue-samples of CC-exposed animals (Fig.1Db), while alveolar and capillary structures were mostly preserved and only several foci of inflammatory cells' infiltrates were observed in the lungs of ENDS-exposed mice (Fig. 1Dc). Less severe lung injury and inflammation were confirmed by significantly lower histological score of ENDS-treated compared to CC-treated mice (Fig.1E, F=19,059, p<0,01)).

In line with these results, serum concentrations of IL-1*β, IL*-6, IL-12, and IL-17 which play crucially important role in the development and progression of inflammation-driven airway pathology, were significantly higher in the samples of CCs and ENDS-exposed animals compared to the mice from control group (Fig.1F). Importantly, serum levels of these inflammatory cytokines were significantly lower in the samples of ENDS-exposed mice when compared to CC-treated animals (Fig.1F, p<0.001 for IL-1β, F=903,370, and IL-6, F=5738,091; p<0.05 for IL-12, F=1310,991, and IL-17, F=181,360), confirming that ENDS induced less severe systemic inflammatory response than CCs. It should be noted that there were no sex differences in the extent of lung injury and inflammation between CC and ENDS-exposed animals.

Reduced number of immune cells were noticed in the inflamed lungs of ENDS-exposed than in CC-exposed animals

Increased presence of neutrophils, macrophages, dendritic cells, T lymphocytes, natural killer (NK) and natural killer T (NKT) cells, was noticed in the inflamed lungs of CC and ENDS-treated than in the lungs of Air-treated mice (Fig.1G-N), suggesting that both CCs and ENDS induced enhanced influx of inflammatory cells in the lungs of experimental animals. Importantly, cellular make-up of inflamed lungs revealed significantly lower number of CD45+leukocytes (Fig.1G, F=29,726, $p<0.001$), CD45+Ly6G/C+ neutrophils (Fig.1H, F=7,699, $p<0.01$), F4/80+ macrophages (Fig.1I, F=78,633, $p<0.001$), CD11c+ DCs (Fig.1J, F=26,174, $p<0.01$), CD49b+ NK and CD3+CD49b+ NKT cells (Fig.1K-L, F=91,180 for CD49b+, F=61,205 for CD3+CD49b+, $p<0.001$), CD4+ (Fig.1M, F=53,798, $p<0.001$) and CD8+ T lymphocytes (Fig.1N, F=37,028, $p<0.01$) in the lungs of ENDS-exposed compared to CC-exposed mice, confirming histological findings that ENDS which heated processed tobacco caused less severe immune cell-driven inflammation in the lungs of experimental animals.

Inflammasome activation and production of inflammatory cytokines were significantly reduced in the lung-infiltrated neutrophils and macrophages of ENDS-exposed compared to CC-exposed animals

IHC staining (Fig.2A) and flow cytometry analysis of lung-infiltrated neutrophils revealed significantly higher number of CD14+, TLR-2+, NLRP3+, iNOS-expressing and TNF-α or IL-1β-producing CD45+Ly6G/C neutrophils in the lungs of CC-exposed and ENDS-exposed mice compared to Air-exposed animals, indicating that both CC and ENDS induced inflammasome activation which led to the increased production of NO and inflammatory cytokines in injured lungs (Fig.2B-J). Reduced presence of Ly6G/C neutrophils was observed in the lung tissue samples of ENDS-exposed compared to CC-exposed mice (Fig. 2A). Additionally, significantly lower expressions of CD14 and TLR-2 (Fig.2B-D, F=8,105 for CD45+Ly6G+CD14+, F=19,181 for CD45+Ly6G+TLR2+, F=29,071 for CD45+Ly6G+CD14+TLR2+, $p<0.05$), reduced activity of NLRP3 (Fig.2F, F=29,140, $p<0.001$) and iNOS (Fig.2G, F=9,589, $p<0.01$) and down-regulated synthesis of IL-1β (Fig.2H-I, F=75,136 for CD45+Ly6G+IL-1β+, F=51,779 for CD45+Ly6G+NLRP3+IL-1β+) and TNF-α (Fig.2J, F=59,951, $p<0.001$) were observed in the lung-infiltrated neutrophils of ENDS-exposed compared to CC-exposed animals, suggesting that ENDS had reduced capacity for the generation of inflammatory phenotype in lung-infiltrated neutrophils than CCs.

Similarly, significant increase in the total number of CD40+, CD86+, TLR2+, CD14+, I-A+, iNOS+ and NLRP3-expressing and IL-1β, IL-12, IL-23, TNF-α-producing F4/80+ macrophages was observed in CCs and ENDS-exposed mice compared to control, Air-exposed animals (Fig.3). IHC staining showed reduced presence of F4/80+ macrophages in the lung tissue samples of ENDS-exposed compared to CC-exposed mice (Fig. 3A). Importantly, F4/80+macrophages from the lungs of ENDS-exposed animals had attenuated capacity for antigen presentation, evidenced by lower expression of MHC class II (I-A) (Fig.3B, F=16,289,

p<0.05) and co-stimulatory CD40 and CD86 molecules (Fig.3C-E, F=20,302 for F4/80+CD40+, F=551,571 for F4/80+CD86+, F=126,486 for F4/80+CD40+CD86+, p<0.001). Also, reduced TLR-2 and CD14-dependent activation of NLRP3-inflammasome (Fig.3F-I, F=3,711 for F4/80+TLR2+, F=4,829 for F4/80+CD14+, F=12,057 for F4/80+CD14+TLR2+, F=19,221 for F4/80+NLRP3+, p<0.01), suppressed iNOS activity (Fig.3J, F=131,308, p<0.01) and down-regulated production of inflammatory cytokines (IL-1β (Fig.3K, F=3,471, p<0.05), TNF-α (Fig.3L, F=112,521, p<0.01), IL-12 (Fig.3M, F=37,957, p<0.05), IL-23 (Fig.3N, F=14,822, p<0.05)) were noticed in the lung-infiltrated macrophages of ENDS-treated compared to CC-treated animals, suggesting that ENDS which heated processed tobacco had less capacity than CCs for inflammasome activation and for generation of inflammatory phenotype in lung macrophages.

DCs from ENDS-exposed animals have reduced antigen-presenting properties and impaired capacity for the induction of Th1 and Th17 cell-driven lung inflammation

Lung DCs of CC and ENDS-treated mice had remarkably improved antigen-presenting properties than DCs of air-exposed animals (Fig.4A-D). Comparison of lung DCs from CC-exposed and ENDS-exposed mice revealed significantly attenuated expression of I-A (Fig.4A, F=11,456, p<0.05), CD40, CD86 (Fig.4B-D, F=2,204 for CD11c+CD40+, F=4,174 for CD11c+CD86+, F=2,987 for CD11c+CD40+CD86+, p<0.05), TLR2, CD14, NLRP3 (Fig.4E-G, F=12,120 for CD11c+TLR2+, F=4,362 for CD11c+CD14+, F=4,667 for CD11c+NLRP3+, p<0.05) and down-regulated production of pro-Th1 cytokines (TNF-α (Fig. 4J, F=18,510, ,p<0.05), IL-12 (Fig.4J, F=8,643, p<0.05)) and pro-Th17 cytokines (IL-1β (Fig.4H, F=11,248, p<0.05), IL-23 (Fig.4K, F=11,653, p<0.05)) in the DCs which were isolated from the lungs of ENDS-treated animals, suggesting that DCs from ENDS-exposed animals have reduced capacity for the generation of effector Th1 and Th17 lymphocytes compared to CC-sourced DCs.

Accordingly, increased influx of inflammatory, CD4+ and CD8+ Th1 and Th17 lymphocytes was observed in the inflamed lungs of CC and ENDS-exposed than in Air-exposed mice (Fig.4L-S). Importantly, remarkably lower number of IFN-γ-producing CD4+ and CD8+ Th1 cells (Fig.4L-M, F=6,251 for CD4+IFNγ+, F=3,054 for CD8+IFNγ, p<0.05) and IL-17-producing CD4+ and CD8+ Th17 cells (Fig.4N-O, F=10,832, p<0.05 for CD4+IL-17+ and F=17,771, p<0.01 for CD8+IL-17+) were observed in the lungs of ENDS-treated compared to CC-treated animals. Similarly, significantly reduced presence of alveolotoxic, TNF-α-producing CD4+ and CD8+T lymphocytes (Fig.4P-FQ F=35,255 for CD4+TNFα+, F=9,266 for CD8+TNFα+, p<0.01), CD49b+NK and CD3+CD49b NKT cells (Fig.4R-S, F=17,140 for CD49+TNFα+ and F=7,053 for CD3+CD49+TNFα+, p<0.05) was noticed in the injured lungs of ENDS-exposed than in the lung parenchyma of CC-exposed mice, supporting the hypothesis that ENDS which heated processed tobacco caused less severe immune cell-driven lung damage and inflammation than CCs.

Discussion

It is well known that frequent use of CCs induces changes in immune cells' phenotype and function paving the way for the development and progression of chronic inflammatory lung diseases [5]. Herewith, we are first to demonstrate that continuous exposition to ENDS which heat processed tobacco also induced potent inflammatory response in the lungs which resulted in the development of lung injury. Importantly, our results showed that ENDS-dependent detrimental effects on respiratory function were significantly less severe than harmful effects caused by CCs. Compared to CCs, ENDS which heated processed tobacco recruited lower number of circulating leukocytes in injured lungs (Fig.1), had less capacity to induce inflammasome activation in lung-infiltrated neutrophils and macrophages (Fig.2-3) and had less potential to elicit DC-dependent activation of NK, NKT, Th1 and Th17 cells (Fig.4) which resulted in the development of less severe pathological changes in the inflamed lungs (Fig.1).

Cigarette smoke generates reactive oxygen species (ROS) and activate transcriptional factors nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) in bronchial epithelial cells (BECs) and lung-infiltrated immune cells [19-21]. Activation of NF-κB and AP-1 signaling pathways leads to the increased production of chemokines, pro-inflammatory cytokines and adhesion molecules, facilitating leukocyte infiltration. In response to CC-derived smoke, BECs massively release IL-6 which modulates phenotype of lung endothelial cells, enabling recruitment of circulating neutrophils and monocytes in the inflamed lungs [22]. IL-6-exposed lung endothelial cells highly express E and P selectins, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) which facilitate leukocyte adhesion to the endothelium and subsequent transmigration into the lung tissue [21]. It is well known that ENDS-treated BECs produce lower amount of neutrophil and monocyte-attracting IL-6 than CC-treated BECs [19]. In line with these findings, lower concentration of IL-6 observed in the serum samples of ENDS-exposed mice (Fig.1F) was accompanied with significantly lower number of CD45+Ly6G/C neutrophils (Fig.1H) and F4/80+monocytes/macrophages (Fig. 1I) in the lungs of ENDS-exposed compared to CC-exposed mice.

CC-sourced smoke enhances expression of CD14 and TLR-2 in alveolar macrophages and lung-infiltrated neutrophils [22]. By triggering CD14 and TLR-2-dependent signaling pathways, CCs activate NLRP3 inflammasome and induce increased production of inflammatory cytokines (IL-1β, TNF-α) which, in turn, promote expression of E and P selectins on lung endothelial cells, enabling massive recruitment of circulating leukocytes in inflamed lungs [21, 23]. In comparison with CCs, ENDS induced reduced CD14 and TLR-2-dependent NLRP3 inflammasome activation in lung-infiltrated neutrophils and macrophages (Fig.2B-F and Fig.3F-I) which led to the down-regulated production of IL-1β and TNF-α (Fig.2H-J and Fig.3K-L) and resulted in attenuated influx of DCs, monocytes and lymphocytes in inflamed lungs of ENDS-exposed compared to CC-exposed mice (Fig.1J-N).

Pulmonary DCs are professional antigen-presenting cells which capture inhaled toxic antigens presented in CCs-derived smoke and ENDS-sourced aerosols, initiating primary T cell-driven immune response in the lungs of CC and ENDS users [1]. Cigarette smoke promotes accumulation of DCs in the inflamed lungs where DCs process inhaled antigens and present

them within MHC class II molecules (I-A) to the antigen-specific naïve CD4+ and CD8+ T lymphocytes, enabling their activation, proliferation and differentiation in effector T cells [24]. Additionally, CCs-exposed DCs highly express co-stimulatory molecules (CD86, CD40) which bind to their ligands on naïve T cells (CD28, CD40L) enabling generation of strong "secondary signal" which is necessary for optimal activation and clonal expansion of T lymphocytes [1]. Chen et al. recently revealed that ENDS-sourced aerosols moderately stimulated activation of pro-inflammatory signaling pathways in DCs *in vitro* [25]. In line with these findings, herewith we showed that ENDS have lower potential for DCs' activation than CCs (Fig.4). Lung DCs from ENDS-exposed animals had reduced antigen-presenting properties (Fig.4A-D) and produced significantly lower amount of Th1 and Th17-related cytokines (IL-12, TNF-α, IL-1β, IL-23) than pulmonary DCs from CC-treated mice (Fig.4H-K).

Lung DCs, in IL-12, IL-1β and IL-23-dependent manner induce activation of naïve T cells, while in TNF-α and IL-1β-dependent manner, recruit circulating effector T lymphocytes in injured and inflamed lungs [26]. In naïve CD4+T and CD8+T cells, DC-derived IL-12 induces increased expression of T-bet and STAT-1 transcriptional factors and promotes synthesis of IFN-γ, enabling generation of Th1 cell-driven lung injury and inflammation [27]. DC-sourced IL-1β and IL-23 are crucially important for the increased expression of RORγT and STAT-3 transcriptional factors which induce generation of CD4+ and CD8+ Th17 cells [1, 20]. In comparison with CCs, ENDS less efficiently induced production of Th1 (IL-12, TNF-α) and Th17-related cytokines (IL-1β, IL-23) in lung DCs, activated lower number of naïve T cells and attracted smaller number of effector Th1 and Th17 lymphocytes in the lungs of ENDS-exposed compared to CC-treated animals.

Th1 and Th17 cells activate lung-infiltrated phagocytes (macrophages and neutrophils), crucially contributing to the progression of on-going lung inflammation [19]. Macrophages, which are the most numerous immune cells within the lung environment under homoeostatic conditions, have crucially important role in the defense against inhaled microbes [28-30]. Upon the phagocytosis of lung-invading pathogens, alveolar macrophages become activated, process antigens and present them within MHC class II (I-A) molecules to the effector CD4+Th1 cells. In CD40, CD86 and IL-12-dependent manner, alveolar macrophages provide additional signals necessary for optimal expansion and activation of IFN-γ-producing CD4+Th1 lymphocytes in inflamed lungs [19]. In turn, macrophages, under the influence of Th1 cell-sourced IFN-γ, massively produced alveolotoxic NO and inflammatory cytokines, which aggravated on-going inflammation [21]. In line with these findings are our results showing massive lung injury and severe respiratory dysfunction of CCs-exposed mice whose lungs were infiltrated with IFN-γ-producing Th1 cells (Fig.4L-M) and CD40, CD86, iNOS-expressing, TNF-α, IL-1β, IL-12 and IL-23-producing macrophages (Fig.3). Importantly, significantly lower number of Th1 cells (Fig.4L-M) and inflammatory macrophages (Fig.3J-N) were observed in the lungs of ENDS-exposed compared to CC-treated mice, suggesting that ENDS which heated processed tobacco had less capacity than CCs for the induction of Th1 cell-driven macrophage activation.

Long-term use of CCs significantly increases total number of Th17 lymphocytes in the blood, bronchial mucosa and lungs of COPD patients [31-33]. Th17 cell-derived IL-17 induces

increased iNOS activity and enhanced NLRP3 inflammasome activation in neutrophils which results in increased NO production in inflamed lungs [5]. Neutrophil-derived NO induces oxidative stress-dependent DNA damage in alveolar epithelial cells and crucially contributes to the development of respiratory dysfunction in CCs users [34]. Accordingly, significant increase in total number of Th17 lymphocytes and iNOS-expressing, lung-infiltrated neutrophils resulted in severe lung injury and respiratory dysfunction in CC-exposed mice, while attenuated production of IL-17 and reduced iNOS activity, observed in ENDS-exposed Th17 lymphocytes and neutrophils (Fig.2G and Fig.4N-O) were accompanied with attenuated lung injury and less severe respiratory dysfunction of ENDS-treated animals (Fig.1A-E).

Inflammatory, lung-infiltrated NK and NKT cells, through the enhanced production of alveotoxic TNF-α, participate in the initial phase of immune cell-driven injury of alveolar epithelial cells [35-37]. Accordingly, we observed increased number of alveolotoxic, TNF-α-producing NK and NKT cells in the lungs of CC-exposed mice (Fig. 4R-S). Importantly, significantly reduced number of TNF-α-producing NK and NKT cells were observed in the lungs of ENDS-exposed compared to CC-treated mice (Fig. 4R-S), indicating that ENDS had less capacity than CCs for the induction of NK and NKT cell-driven lung injury and inflammation.

In summing up, ENDS which heated processed tobacco triggered immune response in the lungs which was less potent than inflammatory response elicited by CCs. While extensive immune cell-driven lung injury and inflammation resulted in severe respiratory dysfunction in CCs-exposed mice, ENDS-dependent activation of lung-infiltrated immune cells induced partial damage of alveolar epithelial cells and caused moderate respiratory dysfunction in experimental animals. Although ENDS-induced detrimental effects on immune cell-driven lung injury were significantly less severe than deleterious effects caused by CCs, ENDS should not be considered as harmless products and their long-term use should be avoided.

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Figures and figure legends

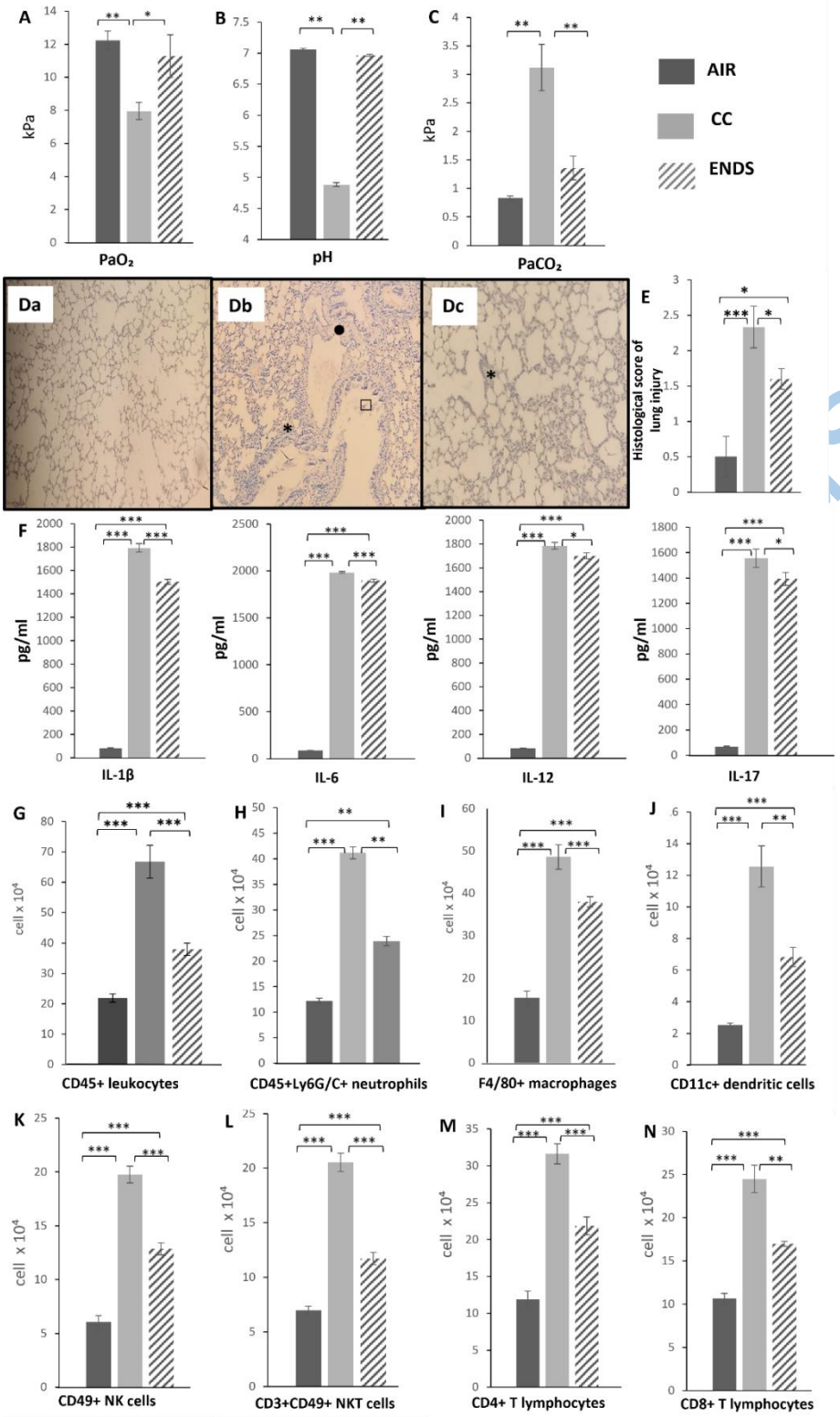

Figure 1. **Less severe lung injury and inflammation were observed in ENDS-exposed than in CC-exposed mice.** Blood-gas analysis showed significantly lower values of PaO2 (A) and pH (B) and higher values of PaCO2 (C) in CC-exposed compared to ENDS and Air-exposed animals. Representative images of hematoxylin-eosin (H&E) stained lung tissue samples (D) demonstrating the most severe inflammation-related pathological changes (* leukocyte infiltration, □ epithelium desquamation, ● epithelium wideness) in the lungs of CC-exposed mice (Db) compared to Air-exposed (Da) and ENDS-exposed animals (Dc) (magnifications (100x)). Significantly higher histological score was observed in CC-treated than in Air- and ENDS-treated mice animals (F). Concentrations of inflammatory cytokines (IL-1β, IL- 6, IL-12, IL-17) were significantly higher in the serum samples of CC-exposed than ENDS and Air-exposed mice (F). Flow cytometry analysis of lung-infiltrated immune cells revealed the highest number of CD45+ leukocytes (G), CD45+Ly6G/C+ neutrophils (H), F4/80+ macrophages (I), CD11c+ dendritic cells (DCs) (J), CD49+ natural killer (NK) cells (K), CD3+CD49+ natural killer T (NKT) cells (L), CD4+ T lymphocytes (M), CD8+ T lymphocytes (N) in the lungs of CCs-exposed mice compared to ENDS-exposed and Air-exposed animals. Values are presented as Mean ± SEM; n = 6 mice per group; *p < 0.05, **p < 0.01, *** p<0,001.

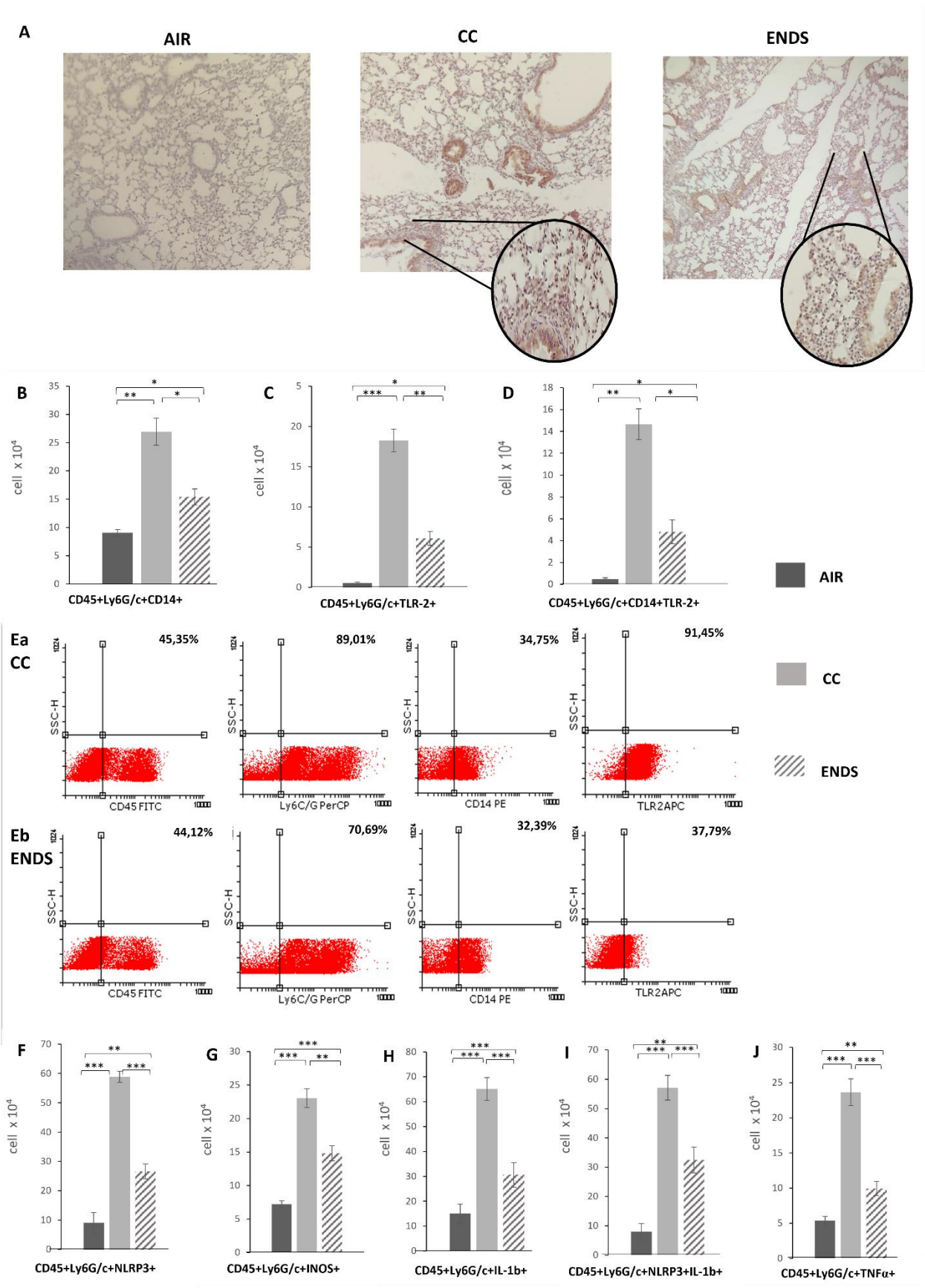

Figure 2. Inflammasome activation and production of inflammatory cytokines were significantly reduced the lung-infiltrated neutrophils of ENDS-exposed than in CC-exposed animals. Immunohistochemical staining revealed reduced presence of Ly6G/c+ neutrophils in the lungs of ENDS-exposed mice compared to CC-exposed animals (A). Flow cytometry analysis of lung-infiltrated neutrophils showed the highest number of CD14+ (B), TLR-2+ (C), CD14+TLR2+ (D), NLRP3+ (F), iNOS+ (G) IL-1β+ (H), NLRP3+IL-1β+ (I), TNF-α+(J) CD45+Ly6G/C+ neutrophils in the lungs of CC-exposed mice compared to ENDS-exposed and Air-exposed animals. Representative dot plots of CD45+Ly6C/G+CD14+TLR-2+ neutrophils isolated from the lungs of CCs-exposed (Ea) and ENDS-exposed mice (Eb) are shown. Values are presented as Mean ± SEM; n = 6 mice per group; *p < 0.05, **p < 0.01, *** p<0,001.

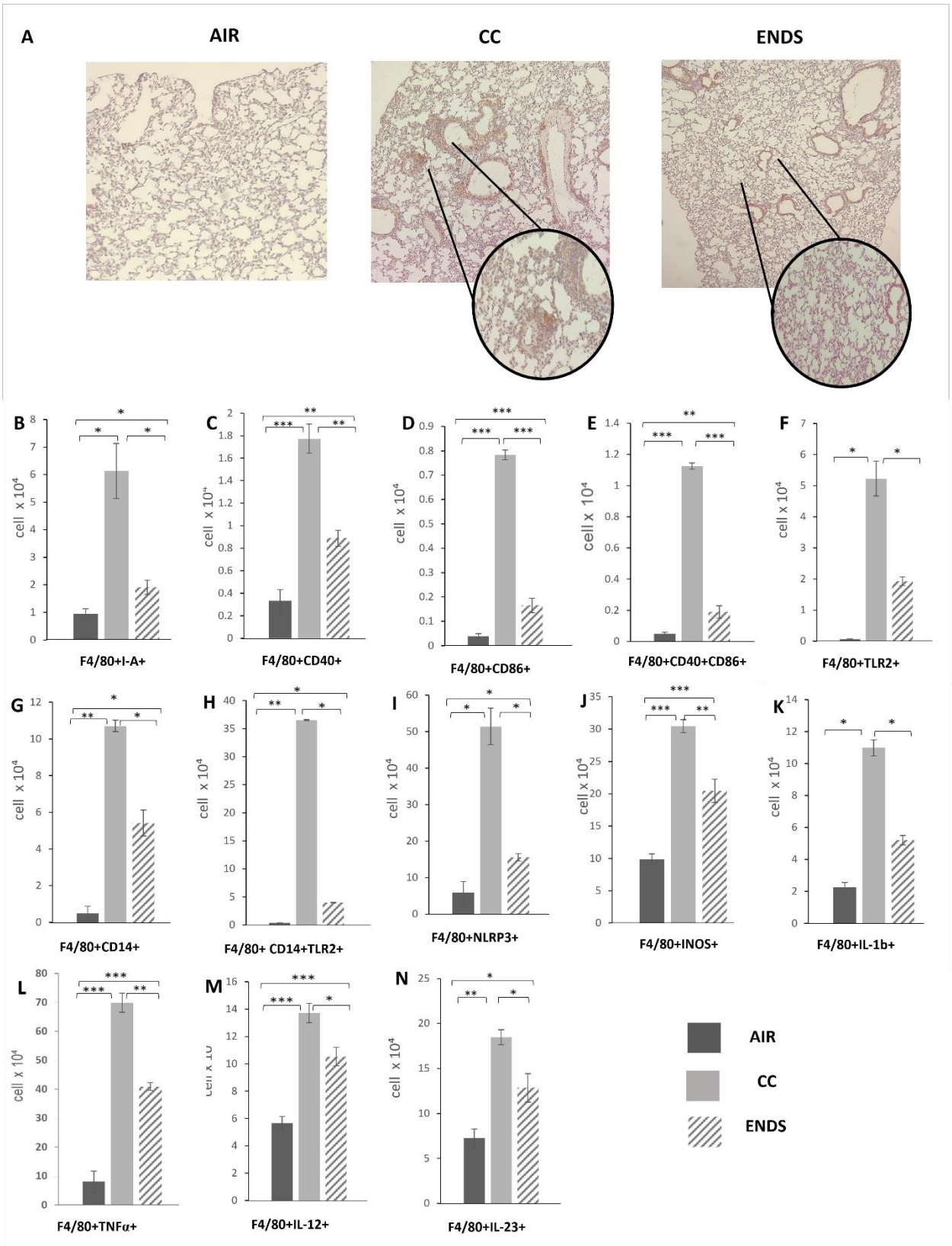

Figure 3. Significantly reduced number of inflammatory macrophages were present in the lungs of ENDS-treated compared to CC-treated mice. Immunohistochemical staining revealed reduced presence of F4/80+ macrophages in the lungs of ENDS-exposed mice compared to CC-exposed animals (A). Cellular make-up of the lungs revealed the highest number of MHC class II (I-A)+(B), CD40+(C), CD86+(D), CD40+CD86+ (E), TLR-2+(F), CD14+(G), TLR2+CD14+ (H), NLRP3 (I)-and INOS (J) expressing, IL-1β (K), TNF-α (L), IL-12 (M), IL-23 (N)-producing F4/80+ macrophages in the lungs of CC-exposed mice compared to ENDS-exposed and Air-exposed animals. Values are presented as Mean ± SEM; n = 6 mice per group; *p < 0.05, **p < 0.01, *** p<0,001.

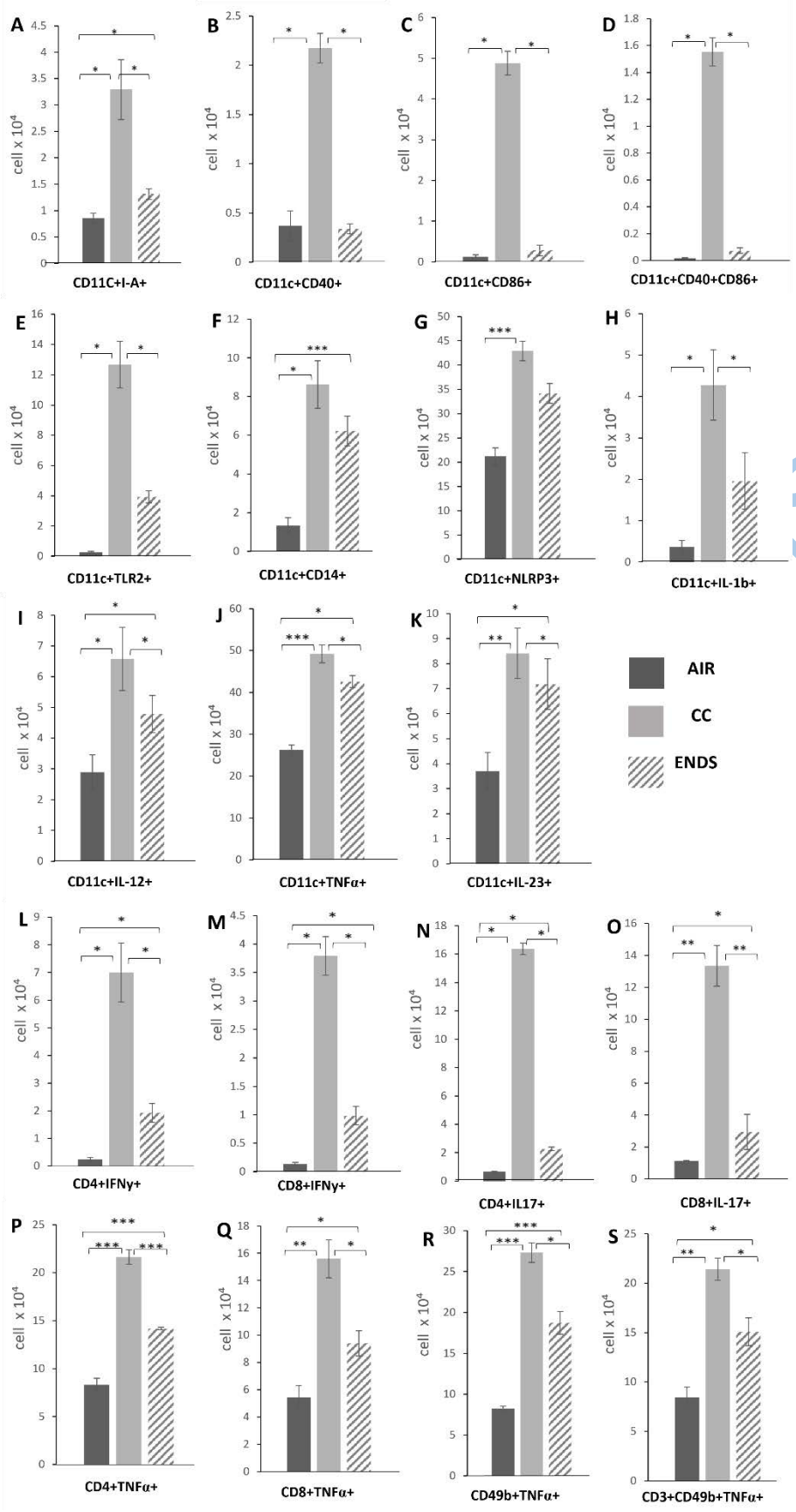

Figure 4. Lung DCs from ENDS-exposed animals have reduced antigen-presenting capacity compared to CC-sourced DCs which led to the attenuated Th1 and Th17 cell-driven lung inflammation. Significantly higher number of inflammatory DCs were observed in the lungs of animals from experimental (CC and ENDS-treated) compared to the mice control (Air-exposed) group. Intracellular staining and flow cytometry analysis of lung DCs showed different phenotype and function of DCs that infiltrated the lungs of CCs and ENDS-exposed mice. Significantly attenuated expression of I-A (A), CD40, CD86 (B-D), TLR2, CD14, NLRP3 (E-G) and down-regulated production of IL-1β (H), TNF-α (I), IL-12 (J) and IL-23 (K) were noticed n the DCs which were isolated from the lungs of ENDS-treated animals compared to CC-exposed mice. Intracellular staining and flow cytometry analysis of T lymphocytes showed increased presence of TNF-α, IFN-γ, IL-17-producing CD4+ and CD8+T lymphocytes, NK and NKT cells in the lungs of CC and ENDS-treated compared to Air-exposed mice. Significantly reduced number of IFN-γ-producing CD4+ (L) and CD8+ Th1 cells (M) and significantly lower number of IL-17-producing CD4+ (N) and CD8+ Th17 cells (O) and remarkably reduced presence of alveolotoxic, TNF-γ-producing CD4+ and CD8+T lymphocytes (P-Q), CD49b+NK and CD3+CD49b NKT cells (R-S) were observed in the lungs of ENDS-treated compared to CC-treated animals. Values are presented as Mean ± SEM; n = 6 mice per group; *p < 0.05, **p < 0.01, *** p<0,001.