

Initial and ongoing tobacco smoking elicits vascular damage and distinct inflammatory response linked to neurodegeneration

Alejandra P. Garza^{a,1}, Lorena Morton^{a,1}, Éva Pállinger^b, Edit I. Buzás^{b,c,d}, Stefanie Schreiber^{e,f,g}, Björn H. Schott^{g,h,i}, Ildiko Rita Dunay^{a,f,*}

^a Institute of Inflammation and Neurodegeneration, Medical Faculty, Otto-von-Guericke University Magdeburg, Germany

^b Department of Genetics, Cell- and Immunobiology, Semmelweis University, Budapest, Hungary

^c HCEMM SU Extracellular Vesicle Research Group, Budapest, Hungary

^d ELKH-SE Translational Extracellular Vesicle Research Group, Budapest, Hungary

^e Department of Neurology, Otto-von-Guericke University, Magdeburg, Germany

^f Center for Behavioral Brain Sciences, Magdeburg, Germany

^g German Center for Neurodegenerative Diseases (DZNE), Magdeburg, Germany

^h Leibniz Institute of Neurobiology, Magdeburg, Germany

ⁱ Department of Psychiatry and Psychotherapy, University Medicine Göttingen, Göttingen, Germany

ARTICLE INFO

Keywords:

Tobacco smoking
Innate immunity
Extracellular vesicles
Monocytes
Vascular damage
Neurodegeneration

ABSTRACT

Tobacco smoking is strongly linked to vascular damage contributing to the development of hypertension, atherosclerosis, as well as increasing the risk for neurodegeneration. Still, the involvement of the innate immune system in the development of vascular damage upon chronic tobacco use before the onset of clinical symptoms is not fully characterized. Our data provide evidence that a single acute exposure to tobacco elicits the secretion of extracellular vesicles expressing CD105 and CD49e from endothelial cells, granting further recognition of early preclinical biomarkers of vascular damage. Furthermore, we investigated the effects of smoking on the immune system of healthy asymptomatic chronic smokers compared to never-smokers, focusing on the innate immune system. Our data reveal a distinct immune landscape representative for early stages of vascular damage in clinically asymptomatic chronic smokers, before tobacco smoking related diseases develop. These results indicate a dysregulated immuno-vascular axis in chronic tobacco smokers that are otherwise considered as healthy individuals. The distinct alterations are characterized by increased CD36 expression by the blood monocyte subsets, neutrophilia and increased plasma IL-18 and reduced levels of IL-33, IL-10 and IL-8. Additionally, reduced levels of circulating BDNF and elevated sTREM2, which are associated with neurodegeneration, suggest a considerable impact of tobacco smoking on CNS function in clinically healthy individuals. These findings provide profound insight into the initial and ongoing effects of tobacco smoking and the potential vascular damage contributing to neurodegenerative disorders, specifically cerebrovascular dysfunction and dementia.

1. Introduction

Tobacco smoking is a major modifiable risk factor for a plethora of pathological conditions. Despite undisputed adverse health effects, the number of smokers continues to rise globally (Gowing et al., 2015; Eriksen M et al., 2012). Currently, 22.3% of the world's population smoke, with detrimental effects on both life expectancy and quality of life (Cheng and Jin 2022). It has been predicted that half of the smokers'

population will succumb prematurely to tobacco-related complications (World Health Organization 2021). Diverse chronic systemic disorders are directly attributable to continuous exposure to tobacco smoke, severely injuring the respiratory and cardiovascular systems, but also affecting the central and peripheral nervous systems (Yanbaeva et al., 2007). Tobacco smoking as a modifiable risk factor for dementia is of particular importance in aging societies since smoking is a known accelerator for vascular dementia and it has recently been linked to

* Corresponding author. Institute of Inflammation and Neurodegeneration, Medical Faculty, Otto-von-Guericke-University Magdeburg, Leipziger Straße 44, 39120, Magdeburg, Germany.

E-mail address: ildiko.dunay@med.ovgu.de (I.R. Dunay).

¹ equal contribution.

<https://doi.org/10.1016/j.bbih.2023.100597>

Received 6 August 2022; Received in revised form 7 December 2022; Accepted 21 January 2023

Available online 29 January 2023

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lower cognitive resilience to Alzheimer's disease (O'Brien et al., 2015; Livingston et al., 2020; Dumitrescu et al., 2020).

Alterations in both innate and adaptive immunity have previously been described in the context of tobacco smoking (Miteva et al., 2018; Piaggieschi et al., 2021; Delgado et al., 2021). T cell activation, differentiation and response is altered in chronic smokers. In particular, the number of CD4+T helper subsets is upregulated, which is accompanied by an increased release of proinflammatory cytokines (Forsslund et al., 2014; Zhang et al., 2014). Regarding innate immunity, previous studies have described elevated levels of circulating blood leukocytes in chronic smokers, including neutrophils and monocytes, as well as increased peripheral inflammatory markers such as fibrinogen, C-reactive protein (CRP) and proinflammatory cytokines IL-6 and IL-1 β (Andersson et al., 2019; Elisia et al., 2020; Pedersen et al., 2019). However, the explicit role of innate immune cell activation in the early pathophysiology of tobacco smoking related-disease has not been fully elucidated. CD36 is a scavenger receptor expressed on the surface of monocytes, mediating uptake of oxidized low-density lipoproteins (oxLDL) from the lumen, thereby facilitating the conversion of healthy peripheral monocytes into foam cells. Foam cells are known for initiating the development of vascular plaques (Febbraio et al., 2001; Park 2014). CD36 has also been described as a potential early biomarker of atherosclerosis due to its role in plaque formation, angiogenesis, and endothelial inflammation (Tian et al., 2020).

The chronic presence of tobacco smoke as a stressor unequivocally leads to inflammation and endothelial dysfunction (Golbidi et al., 2020). Cigarette smoke induces a series of inflammatory mechanisms, activating cell populations from both the innate and the adaptive immunity, which in turn promote the secretion of multiple inflammation-related molecules such as proinflammatory cytokines, chemokines, reactive oxygen species (ROS) and extracellular vesicles (EVs) (Miteva et al., 2018; Elisia et al., 2020; Kodidela et al., 2020). EVs are nanosized membrane-enclosed particles derived from all types of cells in the organism, playing an essential role in intercellular signaling (Buzas et al., 2014; Osteikoetxea et al., 2016; Tóth et al., 2021).

The production of ROS strongly influences oxidative stress, which has a detrimental role in promoting the dysfunction of different barriers, including the blood-brain barrier (BBB) (Hossain et al., 2009; Sajja et al., 2015; Hawkins et al., 2004). In chronic smokers, increased ROS production accelerates endothelial dysfunction predisposing to morphological and functional damage of the BBB, thereby contributing to the pathogenesis of cerebrovascular and neurodegenerative diseases (Cataldo et al., 2010; Chrissobolis et al., 2011; Scicchitano et al., 2019; Zalba et al., 2007). Changes due to chronic cigarette smoking, with consequent alterations in the endothelium and the BBB can lead to neurovascular damage (Cipollini et al., 2019; O'Brien et al., 2015).

Soluble triggering receptor expressed on myeloid cells 2 (sTREM2) is the secreted domain of TREM2, a receptor expressed on microglial cells, and it has been described as a microglial activation marker and constitutes a promising neurodegeneration biomarker in both CSF and peripheral blood of AD patients (Bekris et al., 2018; Ferri et al., 2020; Hu et al., 2014; Park, 2014). Notably, recent evidence points to a gene-environment interaction of smoking and a TREM2 genetic variation contributing to AD risk (Sun et al., 2022). Conversely, with respect to neuronal integrity, plasticity, and survival, brain-derived neurotrophic factor (BDNF) constitutes an established biomarker. BDNF can cross the BBB, and therefore, BDNF levels measured in the periphery correlate with those found in the brain (Pan et al., 1998; Klein et al., 2011; Zuccato and Cattaneo 2009). Results regarding peripheral BDNF levels in smokers vary with some studies reporting reduced plasma concentrations (Bhang et al., 2010), while others detected increased plasma BDNF levels in smokers (Galle et al., 2021). By understanding the initial steps of this process as a consequence of tobacco smoking, we aimed to detect early alterations on the innate and adaptative immune response along with initial neuroinflammatory changes, to decipher and eventually prevent downstream pathologies.

Here we demonstrate the early and ongoing effects of tobacco smoking and related vascular damage in healthy individuals that might contribute to the development of cerebrovascular dysfunction. Our study indicates the rapid release of EVs into blood circulation after an acute tobacco smoking session by the damaged endothelium. Neutrophilia and an elevated number of blood monocytes were detected in chronic smokers. Monocyte subsets increased the expression of CD36, a scavenger receptor involved in atherosclerotic plaque formation. Importantly, decreased levels of brain-derived neurotrophic factor (BDNF) and an elevated sTREM2 in plasma levels in smokers indicate that smoking may contribute to the pathophysiology of neurodegenerative processes in otherwise healthy individuals.

2. Methods

2.1. Participants

The present study prospectively enrolled 76 participants designated as current smokers ($n = 26$) and nonsmokers ($n = 50$) (Table 1). Participants were recruited from June to August 2020 via advertisement at the Otto-von-Guericke University Campus in Magdeburg, Germany. All enrolled patients were ≥ 18 years of age, able to give written informed consent and had no cardiovascular, neurological or psychiatric diseases. They reported negatively to current or recent history of infections, tumors, immunizations, autoimmune disease and pregnancy. Participants had no history of uncontrolled metabolic, respiratory or systemic disorders, and current or recent history of either centrally acting medication (e.g., anticonvulsants, antidepressants, anxiolytics, neuroleptics, opioids), anti-inflammatory pharmacotherapy (e.g., non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics, corticosteroids), or anticoagulants. Smoking status was self-reported and defined as never or current smokers. Never smokers are subjects who never smoked or have smoked less than 100 cigarettes in their lifetime but never on a daily basis. Current smokers are defined as otherwise healthy participants who have smoked more than 100 tobacco cigarettes in their lifetime and currently smoke daily. Subjects agreed to arrive in fasting conditions and to refrain from smoking for at least 8 h before the start of the study. Out of the nonsmokers, 20 participants took part in the acute intervention study (Fig. 1A and Supplementary Fig. 2). The acute setting consisted of blood withdrawal before and 60 min after smoking two cigarettes (12 tar yield mg/cig, 1.0 nicotine yield). Our study was conducted in accordance with the ethical principles for the Declaration of Helsinki with approval from the ethics committee of the Medical Faculty, Otto-von-Guericke Magdeburg (No 07/17; addendum 12/2021). Written informed consent was obtained from all participants enrolled in this study.

2.2. Whole blood lysis and staining

Blood was sampled from the antecubital area via peripheral venipuncture into a sterile BD Ethylenediaminetetraacetic acid (EDTA) Vacutainer blood collection tube with 1.8 mg EDTA per milliliter of blood. All samples were processed within 30 min to 1 h 100 μ L of whole blood was lysed with 1X Red Blood Cell Lysis Buffer (Bio Legend, 10X)

Table 1
Demographic data.

	n	Age median, range	Female n (%)	BMI, SD	Daily cigarettes	Years smoking
Controls	50	57 (20–50)	35 (70)	23.4 $\pm$ 2.7	NA	NA
Smokers	26	40 (21–47)	19 (73)	24.5 $\pm$ 2.5	7.9 $\pm$ 5.6	16 $\pm$ 5.6
Comparison (p-value)		0.1696	0.156	0.1053		

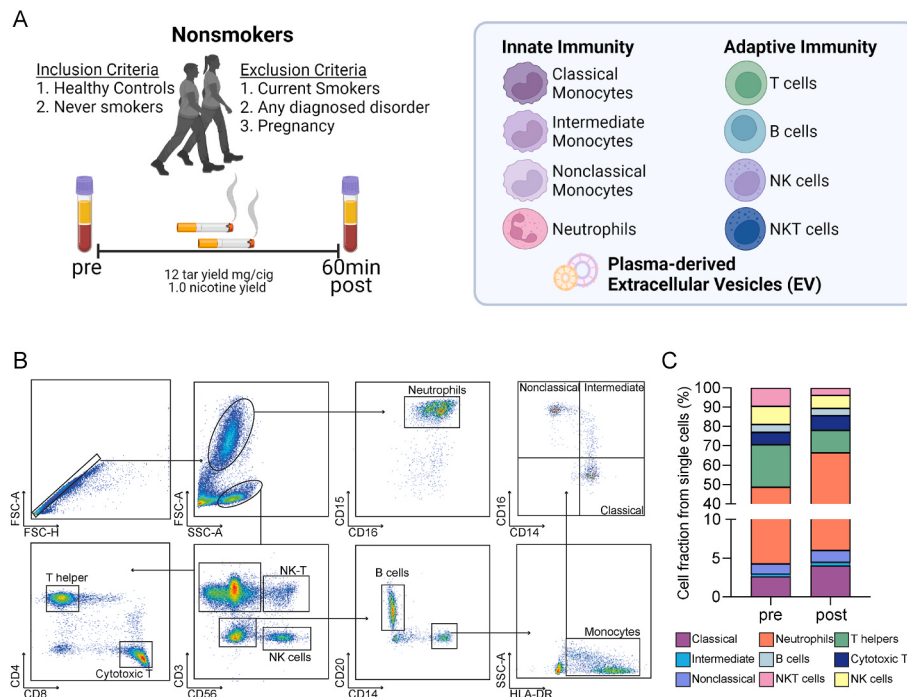


Fig. 1. Subtle shift in the innate and adaptive immune cells in healthy controls after one tobacco smoking session. Clinical and experimental study design and analytical processes showing inclusion and exclusion criteria for nonsmoking healthy controls. Blood was withdrawn before and 60 min after the second and last cigarette consumption and further analysis of immune system cell composition and extracellular vesicles was performed (A). Representative gating strategy to identify granulocytes and mononuclear cells based on their size and granularity. Neutrophils were gated based on surface expression of CD16 and CD15. Mononuclear cells were gated and further identified as T cells, NK cells and NKT cells. The expression of CD4 and CD8 was further assessed in the CD3⁺ T cells. B cells were defined as CD3⁺, CD56⁺ and CD20⁺ cells. Monocytes were identified as CD3⁺, CD56⁺ and CD20⁺, and HLA-DR⁺ and CD14⁺. Gated monocytes were further divided into subpopulations based on their differential expression of CD14 and CD16 (B). Bar chart showing cell fractions of all studied innate immune cell populations before (pre) and 60 min after the short intervention (C).

following the manufacturer's instructions. After lysis, cells were centrifuged at 400 g for 5 min at 18 °C. Supernatant was discarded and cells in the pellet were resuspended in 4 mL PBS with the aforementioned settings. Cells were further resuspended in 300 µL FACS Buffer (1X PBS, 2–5% FBS, 2 mM EDTA and 2 mM NaN₃) and 100 µL were added to a 96-well plate to continue with the staining protocol. To avoid nonspecific binding of antibodies to Fc-receptors, samples were incubated for 10 min in 5 µL of Human TruStain FcX (BioLegend). Each sample was diluted, divided for 2 panels and stained with the following conjugated antibodies: anti-human CD16 (FITC), anti-human HLA-DR (Peridinin chlorophyll protein-Cyanine5.5), anti-human CD86 (Allophycocyanin), anti-human CD3 (Alexa Fluor 700), anti-human CD66b (Alexa Fluor 700), anti-human CD19 (Alexa Fluor 700), anti-human CD56 (Alexa Fluor 700), anti-human CD36 (Allophycocyanin-Cyanine 7), anti-human CD163 (Brilliant Violet 421), anti-human CD15 (Brilliant Violet 510), anti-human HLA-ABC (Brilliant Violet 605), anti-human CCR2 (Phycoerythrin), anti-human CD62L (Phycoerythrin -Dazzle 594), anti-human CD15 (Phycoerythrin -Cyanine5), anti-human CX3CR1 (Phycoerythrin-Cy7), anti-human CD14 (Alexa Fluor 700), anti-human CD15 (APC), anti-human CD4 (Allophycocyanin-Cyanine 7), anti-human HLA-DR (Brilliant Violet 421), anti-human CD3 (Brilliant Violet 510), anti-human CD19 (Brilliant Violet 605), anti-human CD16 (Brilliant Violet711), anti-human CD45 (FITC), anti-human CD56 (Phycoerythrin), CD8 (Phycoerythrin-Dazzle 594) and anti-human CD20 (Peridinin chlorophyll protein-Cyanine5.5). After an incubation period of 30 min at 4 °C, samples were washed twice, and were resuspended in 210 µL of FACS-Buffer for further processing. Fluorescence minus one (FMO) controls were used to determine and assess background fluorescence in the respective detection channel. Data were acquired on the Attune NxT Flow Cytometer (Thermo Fisher Scientific) and analyzed with Flowjo Analysis Software (v10.5.3). The focus of our analysis consisted of the single cell selection and characterization of monocytes (HLA-DR⁺ CD3⁺ CD19⁺ CD56⁺ CD66b⁺). Thereafter, cells were further divided in subsets and identified as classical (CD14⁺⁺CD16⁻), intermediate (CD14⁺⁺CD16⁺), and nonclassical (CD14⁺CD16⁺⁺) monocytes (Fig. 1B) (Guilliams et al., 2018). Analysis of the frequency of all subsets is shown in Fig. 1C. Moreover, neutrophils were characterized as CD15⁺ CD16⁺ and additionally subdivided based

on their expression of CD62L into mature (CD16^{bright}CD62L^{bright}), banded (CD16^{dim}CD62L^{bright}) and hyper segmented (CD16^{bright}CD62L^{dim}) neutrophils (van Staveren et al., 2018) (Fig. 3B).

2.3. Extracellular vesicles isolation

Blood from the participants (n = 20) were collected on ACD tubes to prevent *in vitro* vesiculation of platelets and blood cells (György et al., 2014). Medium-sized vesicles (microvesicles) were separated by differential centrifugation as described previously (Pospichalova et al., 2015). Briefly, 2 mL of platelet-free plasma were first centrifuged at 200 g for 5 min to remove live cells. Thereafter, supernatants were transferred into new sterile tubes and further centrifuged at 1500 g for 10 min for the clearance of large EVs. To extract microvesicles, supernatants were transferred once more for ultracentrifugation at 14,000 g for 70 min at 4 °C. The supernatant was discarded, and the pellet containing microvesicles was resuspended in 0.22 µm (pore size filter) filtered PBS. This last ultracentrifugation step was repeated to improve microvesicle enrichment and purity. Remaining pellets containing microvesicles were stored in 200 µL of filtered PBS at –80 °C until further processing. The guidelines of the International Society for Extracellular Vesicles (Théry et al., 2018) recommend referring to EVs by their physical or molecular characteristics given that they vary in size, and as yet there are no established markers to identify different EVs by their biogenetic routes. Here, we use the term “mEVs” in reference to medium sizes nanoparticles.

2.4. Extracellular vesicle protein concentration

To determine total protein content, aliquots of frozen mEVs were thawed at 37 °C and Bio-Rad Protein Assay (Bradford 1976) was performed following manufacturer's instructions (500-0006 Bio-Rad. München, Germany). Samples were diluted with filtered PBS 1:50 and bovine serum albumin (BioRad Laboratories, USA) was used as standard (1 mg/mL). 200 µL of the diluted sample and 50 µL dye (1x Bradford reagent) were added to a 96-well plate and the absorbance was measured at 595 nm using the SpectraMax® M5 Microplate Reader (Molecular Devices LLC).

2.5. Extracellular vesicle flow cytometry

For the analysis of fluorescently labeled microvesicles via flow cytometry, aliquots of frozen mEVs were thawed up at 4 °C and centrifuged for 10 min at 14,000 g to remove aggregates. Supernatant was removed with caution and pellets were resuspended 200 µL filtered PBS. 100 µL per sample were collected separately for technical replicates and staining controls. mEVs were fluorescently labeled with protein- and lipid-specific dyes (eBioscience™ CFSE and FM™ 4-64FX, respectively) without the necessity of removing the unbound fluorescent dyes by ultracentrifugation, which further facilitates the characterization of microvesicles by flow cytometry (Pospichalova et al., 2015). Samples were first incubated for 20 min in a final concentration of 100 µmol/L Carboxyfluorescein succinimidyl ester (CFSE, Invitrogen by Thermo Fisher Scientific, 65-0850-85) at 37 °C in the dark. Samples were thereafter incubated for another 10 min at 37 °C in the dark in a final concentration of 20 µg/mL FM™4-64FX (Invitrogen by ThermoFisher Scientific, F34653). Antibody labeling was performed subsequent to CFSE/FM4-64FX incubation, samples were stained with anti-human CD3 (Peridinin chlorophyll protein-Cyanine5.5), anti-human CD15 (Allophycocyanin), anti-human CD4 (Alexa Fluor 700), anti-human CD14 (Brilliant Violet 510), anti-human CD19 (Brilliant Violet 605), anti-human CD16 (Brilliant Violet 711), anti-human CD56 (Phycoerythrin) and anti-human CD8 (Phycoerythrin-Dazzle 594). Following incubation, samples were washed, resuspended in filtered PBS and transferred to sterile tubes for data acquisition.

Sample data were acquired on the AttuneNxT Flow Cytometer equipped with 405-, 488-, 561-, and 633-nm lasers and an auto sampler allowing the procession of 96-well plates. To create a stable, slow velocity stream for optimal acquisition recommended for the detection of mEVs, sample collection speed was adjusted to the lowest flow rate (25 µL/min) with SSC-threshold set to 0.2×10^3 . To exclude carryover of fluorescent events between samples, 10% bleach and filtered PBS washes were included after every sample. 100 µL of sample suspension was loaded, and a stopping option was set to 50 µL. Red fluorescent 300 nm, 500 nm and 1000 nm diameter silica particles (Creative Diagnostics) were used for the correct size identification of medium sized EVs. Size reference was determined through Forward and Side Scatter light (FSC-H and SSC-H). Thereafter, CFSE- and FM 4-64FX-positive events with the designated size were identified as microvesicles. Fluorescence minus one (FMO) and unstained controls were used to aid in the gating strategy (Supplementary Fig. 1). Results were exported and analyzed in FlowJo version v10.5.3 (TreeStar). T-distributed stochastic neighbor embedding (tSNE) plots were generated in FlowJo using the DownSample plug-in to normalize the interrogation of events per sample per group. Twenty-five thousand events for each study group were exported and concatenated followed by tSNE algorithm on all compensated parameters for 1000 iterations, perplexity of 30, and learning rate of 4,050, approximate random projection forest – ANNOY as KNN and FFT interpolation as gradient algorithm.

2.6. MACSPlex exosome kit

To study in more detail the surface markers expressed on our EV population, we performed an in depth study of 37 extracellular vesicles surface epitopes (130-108-813, MACSPlex Human Exosome Kit; Miltenyi, Bergisch Gladbach, Germany) as previously described (Koliha et al., 2016). Briefly, this assay uses phycoerythrin and fluorescein isothiocyanat-labeled polystyrene capture beads that permit the discrimination of 39 bead subpopulations based on their different fluorescence intensities. These beads are added to the plasma-separated mEVs and incubated overnight. The next day, allophycocyanin labeled detection antibodies are added, containing anti-CD9, anti-CD63 and anti-CD81 antibodies that allow for the identification of extracellular vesicles. This process creates a structure consisting of the capture beads, EV and the detection antibodies allowing for the detection of the event

and the identification of its surface epitopes. This assay was measured in the Attune NxT flow cytometer collecting the median fluorescence intensity (MFI) of each EV surface marker that was then normalized to the mean MFI of the specific EV markers.

2.7. Western blot

Western Blot analysis was performed with isolated mEV suspensions (30 µg total protein) and human placenta tissue lysate (30 µg total protein) (GTX27695, GeneTex, Germany). Samples were thawed at 4 °C and total proteins were loaded on a 4%–10% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Blocking was performed by incubation of the membrane with 5% non-fat milk for 1 h on an orbital shaker. After washing with TBST for 5 min at RT, membranes were incubated overnight with specific primary antibodies. The antibodies used were GRP94 (1:1000, 100 kDa, Proteintech, 14700-1-AP, IL, USA), CD105 (1:500, 75–90 kDa, Proteintech, 10862-1-AP, IL, USA), CD63 (1:500, 28–35 kDa, Proteintech, 25682-1-AP, IL, USA) and β -actin (1:1000, 42 kDa, Cell Signalling Technology 4970, Cambridge, UK). Membranes were washed the following day with blocking buffer to remove unbound antibodies, followed by incubation with horseradish peroxidase-conjugated host-specific secondary antibodies for 1 h. Proteins were detected via enhanced chemiluminescence using the ChemiDoc XRS + System (Bio-Rad, CA, USA).

2.8. Bead-based cytokine assay

Plasma samples were thawed at 4 °C and centrifuged at 400 g for 10 min at 4 °C to remove debris. Plasma levels of cytokines IL-18, IL-1 β , IL-10, TNF- α , IL-8, IL-33, and of soluble triggering receptor expressed on myeloid cells 2 (sTREM2) and brain-derived neurotrophic factor (BDNF) were assessed using the Human LEGENDplex Multiplex Assay (BioLegend®) was used following manufacturer's instructions (Lehmann et al., 2017). Briefly, the assay contains allophycocyanin-coated beads conjugated with surface antibodies allowing for specific binding to the cytokine of our interest. After incubation of the capture beads with the plasma sample, these compounds were now stained with biotinylated detection antibodies that bind specifically to its analyte on the capture beads. Therefore, a capture bead-analyte-detection antibody sandwich is created, and it's further stained with streptavidin-phycoerythrin. This last staining provides different intensity signals depending on the amount of bound analyte. Samples were measured using the Attune NxT Flow Cytometer. The concentration of each particular analyte is determined on a known standard curve using the LEGENDplex™ Data Analysis Online Software Suit. Half of the limit of detection (LOD) was used to perform statistical analyses when a sample value fell below the LOD.

2.9. Statistical analysis

All statistical analyses were performed using GraphPad Prism 9. We evaluated two groups in the chronic setting (smokers and controls) and two groups on the acute intervention (pre and post intervention). All data were non-normally distributed as shown by D'Agostino & Pearson and Shapiro-Wilk tests, therefore, non-parametric U-tests for independent samples was performed when comparing smokers and controls. For comparison between pre and post intervention groups, Wilcoxon matched-pairs rank test was performed. Graphical representations of data were performed in Graphpad Prism 9 and BioRender. Our analyses took into account potential confounds that could be associated with blood parameters, such as age, sex and BMI. No significant differences were found in relation to these variables within groups nor during group comparisons. Representative gating strategy of Flow Cytometric data was analyzed and performed using FlowJo software. An alpha value of $p < 0.05$ was used for all statistical tests. Thus, p values ≤ 0.05 were considered statistically significant and marked with asterisks as follows:

* for $p \leq 0.05$; ** for $p \leq 0.001$; *** for $p \leq 0.0001$. Data are presented as the arithmetic mean with the corresponding standard error of the mean (SEM).

3. Results

Immune cell populations shift after an acute cigarette smoking intervention in never-smokers. In order to investigate the acute effect of cigarette smoking in healthy nonsmokers, 20 never-smokers (47.24 ± 7.9 years old, 12 females, 23.5 ± 0.7 BMI) smoked two cigarettes (12 tar

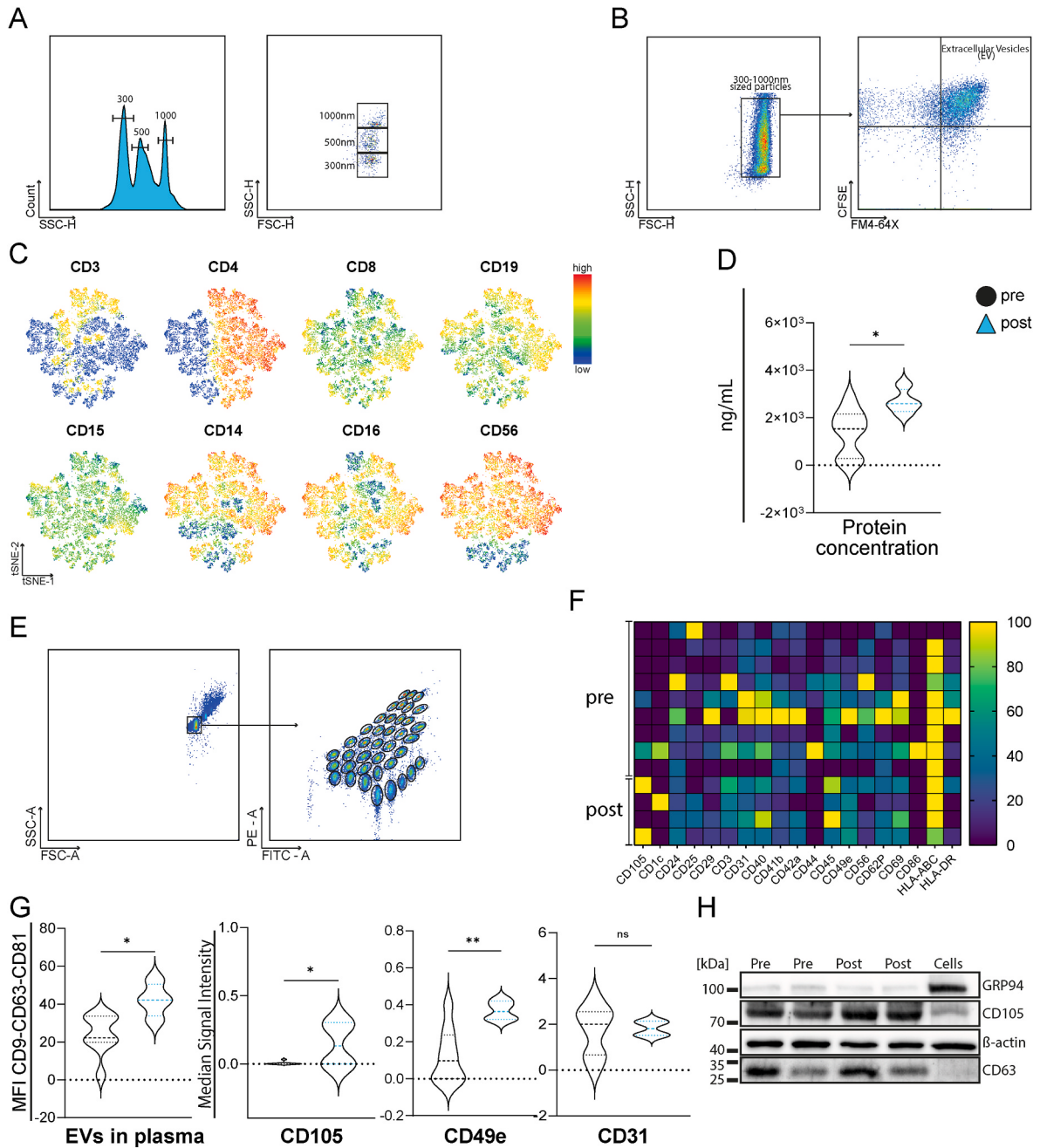
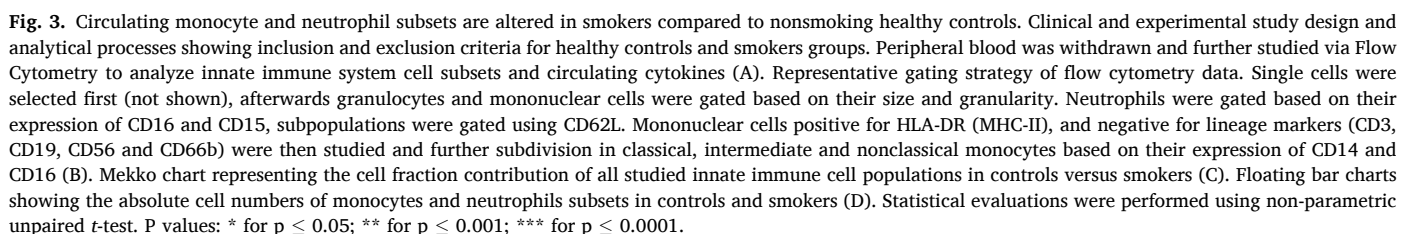


Fig. 2. Plasma extracellular vesicles increase after one tobacco smoking session expressed by immune and endothelial cells. Identification of red fluorescent silica beads via flow cytometry. A size range from 300 to 1000 nm was applied for size calibration (A). EV pellets were labeled by protein-specific fluorescent dye (CFSE), and by lipid-specific (FM 4-64FX) dye prior to measurements. Events within the double positive gate were identified as microvesicles (B). t-distributed stochastic neighbor embedding (t-SNE) plots of the mean fluorescence intensity of CD3, CD4, CD8, CD19, CD15, CD14, CD16 and CD56 surface antigens in a total of 50,000 double positive events (C). Floating bar charts showing the concentration of protein before and after the tobacco smoking intervention, measured via Bradford assay (D). Representative gating strategy of the MACSplex exosome assay, showing the bead size selection (right) and the 37 different dye-labeled capture beads against 37 different EV surface antigens based on FITC and PE fluorescence (left) (E). Heatmap of CD9⁺CD63⁺CD81 normalized MFI in all individual samples, showing 19 differentially expressed surface markers in our cohort both before and after the intervention (F). Violin plots showing the mean MFI of the three main EV markers CD9, CD63 and CD81 in each cohort (far left) and the signal intensity of CD105, CD49e and CD31 surface antigens after detection of CD9, CD63 and CD81 (G). Detection of EV (CD63), endothelial (CD105), cell lysate (GRP94) and loading control (β -actin) markers verified by western blotting before (pre), and 60 min after smoking (post) (H). Statistical evaluations were performed using Wilcoxon matched-pairs rank test. P values: * for $p \leq 0.05$; ** for $p \leq 0.001$; *** for $p \leq 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Extracellular vesicle concentration increases in circulation 1 h after smoking in never-smokers. After the assessment of the immune cell populations, we set to investigate the role of acute smoking in the release of extracellular vesicles. Plasma-derived mEVs were measured before and 1 h after smoking. An overall increased concentration of plasma-derived mEVs was found after the acute smoking intervention (Fig. 2D) (pre, 1383 ± 323.1 ng/mL; post, 2679 ± 258.1 ng/mL, $p = 0.0305$). This result was confirmed by immunostaining of three



canonical tetraspanins found in association with EVs: CD9, CD63 and CD81 (Fig. 2G) (pre, 24.29 ± 3.43 ; post, 42.15 ± 4.4 , $p = 0.0121$). Our findings are supported by previous research where an increased release of EVs was reported from platelets, leukocytes and endothelial cells after tobacco smoking (Mobarrez et al., 2014).

To assess whether these mEVs were from immune cell origin, we performed a comprehensive study to characterize the EV origin based on their expressed surface markers. We utilized multicolor flow cytometry to classify identify microvesicles and by utilizing fluorescent silica beads, its size was determined. Our gating strategy was defined for nanoparticles sized between 300 nm up to 1000 nm (Fig. 2A). Flow cytometry showed predominantly double positive mEVs (Fig. 2B) with signal distribution which indicated $\geq 95\%$ mEVs based on the membrane markers CFSE and FM4-64X (Pospichalova et al., 2015). We further investigated the source of double positive events by using surface markers of the main immune cell populations, namely CD3, CD8 and CD4 for T cells, Cytotoxic T cells and T helper cells respectively; CD19 for B cells, CD15 for neutrophils, CD14 and CD16 for monocytes and CD56 for natural killer cells. Our results showed that the main immune origin of the mEVs after a short cigarette smoking session were monocytes, NK, T, and B cells (Fig. 2C). Afterwards, to assess the non-immune cell-derived mEVs, we studied 37 exosomal surface epitopes using a bead-based assay (Fig. 2E). Nineteen differently expressed markers between pre and post tobacco smoking intervention were selected (Fig. 2F). Out of all the investigated EV surface epitopes in the plasma-isolated mEVs, the endothelial transmembrane glycoprotein Endoglin (CD105) (pre, 0.0040 ± 0.0004 median signal intensity; post, 0.1447 ± 0.0842 median signal intensity, $p = 0.0329$) in addition to the endothelial activation marker CD49e (integrin $\alpha 5$) (pre, 0.1227 ± 0.0482 median signal intensity; post, 0.3681 ± 0.0260 median signal intensity, $p = 0.0080$) were significantly increased upon acute tobacco intervention (Fig. 2G) (Slomka et al., 2018). In addition, following the MISEV2018 criteria (Théry et al., 2018), EV identity was confirmed via Western Blot by the presence of CD63, along with the absence of Golgi membrane stacking protein GRP94, only found in cell lysates (Fig. 2H). Importantly, the presence of CD105 in plasma-derived mEVs was evidenced after a short tobacco smoking session. Taken together, these results point towards an activation of endothelial cells followed by the release of mEVs even after a short tobacco smoking session in healthy nonsmoker individuals.

Clinically asymptomatic current smokers show a decrease in classical and intermediate monocytes accompanied by an increased number of mature neutrophils. To further understand the role of chronic smoking, we now assessed otherwise healthy chronic smokers (Fig. 3). Peripheral monocyte dynamic subsets play an important role in modulating innate immune responses and initiating adaptive immunity (Patel et al., 2017). Here, to expand the understanding on the profile of the peripheral innate immune system of healthy smokers, we studied whole blood from healthy smokers and nonsmokers. No significant differences were observed in the overall monocyte population (controls, 15.35 ± 1.984 cells/ μ L; smokers, 10.43 ± 1.415 cells/ μ L, $p = 0.1921$) however, we found the classical and intermediate monocyte subsets to be significantly decreased in chronic smokers (controls, 5.924 ± 0.8840 cells/ μ L; smokers, 1.852 ± 0.2730 cells/ μ L, $p = 0.0042$ and controls, 4.21 ± 0.5459 cells/ μ L; smokers, 1.926 ± 0.3186 , $p = 0.0284$, respectively). No differences were found within the nonclassical monocytes after comparing never smokers versus chronic smokers (controls, 5.618 ± 0.6217 cells/ μ L; smokers, 4.984 ± 0.6280 cells/ μ L, $p = 0.8469$) (Fig. 3D). We found non-significant lower levels of the CD62L^{dim} (controls, 45.75 ± 11.95 cells/ μ L; smokers 39.66 ± 8.630 cells/ μ L, $p = 0.5134$) and banded (controls, 3.835 ± 0.8854 cells/ μ L; smokers 1.192 ± 0.3863 , $p = 0.1162$) subsets and interestingly, a prominent higher fraction of the mature neutrophils was evidenced in the chronic smokers when compared to nonsmoker controls (controls, 181.4 ± 40.59 cells/ μ L; smokers 1227 ± 212.1 cells/ μ L, $p < 0.0001$) (Fig. 3D). These results indicate that a significantly different innate

immune population composition in current smokers is already present.

Increased CD36 expression by monocytes in asymptomatic chronic smokers. CD36 is a class B scavenger receptor present on an extensive range of cells including monocytes, macrophages, microvascular endothelial cells and adipocytes (Park 2014). However, due to our interest on the peripheral innate immunity and the practicality of a liquid biopsy, we focused our study on the early role of monocyte subsets by analyzing the expression of surface marker CD36 per subset (Fig. 4A). Interestingly, higher levels of CD36 were a general feature detected on classical (controls, 43896 ± 4020 MFI; smokers, 58496 ± 4855 MFI, $p = 0.0453$), intermediate (controls, 42187 ± 4500 MFI; smokers 83370 ± 9488 MFI, $p < 0.0001$) and nonclassical (controls, 17308 ± 2268 MFI; smokers 50839 ± 15929 MFI, $p = 0.0469$) subsets in chronic smokers. CD36 mediates foam cell formation and promotes atherosclerosis (Tian et al., 2020). In line with previous literature, we found an overall higher expression of CCR2 in classical (CD14⁺⁺CD16⁻) and intermediate (CD14⁺⁺CD16⁺) monocytes, which was then decreased in nonclassical monocytes (CD14⁺CD16⁺⁺). Similarly, CX3CR1 was found highly expressed in nonclassical monocytes (CD14⁺CD16⁺⁺) and lessened in (CD14⁺⁺CD16⁻) and intermediate (CD14⁺⁺CD16⁺) monocytes (Kapellos et al., 2019; Hristov and Heine 2015). No significant differences were found in levels of CD62L, CD86, CD163, or HLA-ABC when comparing asymptomatic chronic smokers with never-smokers (Fig. 4B). These results show that the expression of CD36 on circulating monocytes is increased in chronic smokers, suggesting an early shift towards a pro-atherosclerotic environment in otherwise healthy individuals with history of smoking in the absence of clinical symptoms.

Chronic tobacco smoking in healthy individuals influences peripheral cytokine levels. IL-18 has been associated with higher risk for coronary heart disease and tobacco smoking (Jefferis et al., 2011). Our results revealed a significant increase of circulating IL-18 in asymptomatic chronic smokers (controls, 1.559 ± 0.0664 pg/mL; smokers, 1.818 ± 0.2155 pg/mL, $p = 0.0396$) that was accompanied by a decrease in anti-inflammatory cytokine IL-10 (controls, 0.6433 ± 0.1048 pg/mL; smokers, 0.1313 ± 0.0140 pg/mL, $p = 0.0007$) (Fig. 5). Among with these changes, another member of the IL-1 family of cytokines, the alarmin IL33, was found decreased (controls, 1.249 ± 0.1822 pg/mL; smokers, 0.5408 ± 0.0309 pg/mL, $p = 0.0203$) in the chronic smokers groups compared to never smokers (Liew et al., 2016). IL-8 is a pro-inflammatory cytokine that has been described to participate in atherosclerotic plaque destabilization and as a mortality predictor in patients with acute coronary syndrome (Boekholdt et al., 2004; Cavusoglu et al., 2015). In the current smokers' cohort however, the plasma levels of this cytokine were also found to be decreased (controls, 1.938 ± 0.3580 pg/mL; smokers 0.6459 ± 0.1769 pg/mL, $p = 0.0430$).

All previous results from chronic smokers have pointed towards peripheral 'silenced' inflammation. It has been described that peripheral inflammation, evidenced by activation of the innate immune system and release of proinflammatory cytokines can affect the brain (Huang et al., 2021). For this reason, we assessed BDNF and sTREM2 levels in plasma to investigate whether the long exposure to tobacco smoking altered soluble levels of these markers. Our results show significantly lower levels of BDNF in current smokers (controls, 2.324 ± 0.4724 pg/mL; smokers, 0.7674 ± 0.1085 pg/mL, $p = 0.0407$) similarly to previous literature (Bhang et al., 2010), accompanied by a trend of higher soluble TREM2 in plasma of asymptomatic chronic smokers (controls, 4.224 ± 0.4013 pg/mL; smokers, 5.579 ± 0.5345 pg/mL, $p = 0.0562$).

4. Discussion

Tobacco smoking has an evident negative impact on vascular, cardiac, pulmonary and neurological health, including high toxicity that can lead to different types of cancer, chronic obstructive pulmonary disease, and cerebrovascular disease including vascular dementia, among other disorders (Jha et al., 2013; Le Foll et al., 2022; U.S. Department of Health and Human Services). Previous studies have

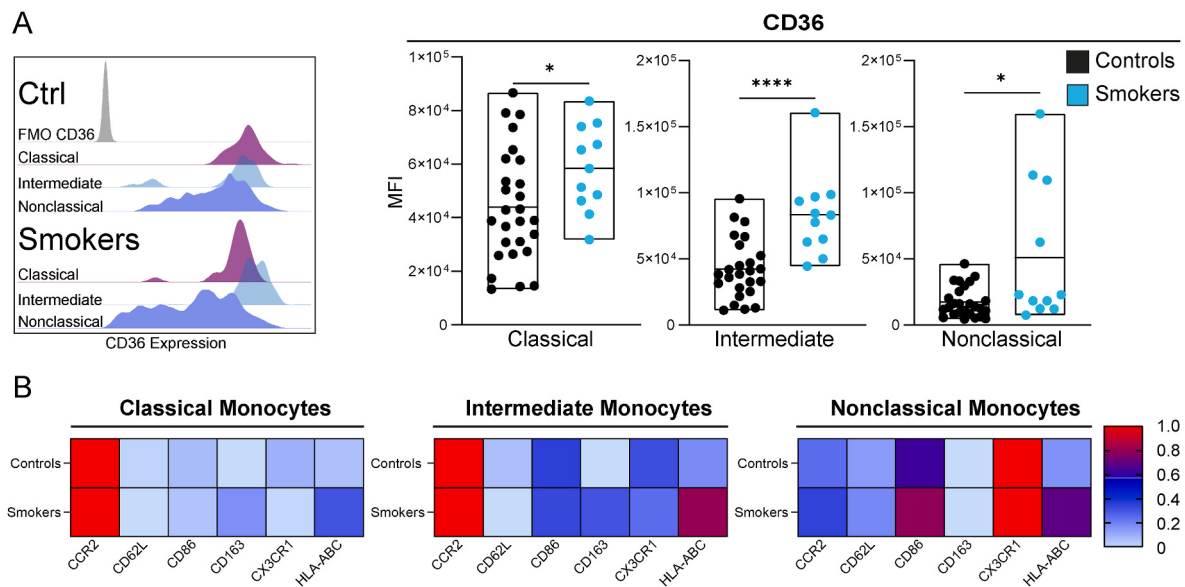


Fig. 4. Chronic tobacco smoking alters CD36 expression in the monocyte subsets. Representative histograms of CD36 median fluorescence intensity (MFI) in monocyte subsets in the two cohorts. Floating bar charts depicting MFI of CD36 in all monocyte subsets from smokers compared to controls. (A). Heatmaps showing the normalized MFI of key activation markers (CCR2, CD62L, CD86, CD163, CX3CR1 and HLA-ABC) across all monocyte subsets (B). Statistical evaluations using non-parametric unpaired *t*-test. P values: * for $p \leq 0.05$; ** for $p \leq 0.001$; *** for $p \leq 0.0001$.

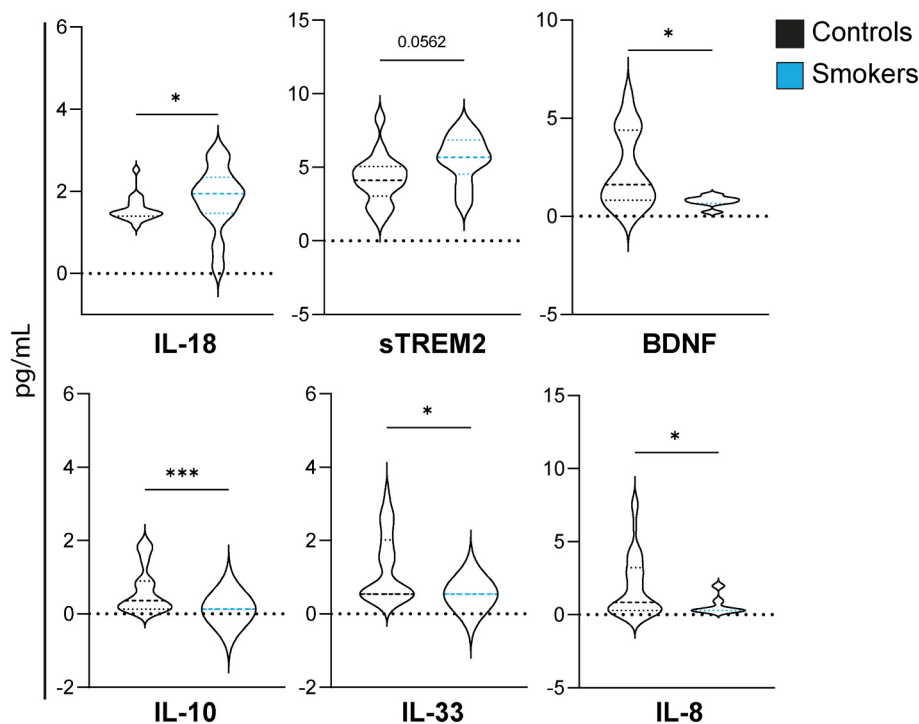


Fig. 5. Circulating cytokine changes in chronic smokers. Levels of IL-18, sTREM2, BDNF, IL-10, IL-33 and IL-8 present in plasma detected by flow cytometry. Values are shown in pg/mL, data points below the Limit of Detection (LOD) were calculated as half the LOD. Statistical evaluation using non-parametric unpaired *t*-test. P values: * for $p \leq 0.05$; ** for $p \leq 0.001$; *** for $p \leq 0.0001$. sTREM2: soluble Triggering receptor expressed on myeloid cells 2. BDNF: brain-derived neurotrophic factor.

detected elevated numbers of circulating leukocytes in the blood of chronic smokers. However, the focus of those studies has been predominantly on patients with diagnosed arterial hypertension, COPD or lung cancer (Andersson et al., 2019; Elisia et al., 2020; Gordon et al., 2011).

Long-term tobacco smoking has been strongly linked to the development of various cardiovascular and neurological disorders, with vascular endothelial damage constituting an important pathomechanism in the development of hypertension and atherosclerosis (Messner and Bernhard 2014). Following the onset of cardiovascular disorders,

chronic smokers endure a higher risk of developing vascular dementia due to the damage on the endothelium (O'Brien et al., 2015). These changes take place after the persistent and chronic stressor that smoking represents. However, the acute effects of smoking on the vasculature and the long-term effects in asymptomatic chronic smokers remained largely unmapped.

In the present study, we focused on a) the acute and transient immune vascular profile of a healthy individual that has not been exposed consistently to tobacco smoking and b) the early and ongoing effects that chronic tobacco smoking elicits on the immune system before any

clinical manifestation of smoking-related cardiovascular diseases. Our results demonstrate that even a short acute tobacco smoking challenge influences the composition of innate and adaptive immune cells circulating in the blood. Activated cells can signal through EVs, that are released by every cell in the organism and can be detected in body fluids (Alberro et al., 2021; Buzas et al., 2014; Panteleev et al., 2017). Studies on EVs in the context of smoking have thus far remained scarce. *In vitro* studies have shown, however, that EVs are released from alveolar macrophages in response to tobacco smoke and that cultivated bronchial epithelial cells increase the production of EV-miRNAs that contribute to lung carcinogenesis (Li et al., 2010; Liu et al., 2016). In our experiment, immune and non-immune cells did release an increased amount of EVs, after the acute tobacco smoking intervention. These data are in line with previous literature in which the amount of EVs (microparticles, an old designation for mEVs) was increased after cigarette smoking (Mobarrez et al., 2014; Heiss et al., 2008).

Investigating the source of mEVs after smoking, we detected that they derived largely from the endothelium. An elevated number of mEVs after acute smoking was previously described *in vitro* in airway basal cells after cigarette smoke extract (CSE) exposure (Saxena et al., 2021). The vascular endothelium is comprised of a monolayer of endothelial cells acting as first barrier in the vessel wall, structurally supporting the circulatory system, and as a versatile endocrine organ producing an array of different molecules (Baumgartner-Parzer and Waldhäusl 2001; Osteikoetxea et al., 2016). In response to smoke exposure, endothelial cells can release inflammatory and proatherogenic cytokines together with reactive oxygen species (ROS), leading to endothelial dysfunction. This has been described in chronic smokers, although the acute *in vivo* effect of smoking in healthy individuals has not been fully elucidated. In 2004, Papamichael et al. reported that healthy individuals who smoked one cigarette had a decrease in flow mediated dilatation (FMD), suggesting a transient vessel wall dysfunction shortly after the intervention (Papamichael et al., 2004). Our results reveal that the endothelium secreted a sizable number of mEVs, characterized by the expression of CD105 and CD49e. CD105 is a transmembrane protein and co-receptor for transforming growth factor β (TGF- β) expressed on endothelial cells, it has been recognized as a marker of angiogenesis, extravasation of leukocytes and associated with tissue injury (Cheifetz et al., 1992; Duff et al., 2003; Rossi et al., 2019). On the other hand, CD49e is the integrin subunit alpha 5 expressed on endothelial cells. The absence of integrin $\alpha 5$ results in leakage of the blood-retinal barrier in mice, highlighting its critical role in barrier integrity (Aylloo et al., 2022).

In order to characterize the innate immune system upon chronic tobacco smoking, we investigated the whole blood of nonsmokers and otherwise healthy current smokers. Our results revealed elevated neutrophil numbers, which can be mechanistically explained by the immediate innate immune response towards the insult (Hidalgo et al., 2019). Even though high numbers of neutrophils have been detected in chronic smokers, their function may be compromised predisposing the host to the development of chronic diseases impairing their immune response against infections (Zhang et al., 2018; Valiathan et al., 2014; Aghapour et al., 2022). Next, we focused on the monocyte subsets, given their critical role in the development of atherosclerosis, driving disease progression via their recruitment into atherosclerotic plaques. We observed significantly lower frequencies of both classical (CD14⁺⁺CD16⁻) and intermediate (CD14⁺⁺CD16⁺) circulating monocytes in chronic smokers. These results suggest a transient adaptation of monocytes due to the chronicity of the stressor and a transitory monocyte suppression in early stages of chronic tobacco smoking.

Given the contribution of CD36 to the dysfunctional development of the endothelium and atherosclerotic plaque formation, we focused our analysis characterizing its expression on the monocyte subsets. CD36 mediates oxidized LDL (oxLDL) uptake and its expression contributes to macrophage foam cell formation (Febbraio et al., 2001) and involvement in angiogenesis (Tian et al., 2020). *In vitro* studies have shown that CD36 depletion from monocytes and macrophages profoundly affected

the ability to uptake oxLDL and prevented foam cell formation, crucial for the development and progression of atherosclerosis (Febbraio et al., 2001; Park 2014). In our study, the expression of CD36 was upregulated by all monocyte subsets, suggesting tissue damage even before the onset of any clinical symptomatology, and yields a myeloid immune specific profile that is observed before tobacco-related disease onset.

Previous studies indicate that pro-inflammatory cytokines such as IL-6 and IL-1 β are elevated in chronic smokers (Elisia et al., 2020; Barbieri et al., 2011). Therefore, our focus was directed towards IL-18, due to its close link with IL-1 β as a byproduct of the NLRP3 inflammasome activation. The NLRP3 inflammasome is highly active in aortic endothelial cells after nicotine treatment *in vitro*, leading consecutively to increased production of IL-18 (Wu et al., 2018; Mehta and Dhawan 2020). We found high levels of IL-18 in the plasma of chronic smokers, which confirmed the significant effect cigarette smoking elicits on the inflammasome-caspase axis (Kang et al., 2007; Eltom et al., 2014). We detected decreased levels of IL-10, a broadly expressed anti-inflammatory cytokine (Saraiva et al., 2010), responding to a pro-inflammatory environment. This finding has been described in the sputum of patients with COPD (Maneechotesuwan et al., 2013), along with observations both *in vitro* and *in vivo* of mice exposed to cigarette smoke or CSE (Higaki et al., 2015). IL-33, is an alarmin regularly released by damaged barrier cells such as endothelial cells (Liew et al., 2016). One of the particularities of IL-33 is that it acts as a traditional cytokine through a receptor complex but also plays an important role as intracellular nuclear factor which regulates the transcription of the p65 subunit of the NF- κ B complex, involved in endothelial cell activation (Choi et al., 2012; Ali et al., 2011). This cytokine was increased in plasma of patients with COPD (Xia et al., 2015) and decreased in mini-bronchoalveolar lavage (mini-BAL) from chronic smokers. Another study detected increased intracellular mRNA levels of IL-33 in bronchial epithelial cells after induction with CSE *in vitro* (Pace et al., 2014). IL-33 is known for its proinflammatory effect, however, in ApoE $-/-$ mice, IL-33 helped reduce the presence of atherosclerotic plaques (Miller et al., 2008). In our study, decreased levels of IL-33 were detected in the plasma of chronic asymptomatic smokers, suggesting an IL-33 intracellular arrest due to its role as a transcription factor. These low levels of IL-33 can potentially be prognostic, given that its absence supports the formation of atherosclerotic plaques in healthy humans with history of smoking. Taken together, low levels of IL-33 and increased CD36 expression in monocyte subsets create a favorable environment for the formation of atherosclerotic plaques.

Smoking increases the risk for vascular dementia, the second most common cause of dementia after Alzheimer's disease (Cipollini et al., 2019). The effects of smoking correlated to dementia vary from toxic to vascular damage (Livingston et al., 2020). It has been widely described that chronic endothelial injury leads to blood-brain barrier dysfunction affecting directly the brain parenchyma (Wardlaw et al., 2017; Rajeev et al., 2022). Recently, tobacco smoking has been linked to a decrease on the resistance and resilience towards Alzheimer's disease (Dumitrescu et al., 2020). Therefore, we aimed to determine plasma levels of soluble triggering receptor expressed on myeloid cells 2 (sTREM2) and brain-derived neurotrophic factor (BDNF) as CNS biomarker candidates in asymptomatic chronic smokers. sTREM2 is a receptor expressed by activated microglial cells, and it has been described as a reliable neurodegeneration biomarker both in CSF and blood of AD patients and in pre-symptomatic individuals with AD-related Tau pathology (Brosseron et al., 2022). Our results revealed elevated sTREM2 in chronic smokers, suggesting neuroinflammatory changes, reflecting microglia enhanced activation during the course of chronic smoking. BDNF is the most prevalent growth factor in the brain regulating neuronal plasticity and survival (Pan et al., 1998; Klein et al., 2011; Zuccato and Cattaneo 2009). It is of relevance to recognize that, unlike serum BDNF, plasma BDNF is not affected by platelet-stored BDNF due to the absence of a clotting process, and this measurement allows for the prediction of BDNF levels in the CNS without an invasive procedure (Gejl et al., 2019;

Fujimura et al., 2002). In line with previous literature (Bhang et al., 2010), we detected lower levels of peripheral BDNF in plasma of chronic tobacco smokers. Reduced BDNF availability upon exposure to chronic stressors increases the risk for accelerated aging and development of neurodegenerative and cardiovascular diseases (Miranda et al., 2019; Kaess et al., 2015). Together with the observation that stress-dependent BDNF suppression may be dependent on neuroinflammatory signaling (Schott et al., 2021), the reduction of BDNF levels accompanied by an increased sTREM2 levels in our study, suggests the presence of a pre-clinical impact of tobacco smoking on CNS function in clinically healthy individuals.

Collectively, we demonstrate the effects of a single tobacco smoking session on the endothelial signaling in healthy never-smokers. Even though the characterization of innate immune cell subsets has been attempted previously in smokers, to our knowledge, this is the first study that provides an in-depth characterization of monocyte subsets and their activation profile comparing disease-free and clinically asymptomatic chronic smokers. Furthermore, we provide evidence of altered cytokine and protein levels related to CNS pathology in clinically asymptomatic chronic smokers. These findings provide further insight into the profound effects of tobacco smoking on the immune system and the potential vascular damage, contributing to neurodegenerative disorders, specifically cerebrovascular dysfunction lowering the threshold for dementia. These results provide a reference to further classify smokers into risk groups, eventually fueling their ambition to quit tobacco consumption, and elucidating potential biomarkers to diagnose vascular disorders before the onset of symptoms (O'Brien et al., 2015; Sachdev et al., 2014). In conclusion, we propose an immuno-vascular dysregulated profile in chronic tobacco smokers who are considered otherwise healthy individuals, contributing to the pathogenesis of early preclinical stages of vascular dementia.

5. Strengths and limitations

The strengths of our study include the comparison between two different disease-free asymptomatic groups, and the in-depth characterization of monocyte subsets and their activation, accompanied by peripheral cytokine levels and extracellular vesicles with one liquid biopsy. Our study also included older adults, who are often excluded from this type of research. Healthy nonsmokers were also included in our research, whereas in previous literature, chronic smokers with symptoms and/or diagnoses of tobacco-related and non-tobacco-related diseases such as COPD, cancer, ischemic heart disease, arterial hypertension and diabetes mellitus were present in a relevant fraction of the participants included (Pedersen et al., 2019; Elisia et al., 2020; Gordon et al., 2011). A limitation was that the majority of our participants were of European ancestry, and therefore, it is impossible to extrapolate our results to other ethnicities. In addition, the smoking status of the participants was self-reported and we did not conduct any cotinine or nicotine blood assessment.

Author contribution

IRD conceived the presented idea, designed the study, supervised analyses and edited the manuscript. IRD, SS and BHS were responsible for supervision and project administration. EIB and EP supervised the isolation and processing of plasma-derived mEVs and edited this manuscript. APG planned and carried out the experiments, data analyses, data visualization and wrote the manuscript. LM contributed to sample preparation, measurements, analyses and wrote the manuscript. BHS contributed to statistical analysis and edited the manuscript. APG, IRD, SS, BHS and LM contributed to the interpretation of the data. All authors approved the submitted version.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

Acknowledgments

This research was supported by DGM Sc 26/1, RTG2413 Synage. A.P. G., B.H.S., and I.R.D. further received support from the European Fund for Regional Development and the State of Saxony-Anhalt (Research Alliance "Autonomy in Old Age"). The project has also received funding from the EU's Horizon 2020 research and innovation program under grant agreement No. 739593 and by the Hungarian Thematic Excellence Program No. TKP2020-NKA-26 and the National Cardiovascular Laboratory program.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbih.2023.100597>.

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