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Cigarette smoke inhibits the NLRP3 inflammasome and leads to caspase-1 activation via the TLR4-TRIF-caspase-8 axis in human macrophages

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Abstract

The NLRP3 inflammasome is formed by the sensor NLRP3, the adaptor ASC, and pro-caspase-1. Assembly and activation of the inflammasome trigger caspase-1-dependent cleavage of pro-IL-1β and pro-IL-18 into their secreted forms. Cigarette smoke is a risk factor for chronic inflammatory diseases and is associated with macrophage dysfunction. The impact of cigarette smoke on NLRP3-dependent responses in macrophages is largely unknown. Herein, we investigated the effects of cigarette smoke extract (CSE) on the NLRP3 inflammasome in human monocyte-derived macrophages (MDMs) and THP-1 cells stimulated with lipopolysaccharide (LPS) and LPS plus the NLRP3 inflammasome activator ATP. We found that CSE inhibited the release of IL-1β and IL-18 as well as the expression of NLRP3 acting mainly at the transcriptional level. Interestingly, we found that CSE increased the caspase-1 activity via an NLRP3-independent and TLR4-TRIF-caspase-8-dependent pathway. Activation of caspase-1 by CSE led to a reduction of the basal glycolytic flux and impaired glycolytic burst in response to LPS. Overall, our findings unveil novel pathways leading to immune-metabolic alterations in human macrophages exposed to cigarette smoke. These mechanisms may contribute to macrophage dysfunction and increased risk of infection in smokers.

KEYWORDS

caspase-8, inflammation, IL-1β, IL-18, metabolism, smoking

1 | INTRODUCTION

The NLRP3 (NACHT, LRR, and PYD domains-containing protein 3) inflammasome is a macromolecular platform composed of the innate immune sensor NLRP3, the adaptor protein ASC, and pro-caspase-1.[1] NEK7, a mitotic Ser/Thr kinase, is also required for NLRP3 activation via direct NLRP3-NEK7 interaction.[2] Assembly and activation

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of the inflammasome occur in response to several stimuli including pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) as well as environmental pollutants.[1,3] Upon the assembly of the complex, caspase-1 becomes active via autoproteolytic cleavage and in turn cleaves pro-IL-1β and pro-IL-18 into their released form and gasdermin D leading to pore formation.[1] Cigarette smoke is a major risk factor for several chronic diseases, such as chronic obstructive pulmonary disease (COPD). However, the role of the NLRP3 inflammasome in cigarette smoke-related diseases is still a matter of debate and available data are limited.[3-7] It is well recognized that cigarette smoke leads to the abnormal inflammatory activation of the bronchial epithelium and to impaired macrophage responses.[8-10] With respect to the NLRP3 inflammasome, growing evidence suggests that cigarette smoke activates the NLRP3 inflammasome in the lung epithelium, with increased expression of NLRP3, pro-IL-1β and caspase-1, higher caspase-1 activity and increased release of inflammasome-related cytokines IL-1β and IL-18.[11-13] However, there is very limited evidence on the impact of cigarette smoke on the NLRP3 inflammasome in macrophages.[14,15] It has been reported that the expression of NLRP3 and the release of IL-1β are reduced in macrophages exposed to cigarette smoke extract.[15,16] In order for the NLRP3 inflammasome complex to be formed and mature IL-1β and IL-18 to be released, several events must take place including (i) transcriptional upregulation of NLRP3 and pro-IL-1β, the so-called "priming" step; (ii) posttranslational modifications of NLRP3 such as phosphorylation and ubiquitylation[17]; (iii) assembly of the active inflammasome complex requiring the core unit including NLRP3, ASC and caspase-1; (iv) autoproteolytic cleavage and activation of caspase-1; (v) cleavage of caspase-1 substrates. An extensive investigation of the impact of cigarette smoke on all these events in macrophages has never been performed.

With the present work, we aimed at assessing the impact of cigarette smoke on NLRP3 inflammasome-dependent responses in human macrophages. Data, herein, reported confirm and expand previous findings on the downregulation of NLRP3 expression and the inhibition of IL-1β and IL-18 release in response to cigarette smoke extracts (CSE). Our data show that CSE inhibits the expression of NLRP3, IL-1β, and IL-18 mainly at the transcriptional level. Interestingly, we report that despite inhibiting the NLRP3 inflammasome, CSE leads to the activation of caspase-1, via an NLRP3-independent and TLR4-TRIF-caspase-8-dependent mechanism. CSE-induced activation of caspase-1 caused a reduction of the glycolytic flux; therefore, unveiling new mechanisms leading to macrophage dysfunction upon exposure to cigarette smoke.

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

The following reagents were purchased from EuroClone, Milan, Italy: RPMI 1640 medium (ECB9006L), L-glutamine (ECB3000B), fetal bovine serum (ECS0180L), sodium pyruvate (ECM0542D), HEPES (ECM0180D), and penicillin–streptomycin (ECB3001D). Human M-CSF (130-096-492) was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). The following chemicals were purchased from Sigma-Aldrich: phorbol 12-myristate 13-acetate (PMA, P8139), lipopolysaccharides from Escherichia coli 0111:B4 (LPS, L3012), adenosine 5′-triphosphate disodium salt hydrate (ATP, A6419), and CP-456773 sodium salt (MCC950, PZ0280). The following antibodies were purchased from Santa Cruz Biotechnology, Dallas, TX, USA: caspase-1 (sc-515), ASC (sc-271054), and actin (sc-81178). Antibodies against NLRP3 and caspase-1 were from Adipogen (AG-20B-0014-C100 and AG-20B-0048-C100), antibody against IL-1β was from R&D (af-201-na) and antibody against caspase-8 was from Enzo Life Sciences (ALX-804-242-C100) and from PharMingen, BD (66231A). The following secondary antibodies were purchased from LI-COR (Lincoln, Nebraska USA): Goat anti-Mouse IRDye 800CW (926-32210), Donkey Anti-Rabbit IRDye 800CW (926-32213), and Donkey anti-Goat IRDye 680CW (926-68074). The anti-mouse HRP secondary antibody was purchased from Dako (P0447). Z-YVAD-fmk was purchased from Abcam (ab141388) and Z-IETD-fmk was purchased from Calbiochem (218759). CLI-095 (tlrl-cli95), Pepinh-MYD (tlrl-pimyd), and Pepinh-TRIF (tlrl-pitrif) were purchased from InvivoGen (San Diego, California, USA).

2.2 | Cell culture

Primary human CD14 + monocytes isolated from peripheral blood mononuclear cells (PBMCs) were purchased from Voden Medical Instruments spa. Human macrophages were obtained by culturing monocytes for 7 days in the presence of 50 ng/mL of human M-CSF in complete RPMI 1640 medium supplemented with 10% FBS. The medium was replaced after 3 days of culture. The day before each experiment, monocyte-derived macrophages (MDMs) were treated with trypsin for 1 minute, scraped, plated in complete medium without M-CSF into 96-well plates (5×10^4 cells/well) or 6-well plates (1.5×10^6 cells/well), and incubated at 37°C, with 5% CO_2. The human monocytic cell line THP-1 (ATCC TIB-202, kindly provided by Dr Angelo Sala) was grown in complete RPMI 1640 medium supplemented

with 10% FBS and differentiated with phorbol 12-myristate 13-acetate (PMA, 5 ng/mL) for 48 hours. The day of stimulation, culture medium was changed to 1% FBS or no serum (as indicated) and cells were stimulated as follows: cells were treated with LPS (1 μg/mL), in the presence or not of 20% CSE and samples were collected after 5 hours. Where indicated, after 3 hours of LPS priming, the inflammasome activator adenosine triphosphate (ATP, 5 mM) was added in the culture medium for 2 hours. Where indicated, the inhibitors Z-YVAD-fmk, Z-IETD-fmk, and MCC950 were added 1 hours before stimulation and the inhibitors CLI-095, Pepinh-MYD, and Pepinh-TRIF were added 6 hours before stimulation.

2.3 | Preparation of cigarette smoke extract (CSE)

Cigarette smoke extract was obtained using the 3R4F reference cigarettes (The Tobacco Research Institute, University of Kentucky) by dissolving in PBS the water-soluble gas and particle phases of cigarette smoke as previously reported.[18] Mainstream smoke as well as CSE composition from 3R4F cigarettes have been previously analyzed.[19,20] Briefly, each cigarette was smoked for 5 minutes and two cigarettes were used for 20 mL of PBS to generate a CSE solution. The CSE solution was filtered, adjusted to pH 7.4, and used within 30 minutes of preparation. This solution was considered to be 100% CSE and diluted to obtain the desired concentration for each experiment. The concentration of CSE was verified spectrophotometrically at a wavelength of 320 nm. The concentration of CSE that we used for cell treatments was selected after performing a dose–response experiment evaluating the effect of increasing concentrations of CSE on cell viability (Figure S1A). About 20% of CSE displayed maximum inhibition of IL-1β release without any toxic effects and therefore was selected for all the following experiments (Figure S1B). The absence of LPS contamination in undiluted CSE was assessed as previously described.[18]

2.4 | Western blot

Cells were lysed using a lysis buffer (10 mM Tris–HCl, 50 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, protease inhibitor (P8340, Sigma-Aldrich, St. Louis, MO), and phosphatase inhibitor (P0044, Sigma-Aldrich, St. Louis, MO). The total protein content was determined using the Bradford protein assay kit (Thermo Fisher, Waltham, MA). A total of 30 μg protein was loaded and resolved by SDS-PAGE and blotted onto nitrocellulose membranes (Bio-Rad, Hercules,

CA). For supernatant precipitation, cells were stimulated in the medium without FBS and samples were processed as previously described.[21] Blots were incubated overnight with primary antibodies at 4°C. Secondary antibodies were used at the following dilutions: 1:2000 for anti-mouse and 1:5000 for anti-rabbit or anti-goat. The blots were analyzed using the Odyssey Imaging System (LI-COR, Lincoln, NE). Western blot of supernatant precipitates was developed using LiteAblot PLUS (EuroClone) and Pierce ECL Western Blotting Substrate (Thermo Scientific) for caspase-1 and caspase-8, respectively. Densitometric analysis of western blot images for the quantification of protein expression relative to actin was performed using the NIH Image J software.

2.5 | RT-PCR

For RT-PCR analysis, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA,) following the manufacturers' instruction. For reverse transcription of NLRP3, IL-1β, and IL-18 mRNA, the equivalent volume to 1 μg RNA was reverse-transcribed to cDNA, using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time quantitative PCR of NLRP3, IL-1β, and IL-18 mRNA was carried out on Step One Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using specific FAM-labeled probe and primers (prevalidated TaqMan Gene expression assay for NLRP3 (Hs00918082-m1), IL-1β (Hs01555410-m1), and IL-18 (Hs01038788-m1), Applied Biosystems). Gene expression was normalized to GAPDH (prevalidated TaqMan Gene expression assay for GAPDH, Hs03929097g1, Applied Biosystems) endogenous control gene. Relative quantification of mRNA was carried out with the comparative CT method (2^$-\Delta\Delta$CT) and was plotted as a relative fold-change compared to untreated cells that were chosen as the reference sample.

2.6 | Immunofluorescence

After stimulation, at the indicated time, cells were stained using the Fam-Flica Caspase-8 assay kit (Immunochemistry Technologies, #98) following the manufacturers' instructions. Images were acquired using the Operetta CLS system (PerkinElmer).

2.7 | ELISA assay

IL-1β, IL-18, and TNFα were measured in culture supernatants using ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturers' instructions.

2.8 | Caspase-1 and caspase-8 enzymatic assay

The intracellular and extracellular activities of caspases were determined using Caspase-Glo 1 homogeneous luminescent assay kit (Cat# G9951, Promega Corporation, Madison, WI), and Caspase-Glo 8 (Cat# G8201, Promega Corporation, Madison, WI) following the manufacturers' instructions.[22]

2.9 | Quantification of intracellular ATP and extracellular lactate

Intracellular ATP was measured using the ATPlite Luminescence Assay System (PerkinElmer) according to the manufacturers' instructions. Extracellular lactate was measured using Lactate Colorimetric/Fluorometric Assay Kit (BioVision Inc) following the manufacturers' instructions.

2.10 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 software. Data were expressed as mean ± SEM. Differences were identified using one-way repeated measures ANOVA with Bonferroni post hoc test. Differences with $P < .05$ were considered significant.

3 | RESULTS

3.1 | Cigarette smoke extract (CSE) inhibits the release of IL-1β and IL-18

The impact of CSE on the release of mature IL-1β and IL-18 was evaluated in human monocyte-derived macrophages (MDMs) and PMA-differentiated THP-1 cells under basal conditions and upon stimulation with LPS alone and LPS followed by the inflammasome activators ATP and Nigericin (Figure 1 and S2). In MDMs, IL-1β and IL-18 were secreted following the inflammasome activation by LPS + ATP or LPS + Nigericin and exposure to CSE significantly reduced their release (Figure 1A,B and S2A,B). Exposure to CSE also inhibited the LPS-induced release of TNFα (Figure S2C). In THP-1 cells, IL-1β was released both in LPS- and LPS + ATP-treated cells (Figure 1C), while IL-18 was released only upon treatment with LPS + ATP (Figure 1D). CSE inhibited the release of both cytokines (Figure 1C,D).
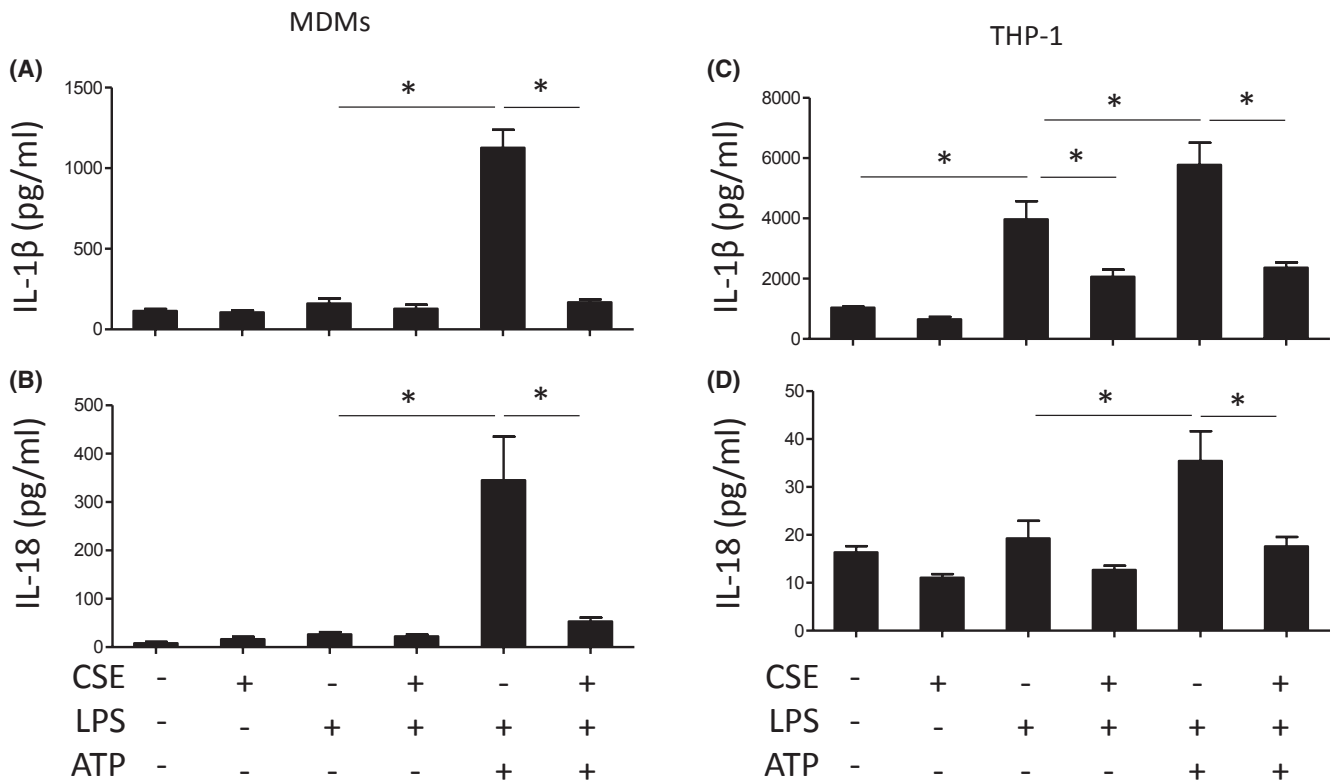

FIGURE 1 CSE reduces LPS- and LPS + ATP-induced the release of IL-1β and IL-18 in MDMs and THP-1 macrophages. Cytokine release by (A, B) MDMs (N = 6 independent donors) and (C, D) THP-1 cells (N = 6 independent experiments for IL-1β and N = 4 independent experiments for IL-18) was determined by ELISA assay. Cells were primed with 1 μg/mL of LPS for 3 hours and then 5 mM ATP was added for 2 hours. Where indicated, 20% CSE was added at the same time of LPS for 5 hours. Mean with SEM is reported. Repeated measures ANOVA with Bonferroni post hoc test was applied

3.2 | CSE reduces the protein expression of NLRP3 and pro-IL-1β

To assess whether the inhibition of cytokine release by CSE was associated with decreased availability of the IL-1β precursor or altered expression of inflammasome components, the expression of pro-IL-1β, NLRP3, ASC, and pro-caspase-1 was evaluated by western blot. In THP-1 cells, the expression

of pro-IL-1β and NLRP3 was induced by LPS and this increase was inhibited by CSE (Figure 2A-C and S3A). The addition of ATP after LPS priming had no effect on the expression of pro-IL-1β and NLRP3. In MDMs, CSE inhibited LPS-induced increase of both NLRP3 and pro-IL-1β (Figure 2D-F and S3B). The expression of ASC and pro-caspase-1 was not modulated by any of the treatments in both cell types (Figure 2A,D and S3, S4A-D).
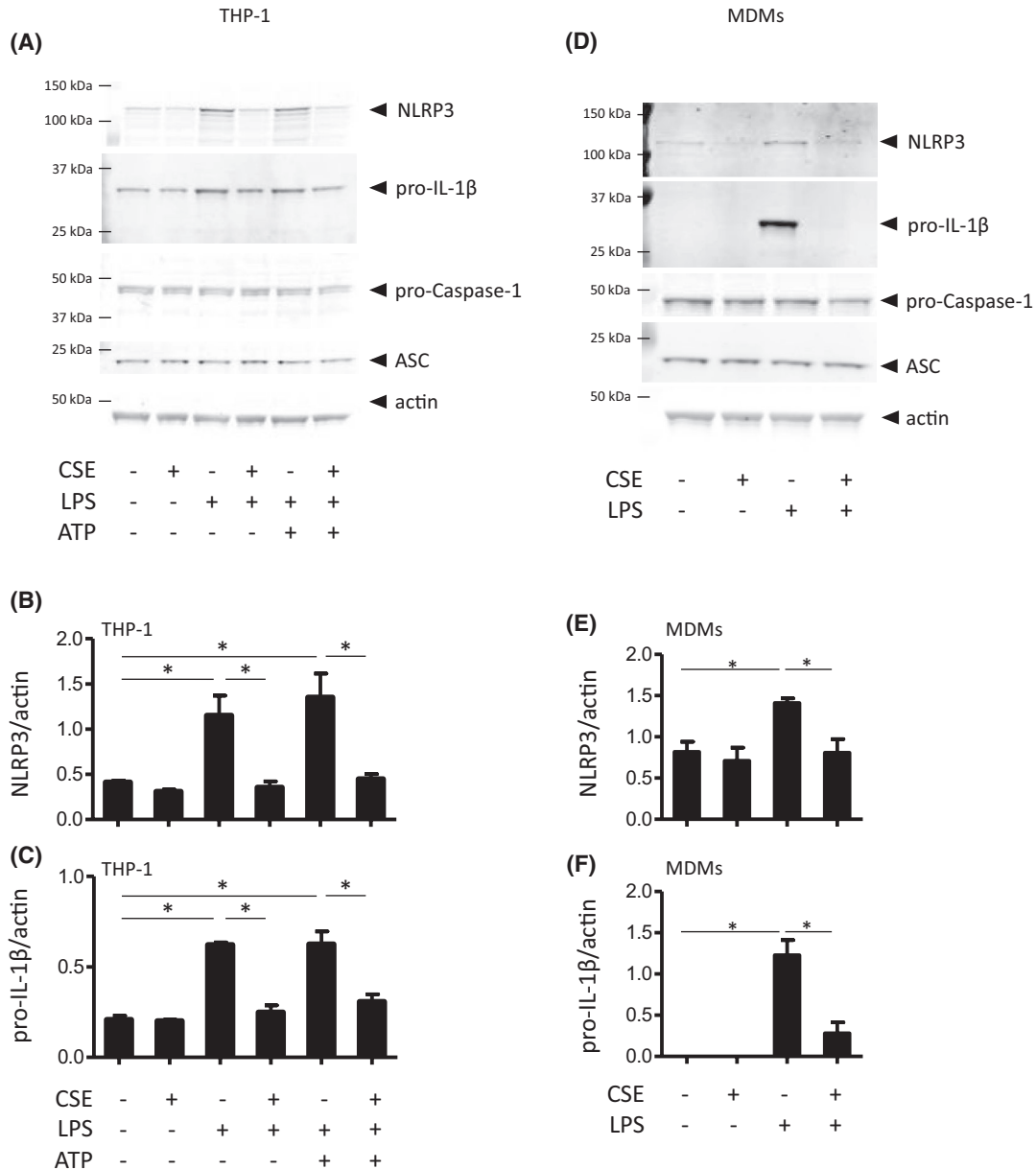

FIGURE 2 CSE inhibits the protein expression of NLRP3 and pro-IL-1β in THP-1 macrophages and MDMs. PMA-differentiated THP-1 cells and MDMs were primed with 1 μg/mL of LPS for 3 hours. Where indicated, 5 mM ATP was added for 2 hours and 20% CSE was added at the same time of LPS for 5 hours. After stimulation, cell extracts were obtained for western blot analysis. A,D, Representative Western Blot images; (B, C, E, F) Densitometric analysis (N = 4 independent experiments for THP-1 cells and N = 3 independent donors for MDMs) for the relative expression of NLRP3 (B, E) and pro-IL-1β (C, F). Data are expressed as mean with SEM. For statistical analysis, repeated measures ANOVA with Bonferroni post hoc test was applied

3.3 | CSE reduces the expression of NLRP3, pro-IL-1β, and pro-IL-18 at the transcriptional level

We next evaluated whether the effects of CSE were due to transcriptional downregulation. Since the addition of ATP after the LPS priming had no effect on the expression of pro-IL-1β and NLRP3 (Figure 2A-C), we focused on the effects of CSE under basal conditions and in cells stimulated with LPS alone. In MDMs, CSE significantly reduced the LPS-dependent transcriptional upregulation of NLRP3, pro-IL-1β, and pro-IL-18 mRNA (Figure 3A-C) as well as the basal expression of NLRP3 (Figure 3A). In THP-1 cells, LPS led to the transcriptional upregulation of NLRP3 and pro-IL-1β, but not of pro-IL-18 (Figure 3D-F). Exposure to CSE inhibited LPS-induced transcriptional upregulation of NLRP3 and pro-IL-1β and significantly reduced IL-18 mRNA constitutive levels. It has been reported that the NLRP3 inflammasome is negatively controlled by miR-223, a microRNA that binds to the NLRP3 3'-untranslated region and that is highly expressed in human macrophages.[23,24] We, therefore, investigated whether the modulation of miR-223 by CSE could contribute to the downregulation of NLRP3 levels. No modulation of miR-223 expression in response to CSE was observed in THP-1 cells (Figure S5); therefore, ruling out the

involvement of this miRNA in the control of NLRP3 levels upon exposure to CSE.

3.4 | CSE increases extracellular active caspase-1

Assembly of the active NLRP3 inflammasome triggers the activation of caspase-1.[1] We, therefore, wondered whether the downregulation of NLRP3 caused by exposure to CSE was associated with a reduction of caspase-1 activity. Surprisingly, CSE caused a significant increase of extracellular caspase-1 activity both in MDMs and in THP-1 macrophages (Figure 4A and S6A). The increase of caspase-1 activity in response to CSE was not associated with cell death (Figure S7A,B). Interestingly, activation of caspase-1 was higher when LPS was added in combination with CSE and was completely abrogated by the caspase-1 inhibitor z-YVAD-fmk; therefore, confirming the selectivity of the assay. The increase of extracellular active caspase-1 in response to CSE, alone or in combination with LPS, was further confirmed by the western blot analysis of supernatant precipitates showing the processing of pro-caspase-1 into the active p20 subunit (Figure 4B). Since the exposure to CSE led to a very strong reduction of NLRP3 levels, we
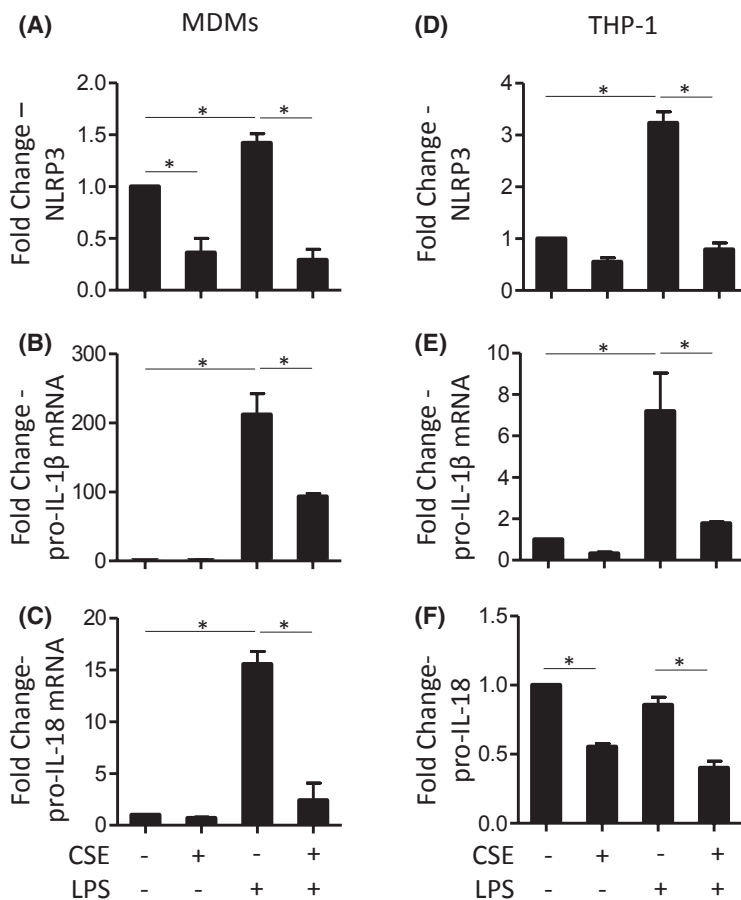

FIGURE 3 CSE downregulates mRNA levels of NLRP3, pro-IL-1β, and pro-IL-18 in MDMs and THP-1 cells. MDMs (A-C, N = 3 independent donors) and PMA-differentiated THP-1 cells (D-F, N = 3 independent experiments) were treated with 1 μg/mL of LPS for 5 hours (18 hours in the case of IL-18 in THP-1 cells). Where indicated, 20% CSE was added at the same time of LPS. After stimulation, total RNA was extracted and NLRP3 (A, D), pro-IL1-β (B, E), and pro-IL-18 (C, F) mRNA levels were measured by RT-PCR. Mean with SEM is reported. For statistical analysis, repeated measures ANOVA with Bonferroni post hoc test was applied
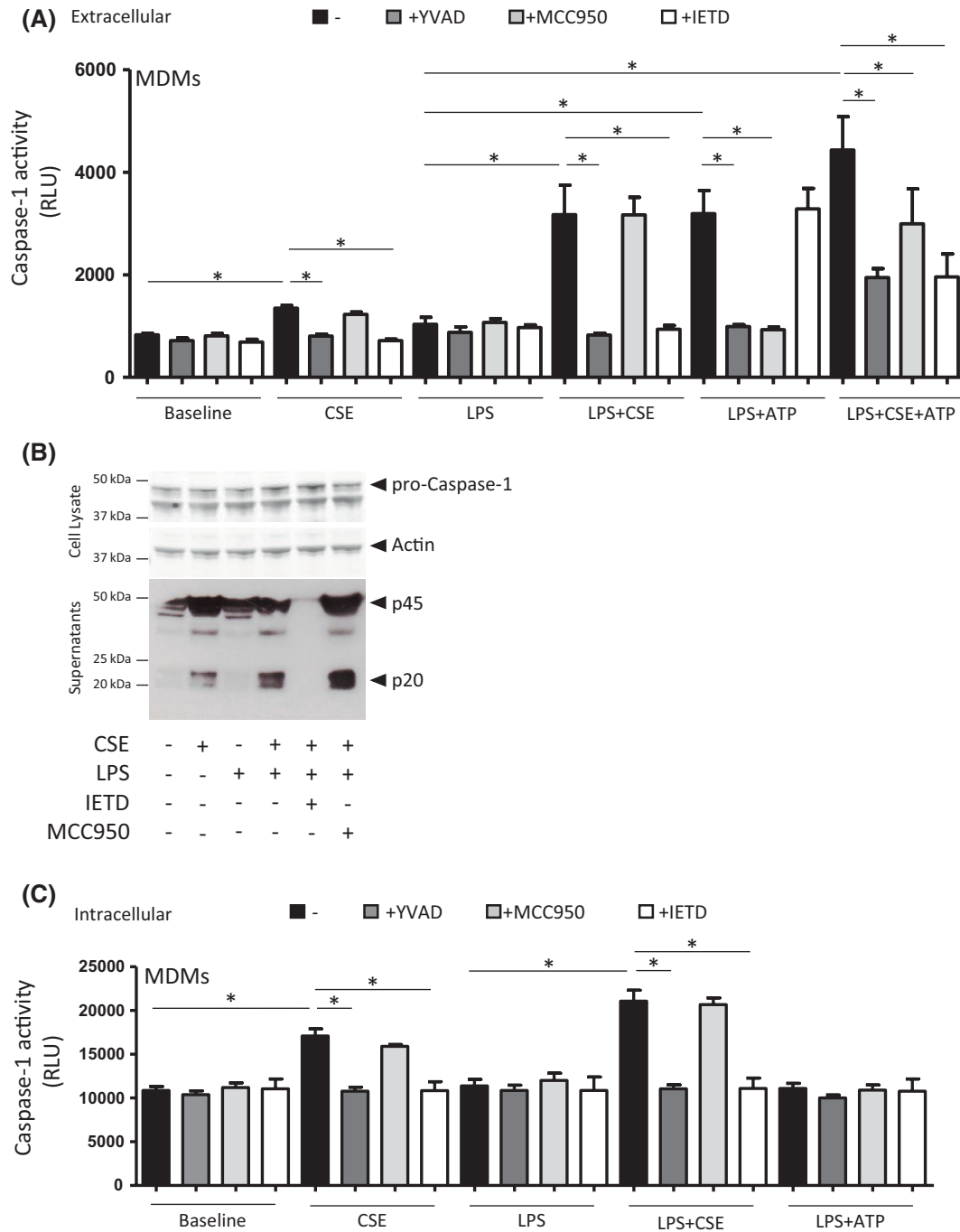
FIGURE 4 NLRP-3-independent activation of caspase-1 by CSE requires caspase-8. MDMs were primed with 1 μg/mL of LPS for 3 hours. Where indicated, 5 mM ATP was added for 2 hours and 20% CSE was added at the same time of LPS. Inhibitors were added 1 hour before the addition of LPS (10 μM Z-YVAD-fmk, 0.1 μM Z-IETD-fmk and 10 μM MCC950). Then, extracellular (A, N = 5 independent donors) or intracellular (C, N = 6 independent donors) activity of caspase-1 was measured using the Caspase-Glo-1 homogeneous luminescent assay kit. Mean with SEM is reported. For statistical analysis, repeated measures ANOVA with Bonferroni post hoc test was applied. B, Supernatants were precipitated and processing of caspase-1 was evaluated by western blot. Western blot images from cell lysates deriving from the same experiments are reported

hypothesized that the observed activation of caspase-1 in response to CSE occurred via an NLRP3-independent mechanism. To test this hypothesis, cells were treated with MCC950, a selective inhibitor of NLRP3. MCC950 did not inhibit the increase of caspase-1 activity in response to

CSE; therefore, confirming our hypothesis (Figure 4A,B and S6A). In contrast, MCC950 inhibited the increase of active caspase-1 observed in response to LPS + ATP, confirming that the classical activation of the NLRP3 inflammasome by LPS + ATP requires NLRP3.

3.5 | NLRP3-independent activation of caspase-1 by CSE occurs via TLR4-TRIF-caspase-8-dependent pathway

It has been previously reported that caspase-8 can mediate the NLRP3-independent activation of caspase-1 in macrophages.[25] Furthermore, activation of caspase-8 is increased in the lung of mice exposed to cigarette smoke and in human fibroblasts exposed to CSE[26] and growing evidence supports that caspase-8 plays a non-apoptotic role in modulating inflammatory responses.[27-30] Therefore, we hypothesized that activation of caspase-1 in response to CSE could depend on the activity of caspase-8. To test our hypothesis, we first evaluated the effects of CSE on caspase-8 activation. Fluorescent staining of MDMs with the FAM-LETD-fmk probe (specific for active caspase-8) showed that CSE, alone or in combination with LPS, caused the activation of caspase-8 (Figure 5A and S8). The observed activation was inhibited by the caspase-8 inhibitor Z-IETD-fmk at 0.1 μM; therefore, confirming the selectivity of the assay. The low concentration of Z-IETD-fmk (0.1 μM) was selected based on a dose–response experiment aimed at evaluating the cross-reactivity of Z-IETD-fmk toward caspase-1. As reported in Figure S9A, when testing the activity of Z-IETD-fmk toward human recombinant caspase-1 some cross-reactivity was observed at concentrations ranging from 0.2 to 0.4 μM. At 0.1 μM, Z-IETD-fmk did not inhibit the caspase-1 activity. Therefore, this concentration was selected for our experiments. Caspase-8 activation was further confirmed by western blot of cell supernatants showing proteolytic cleavage (Figure 5B) as well as using an enzymatic luminescent assay (Figure 5C). Activation of caspase-8 was significantly higher in the presence of LPS + CSE compared to CSE alone (Figure 5C). To test whether the activation of caspase-1 observed in response to CSE may depend on caspase-8, cells were treated with the caspase-8 inhibitor Z-IETD-fmk prior to CSE exposure and activation of caspase-1 was evaluated. As reported in Figure 4A,B, Z-IETD-fmk inhibited the activation of caspase-1 in response to CSE thus indicating the involvement of caspase-8. In contrast, Z-IETD-fmk did not inhibit the activation and processing of caspase-1 in response to LPS + ATP (Figure 4A and S6A, S9B). When cells were exposed to CSE and treated with LPS + ATP, the activation of caspase-1 was inhibited by both MCC950 and Z-IETD-fmk (Figure 4A). Caspase-8 requirement for the activation of caspase-1 in response to CSE was further confirmed by a siRNA experiment showing that downregulating caspase-8 by targeting siRNA inhibited caspase-1 activation in response to CSE (Figure S6C-D).

Previous reports have shown that caspase-8 activation can occur in response to endoplasmic reticulum stress via a TLR4-TRIF-dependent pathway, independent of the TLR4-MyD88 axis, in bone marrow-derived macrophages.[29] To evaluate whether the activation of caspase-8 in response to cigarette smoke occurred via TLR4-dependent mechanisms, MDMs were treated with the TLR4-inhibitor CLI-095 prior to exposure to LPS + CSE and caspase-8 proteolytic cleavage was evaluated by western blot (Figure 5B). CLI-095, also known as TAK-242, specifically suppresses TLR4 signaling by blocking the signaling mediated by the intracellular domain of TLR4. We found that CLI-095 blocked the activation of caspase-8 in response to LPS + CSE; therefore, confirming our hypothesis. To assess whether the activation of caspase-8 downstream of the TLR4 occurred via the TRIF or the MyD88 pathway, MDMs were treated with the TRIF inhibitor Pepinh-TRIF or the MyD88 inhibitor Pepinh-MYD. Pepinh-TRIF is a 30 aa peptide that blocks TRIF signaling by interfering with TLR-TRIF interaction. Pepinh-MYD is a 26 aa peptide that blocks MyD88 signaling by inhibiting its homodimerization through binding. Afterward, cells were exposed to CSE and LPS, alone or in combination, and activation of caspase-8 was quantified using a luminescent enzymatic assay. Data reported in Figure 5B and C show that the activation of caspase-8 in response to CSE and LPS + CSE did not depend on MyD88 but was blocked in the presence of the TRIF inhibitor Pepinh-TRIF.

3.6 | CSE increases intracellular activation of caspase-1

We next investigated whether the intracellular activity of caspase-1 was modulated by CSE and found that, unlike LPS + ATP, CSE led to a significant NLRP3-independent and caspase-8-dependent increase of intracellular caspase-1 activity (Figure 4C and S6B). The increase of intracellular caspase-1 activity was further confirmed using an independent colorimetric assay performed in fresh THP-1 cell extracts (Figure S10) as well as by immunofluorescence in MDMs (Figure S11).

3.7 | Exposure to CSE causes the caspase-1-dependent reduction of intracellular ATP and glycolytic flux

Exposure to cigarette smoke impacts on cell metabolism and energy homeostasis.[31,32] A previous study showed that caspase-1 is able to cleave a number of substrates that participate in the control of cell bioenergetics and glycolysis.[33] Therefore, we hypothesized that the activation of caspase-1 upon exposure to CSE could contribute to the impairment of energy homeostasis. At first, the impact of CSE on the intracellular levels of ATP was evaluated in the presence or not of the caspase-1 inhibitor Z-YVAD-fmk. Treatment with CSE significantly reduced the intracellular levels of ATP in
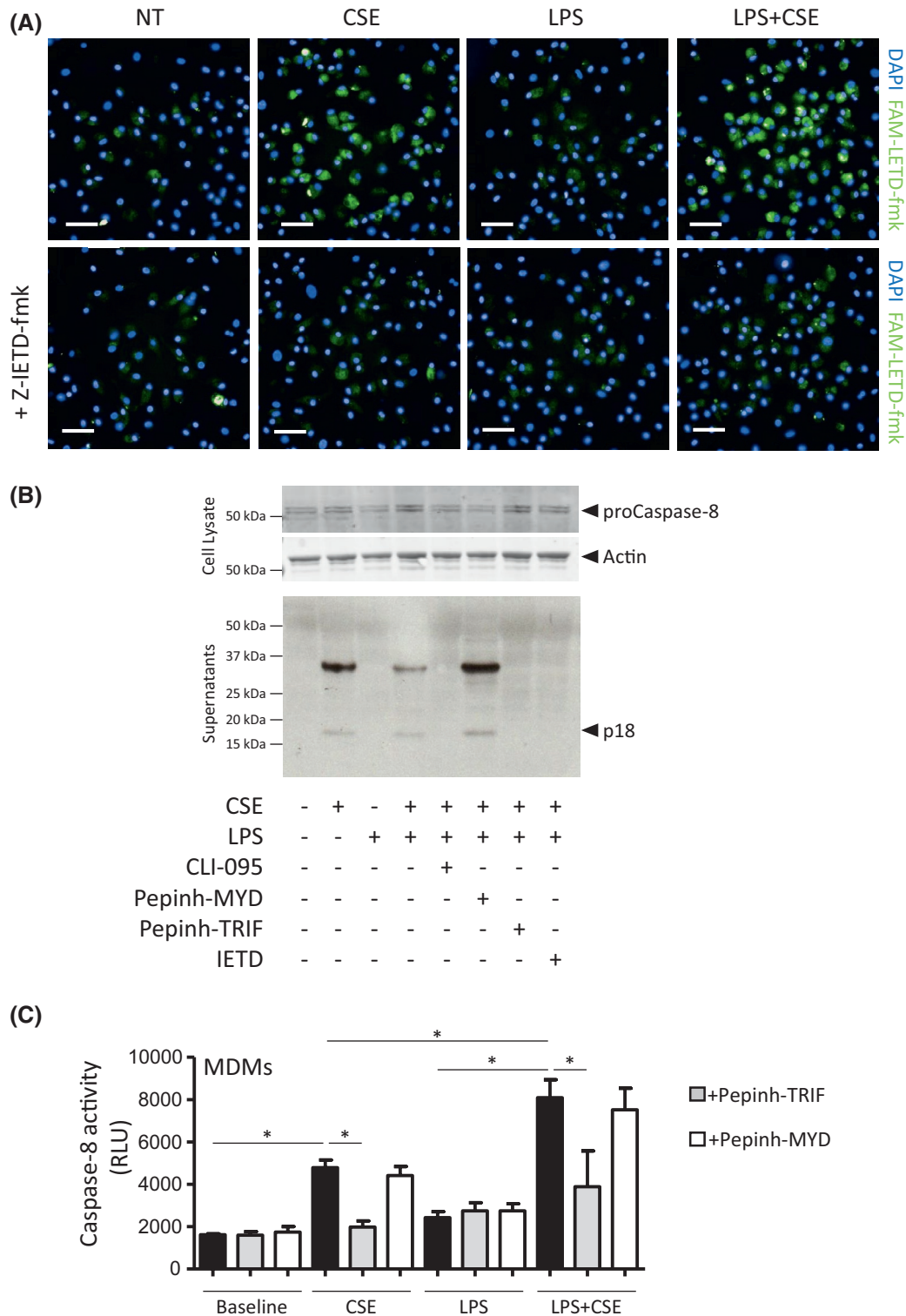
FIGURE 5 Exposure to CSE increases caspase-8 activity in MDMs. MDMs were treated with 1 μg/mL of LPS and 20% CSE, alone or in combination, for 5 hours. Where indicated, inhibitors were added 1 hour (0.1 μM Z-IETD-fmk) or 6 hours (1 μg/mL of CLI-095, 25 μM Pepinh-TRIF and 25 μM Pepinh-MyD88) before the addition of LPS. A, After treatment, cells were stained with the fluorescent probe FAM-LETD-fmk to detect active caspase-8 by fluorescence microscopy and incubated for 1 hour at 37°C. Images are the representative of three independent experiments. Scale bar: 50 μm. B, Supernatants were precipitated and processing of caspase-8 was evaluated by western blot. Western blot images from cell lysates deriving from the same experiments are reported. C, Extracellular activity of caspase-8 was measured using the Caspase-Glo-8 homogeneous luminescent assay kit. Mean with SEM is reported (N = 3 independent donors). For statistical analysis, repeated measures ANOVA with Bonferroni post hoc test was applied

MDMs and THP-1 cells (Figure 6A and S12A). Inhibition of caspase-1 by Z-YVAD-fmk completely reverted this effect.

Macrophages mostly rely on glycolysis for energy generation and their activation by LPS is dependent on glycolysis.[34,35] Therefore, we hypothesized that caspase-1-dependent decrease of intracellular ATP levels upon exposure to CSE was associated with a reduction of the glycolytic flux. The release of extracellular lactate correlates with glucose consumption and glycolytic rate [35,36] and therefore lactate production was measured to evaluate the impact of CSE on the glycolytic flux. We found that LPS increased the release of lactate in MDMs (Figure 6B) but not in THP-1 cells (Figure S12B). Exposure to CSE significantly decreased both basal as well as LPS-induced lactate release in MDMs and THP-1 cells (Figure 6B and S12B). Inhibition of caspase-1 by Z-YVAD-fmk reverted CSE-dependent reduction of lactate release in both cell types (Figure 6B and S12B).

4 | DISCUSSION

Our current work unveils novel findings on the effects of cigarette smoke exposure on human macrophages. We found that CSE inhibited the release of IL-1β and IL-18 and the expression of NLRP3, pro-IL-1β, and pro-IL-18 acting at the transcriptional level. It was unexpected to find that CSE caused the NLRP3-independent activation of caspase-1. Our investigation unveiled that this occurred via the TLR4-TRIF-caspase-8 axis and also led to the reduction of the glycolytic flux (Figure 7).

Cigarette smoke is a major risk factor for several chronic inflammatory disorders and promotes the impairment of macrophage function, leading to increased risk of infection.[16,37-39] The role of the NLRP3 inflammasome in diseases associated with cigarette smoke exposure is still unclear. Herein, we investigated the impact of CSE on the NLRP3 inflammasome and NLRP3-dependent responses in human macrophages.

With regard to transcriptional and post-transcriptional regulatory mechanisms governing the NLRP3 inflammasome and its downstream cytokines, our data revealed key differences between MDMs and THP-1 cells. In MDMs, both pro-IL-1β and pro-IL-18 were transcriptionally upregulated in response to LPS but the release of their mature forms strictly depended on inflammasome activation, consistent with previous reports.[40,41] In THP-1 macrophages, pro-IL-18 displayed a constitutive expression and mature IL-18 was released only upon inflammasome activation as previously shown.[42] With regard to pro-IL-1β, LPS induced both transcriptional upregulation and release of the active form in THP-1 cells. Transcription of NLRP3 was induced by LPS in both cell types. Despite the differences, both cell types responded similarly to CSE exposure. We found that
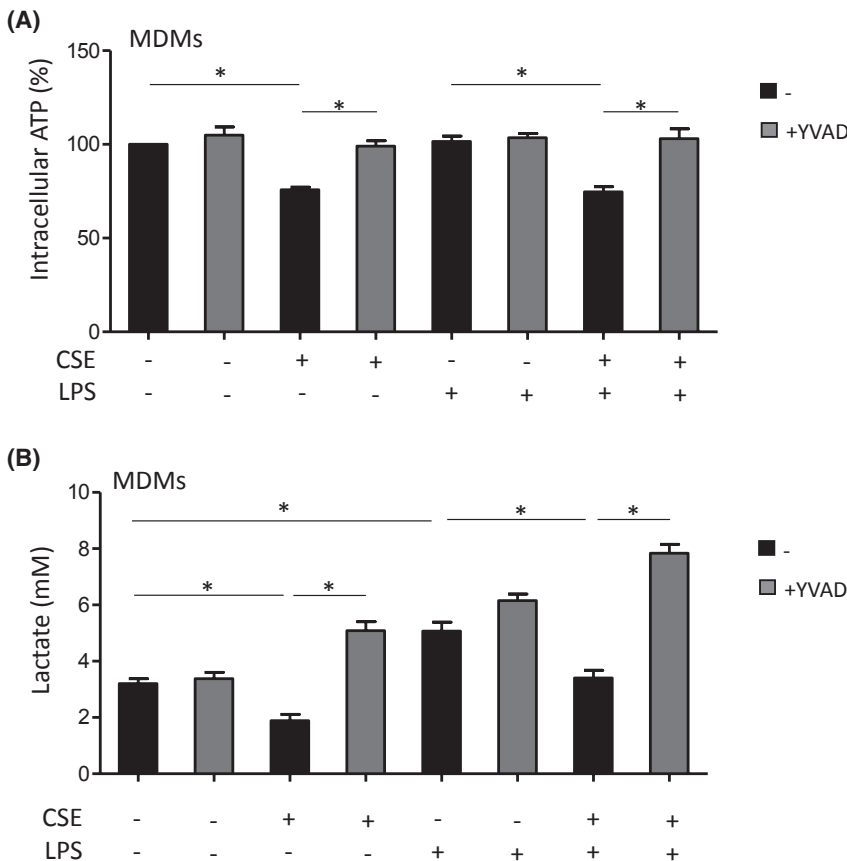

FIGURE 6 Exposure to CSE leads to the caspase-1-dependent reduction of intracellular ATP and extracellular lactate in MDMs. MDMs were treated with 1 µg/mL of LPS and 20% CSE, alone or in combination, for 5 hours. Where indicated, 10 µM Z-YVAD-fmk was added 1 hour before stimulation. A, After treatment, intracellular ATP was measured. Intracellular ATP levels are reported as the percentage of control. Mean with SEM is reported (N = 4 independent donors). B, After treatment, supernatants were collected for lactate measurement. Mean with SEM is reported (N = 8 independent donors). For statistical analysis, repeated measures ANOVA with Bonferroni post hoc test was applied
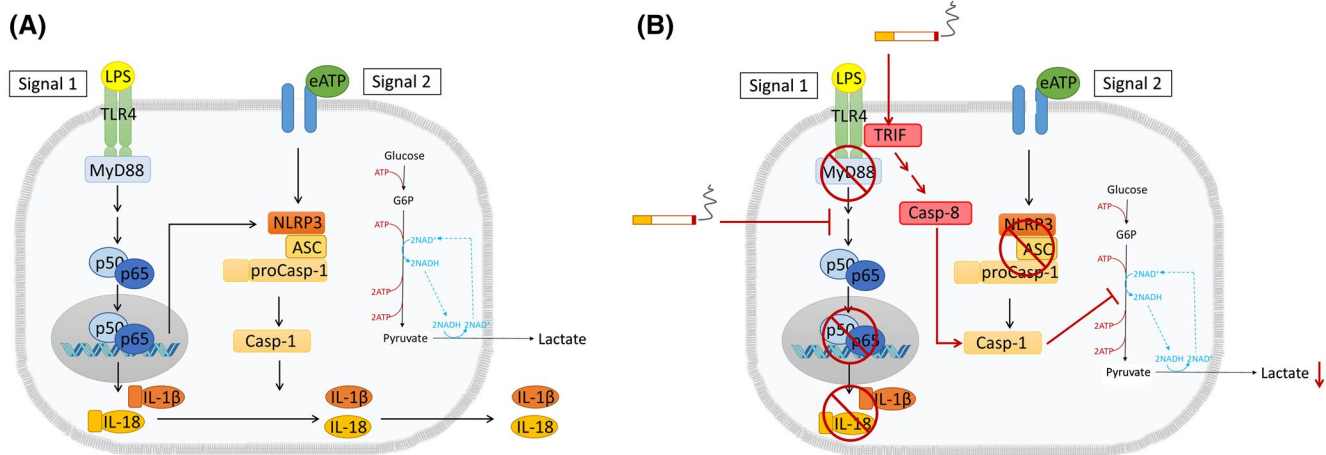
FIGURE 7 Proposed model. A, Following LPS transcriptional priming (Signal 1) and treatment with extracellular ATP (Signal 2) the NLRP3 inflammasome becomes active, leading to the activation of caspase-1 and cleavage and release of mature IL-1β and IL-18 (B) When macrophages are exposed to CSE, the response to LPS is altered: transcription of NLRP3, pro-IL-1β, and pro-IL-18 is inhibited and no NLRP3 inflammasome complex can be formed. Also, the release of IL-1β and IL-18 is reduced due to the lack of precursors. CSE leads to the activation of caspase-1 via the TLR4-TRIF-caspase-8axis. Active caspase-1 in turn inhibits glycolysis

CSE inhibited LPS-induced transcription of pro-IL-1β as previously reported[16] and for the first time show that CSE inhibited basal and LPS-induced transcription of NLRP3 as well as LPS-induced transcription of pro-IL-18 in MDMs. We also show that CSE inhibited the constitutive expression of pro-IL-18 in THP-1 cells and LPS-induced expression of pro-IL-1β and NLRP3 in THP-1 cells. The protein levels of NLRP3 and pro-IL-1β paralleled the pattern of transcriptional regulation therefore supporting that the effects of CSE were mainly due to transcriptional downregulation. We also report that CSE inhibited LPS-induced transcription of TNFα. Taken together, data herein presented indicate that the transcriptional inhibition observed in response to CSE was not specific to the NLRP3 inflammasome. These findings are in agreement with previous reports showing that human macrophages exposed to cigarette smoke display a general inhibition of the pro-inflammatory transcriptional response triggered by LPS due to a reduction of NF-κB activation.[16,43]

Activation of caspase-1 is the established readout of inflammasome activation. Therefore, we were surprised in finding that exposure to CSE significantly increased the activity of caspase-1 despite strongly inhibiting the expression of NLRP3. The use of the potent NLRP3 inhibitor MCC950 at 10 μM ruled out any suspect that the observed caspase-1 activation could be mediated by any residual activity of NLRP3. Increased expression and activity of caspase-1 has been reported in BAL from smokers, in lung tissue of COPD patients and in bronchial epithelial cells exposed to cigarette smoke.[11,44] Searching for pathways that could lead to NLRP3-independent activation of caspase-1 in response to CSE we found that caspase-8 is able to mediate processing of caspase-1 via inflammasome-independent mechanisms in macrophages.[25] Growing evidence supports a non-apoptotic

role for caspase-8, for example, as the downstream effector of TLR4-dependent signaling.[27-29] Furthermore, it was reported that the activity of caspase-8 increased in the lung of mice exposed to cigarette smoke.[26] Taken together, these findings led us to hypothesize that caspase-8 may become active upon exposure to CSE and in turn promote the activation of caspase-1. Data herein reported confirmed this hypothesis. The NLRP3-independent and caspase-8-dependent activation of caspase-1 in response to CSE were also confirmed in murine macrophages RAW264.7 (data not shown). Interestingly, when added in combination with CSE, LPS led to a stronger activation of caspase-8 prompting us to evaluate the involvement of TLR4-dependent pathways. It has been previously reported that CSE can activate TLR4-dependent responses in bronchial epithelial cells.[38] Inspired by recent findings showing that the activation of caspase-8 in macrophages can be triggered via the TLR4-TRIF pathway,[27] independent of MyD88, we evaluated the involvement of TLR4 and TRIF in our model. Our data confirm that CSE causes dysregulation of the TLR4 pathway and add novel information in this respect. More specifically, we show for the first time that CSE promotes the activation of the TLR4-TRIF axis leading to caspase-8 activation via a MyD88 independent mechanism. When combined with LPS, CSE causes a shift toward the TLR4-TRIF axis, which becomes strongly activated, while the TLR4-MyD88 pathway (leading to NF-κB activation) is inhibited by CSE. Interestingly, activation of caspase-8 did not lead to cell death. This was in agreement with previous reports showing that when caspase-8 is activated in response to TLR4 activation, it displays a non-apoptotic role and participates in inflammatory reactions.[27,28,30]

Another interesting finding that we present in this study is the increased intracellular activation of caspase-1 upon

exposure to CSE. This is different compared to what is observed after treatment with LPS + ATP, when active caspase-1 is detected only in the extracellular space. These data suggest that, unlike the inflammasome activator ATP, CSE may activate caspase-1 with alternative mechanisms that do not lead to cell death. Previous work has shown that activation of caspase-1 and secretion of IL-1β can occur in living cells when alternative activation of the inflammasome takes place.[30] Furthermore, it has been recently shown that caspase-1-dependent formation of gasdermin D pores on cell membranes is a reversible mechanism, that can lead to the unconventional secretion of cytoplasmic proteins from living cells.[45] By combining previous findings and results, herein, presented, we propose a model where exposure to CSE leads to a gradual increase of caspase-1 activation without committing cells to death. This may, in turn, generate dysfunctional macrophages that promote tissue damage and low-grade inflammation.

In this respect, we further expanded our investigation exploring the effects downstream of caspase-1 activation. Previous evidence has shown a role for caspase-1 in the regulation of central carbon metabolism in addition to its role in the maturation of IL-1-family cytokines.[33] Furthermore, we have found that the classical substrates of caspase-1, that is, IL-1β and IL-18, were strongly reduced in CSE-treated macrophages. Taken together, these findings led us to hypothesize that in CSE-treated macrophages active caspase-1 may transduce non-canonical downstream effects associated with its actions on substrates different from IL-1β and IL-18. Optimal activation of macrophages is sustained by the increase of the glycolytic flux that generates, among others, a positive crosstalk between glycolysis and the NLRP3 inflammasome that is required for proper response to infections.[46] For example, hexokinase-1 (HK1) as well as pyruvate kinase M2 (PKM2)-dependent glycolysis are critical for the optimal activation of the NLRP3 inflammasome and optimal secretion of IL-1β and IL-18 in macrophages.[47,48] On the other side, activation of the NLRP3 inflammasome increases the glycolytic flux via an IL-1β-dependent mechanism further sustaining the inflammatory response and improving host defenses.[46,49] As an adaptive response during infection, activation of caspase-1 reduces the cellular glycolytic rate by promoting the cleavage of glycolytic enzymes.[33,48] Interestingly, exposure to cigarette smoke has been associated with reduced glycolytic rate and decreased lactate production in macrophages during infections.[32] Our data bring together all these findings unveiling the missing link that connect exposure to cigarette smoke with the impairment of the glycolytic flux: We show for the first time that activation of caspase-1 via a NLRP3-independent and TLR4-TRIF-caspase-8-dependent pathway is the key mechanism transducing the inhibitory action of cigarette smoke towards glycolysis. Furthermore, our data suggest that the inhibition of the NLRP3 inflammasome and IL-1β released by

CSE eliminates the positive feedback of the inflammasome toward glycolysis under physiological conditions. This generates a vicious circle where the glycolytic burst that would be required for optimal macrophage activation cannot take place when cells are exposed to cigarette smoke. With respect to data reported in Figure 7 and S12 showing that inhibition of caspase-1 by z-YVAD-fmk reverts the phenotype induced by CSE it is important to note that, in addition to caspase-1, caspase-4 recognizes and cleaves the peptide Ac-YVAD-pNA, although with much lower efficiency compared to caspase-1.[50] Therefore, a role for caspase-4 in transducing CSE-dependent effects cannot be completely ruled out.

Finally, it is worth noting that THP-1 cells responded differently compared to MDMs with regard to metabolic reprogramming in response to LPS. In fact, THP-1 cells produced higher levels of lactate compared to MDMs under basal conditions (10.2 vs 3.2 mM) and failed to increase lactate production upon stimulation with LPS, contrarily to MDMs. This was consistent with the tumoral origin of the THP-1 cells and suggests that this cell line is not a suitable model for metabolic studies.

Overall, our data unveil novel mechanisms that may contribute to macrophage dysfunction and increased risk of infection following cigarette smoke exposure. Future studies in samples isolated from healthy individuals, smokers, and smokers with/without CODP will be required for a better understanding of the real pathophysiological relevance of these mechanisms in cigarette smoke-associated diseases.

CONFLICT OF INTEREST
The authors have no financial conflicts of interest.

AUTHOR CONTRIBUTIONS
M. Buscetta, C. Cipollina, S. Di Vincenzo, and E. Pace designed research; M. Buscetta, S. Di Vincenzo, M. Miele, and E. Badami performed research; M. Buscetta, S. Di Vincenzo, and C. Cipollina analyzed the data; M. Buscetta, C. Cipollina, S. Di Vincenzo, and E. Pace wrote the paper.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.