Received: 15 October 2020 | Revised: 25 February 2021 | Accepted: 26 February 2021

DOI: 10.1096/fj.202001966RR

RESEARCH ARTICLE

THE FASEB JOURNAL

Suppression of cigarette smoke induced MMP1 expression by selective serotonin re-uptake inhibitors

Gerber Adam[1] | **Takayuki Shiomi**[2] | **Goldklang Monica**[1] | **Sonett Jarrod**[1] | **Anguiano Vincent**[3] | **Mercer Becky**[4] | **Zelonina Tina**[1] | **D'Armiento Jeanine**[1]

[1]Department of Medicine, Anesthesiology, Physiology and Cell Biology, College of Physicians and Surgeons, Columbia University Irving Medical Center, New York, NY, USA

[2]Center for Basic Medical Sciences, Graduate School, International University of Health and Welfare, Chiba, Japan

[3]Department of Oncological Sciences, Icahn School of Medicine, Mount Sinai Hospital, New York, NY, USA

[4]Office of Academic Affairs, Palm Beach State College, Florida, USA

Correspondence
D'Armiento Jeanine, Department of Medicine, Anesthesiology, Physiology and Cell Biology, College of Physicians and Surgeons, Columbia University Irving Medical Center, New York, New York, USA.
Email: jmd12@cumc.columbia.edu

Funding information
NHLBI, Grant/Award Number: R01 HL086936

ABSTRACT

Globally, COPD remains a major cause of disability and death. In the United States alone, it is estimated that approximately 14 million people suffer from the disease. Given the high disease burden and requirement for chronic, long-term medical care associated with COPD, it is essential that new disease modifying agents are developed to complement the symptomatic therapeutics currently available. In the present report, we have identified a potentially novel therapeutic agent through the use of a high throughput screen based on the knowledge that cigarette smoke induces the proteolytic enzyme MMP1 leading to destruction of the lung in COPD. A construct utilizing the cigarette responsive promoter element of MMP-1 was conjugated to a luciferase reporter and utilized in an in vitro assay to screen the NIH Molecular Libraries Small Molecule Repository to identify putative targets that suppressed luciferase expression in response to cigarette smoke extract (CSE). Selective serotonin reuptake inhibitors potently inhibited luciferase expression and were further validated. SSRI treatment suppressed MMP-1 production in small airway epithelial cells exposed to (CSE) in vitro as well as in smoke exposed rabbits. In addition, SSRI treatment inhibited inflammatory cytokine production while rescuing cigarette smoke induced downregulation in vivo of the anti-inflammatory lipid transporter ABCA1, previously shown by our laboratory to be lung protective. Importantly, SSRI treatment prevented lung destruction in smoke exposed rabbits as measured by morphometry. These studies support further investigation into SSRIs as a novel therapeutic for COPD may be warranted.

1 | INTRODUCTION

COPD is a highly prevalent disease, which is a major cause of disability and death in most countries. In the United States, it is estimated that over 14 million people suffer from this disease.[1] The major etiological agent in COPD is cigarette smoke. Thus, it is essential that COPD research focus on improving our understanding of the specific cellular and biochemical injury induced by smoke within the lung. Although cigarette smoke is the principal cause of emphysema, proteases are ultimately responsible for the destruction of the lung architecture.[2] Induction of lung inflammation together with oxidative stress is thought to tip the balance of proteolytic

Abbreviations: ABCA1, ATP binding cassette 1; BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; CSE, cigarette smoke extract; ECM, extracellular matrix; LXR, liver receptor X; MMP, matrix metalloproteinase; NMLSMR, NIH molecular libraries small molecule repository; SAEC, small airway epithelial cell; SSRI, selective serotonin reuptake in.

enzymes and their inhibitors towards breakdown of the extracellular matrix (ECM). Despite the complexity of the cellular response to smoke exposure, successful attenuation of lung injury secondary to protease blockade has not been achieved.

Our laboratory revealed a precise role for MMPs in emphysema causation through the generation of several transgenic mouse lines that express human MMP1 in lung epithelial cells.[3] These MMP1 transgenic mice develop progressive, adult-onset emphysema with physiological changes in lung compliance directly demonstrating that increased local expression of a single MMP resulted in lung destruction similar to that seen in the human disease. Subsequent studies in our laboratory established that cigarette smoke induces expression of MMP1 in resident lung cells,[4] particularly small airway epithelial cells where the initial damage to lung architecture is seen in emphysema.[5,6] Subsequently, we identified a cigarette smoke responsive element in the MMP1 promoter region induced through the TL4 pathway signaling through MAPKinase.[7]

In the present report, we identify SSRIs as potential modulators of the destructive process in emphysema in response to cigarette smoke via a novel screening technique of the NIH Molecular Libraries Small Molecule Repository (NMLSMR) utilizing the cigarette smoke responsive promoter element of MMP1 linked to a luciferase reporter. MMP-1 induction in response to cigarette smoke extract (CSE) in vitro and smoke exposure in vivo is inhibited by SSRI treatment. Furthermore, SSRI treatment in vivo mitigates smoke-induced lung damage. Concomitant decreases in inflammatory cytokine expression were also observed along with the restoration of ABCA1, a lipid transporter that has been previously demonstrated by our laboratory to correlate with a protection from lung destruction. Taken together, these data suggest SSRI therapy as a potential treatment modality with the ability to alter lung destruction in COPD patients.

2 | METHODS

2.1 | Smoke exposure studies

All animal studies were performed with the approval of the Institutional Animal Care and Use Committee of Columbia University. Rabbits were smoke exposed as previously described.[8] Briefly, female New Zealand White Rabbits (Charles River Laboratories, Wilmington, MA) were smoke exposed 4 hours per day, 5 days per week for a total of 20 weeks with total particulate matter (TPM) maintained at 100–150 g/m^3. At week 10, a cohort of animals received oral duloxetine (10 mg/kg) for the remainder of the smoke exposure period. C57BL/6 mice were chronically exposed to smoke in a TE-10 Teague Smoking Apparatus (Teague Enterprise). Eight-week-old mice were smoke exposed for 5 hours a day, 5 days a week for 10 days. The TPM within the smoking chamber was regulated so that the mice receive a TPM of 100–150 mg/m^3. TPM was determined by a gravimetric analysis of filter samples taken daily during the exposure period. After 6 days of smoke, the mice were treated twice per day with oral gavage of fluoxetine at a concentration of 10 mg/kg body weight.

2.2 | Cell culture and treatment

Small airway epithelial cells (SAEC) on were plated on a 12-well plate at a seeding density of 2500 cells/cm^2 and allowed to sit for 24 hours. The cells were cultured with small airway growth medium (SAGM; Lonza) supplemented with SAGM bullet kit (LONZA). The plate was then treated with 5% cigarette smoke extract and 10-μmol/L fluoxetine (TOCRIS Bioscience). Cigarette smoke extract (CSE) was prepared using a modified protocol.[9] Briefly, a Barnant vacuum pump operating at constant airflow was used to draw the smoke of one unfiltered 2R1 reference cigarette (University of Kentucky) through 25 mL of Dulbecco's phosphate-buffered saline. This solution (100% CSE) was adjusted to pH 7.4, filtered, added to cell medium to a final concentration of 5%, and added to the cells immediately. Cultures were harvested for analysis 24-hour following treatment. Alveolar macrophages were obtained from New Zealand White rabbits. Briefly, BALF was obtained, and a cell pellet was obtained by centrifugation at 3000 *g*. The pellet was resuspended in RPMI and plated for 5 hours to allow adherence of macrophages at a concentration of 1×10^5 cells/mL. Nonadherent cells were removed with a PBS wash, and fresh RPMI was added back to the culture. Macrophages were treated with 5% cigarette smoke extract and 10-μmol/L fluoxetine and harvested for analysis 24 hours later. All protocols involving animals were approved by the Columbia University IUCAC.

2.3 | Immunoblotting

For protein harvesting from SAEC cell culture, cells underwent treatment with 5% CSE, ±fluoxetine. Following treatment, cultures were lysed in ice-cold RIPA buffer (Sigma-Aldrich) containing freshly added protease inhibitor cocktail, sodium orthovanadate, and phenyl sulfonyl fluoride (SC-24948A, Santa Cruz Biotechnology). To obtain protein from mouse lung, the right lung was removed and ~10–20 mg of tissue were cut off and places immediately into ice-cold RIPA buffer, the remainder of the lung was frozen in liquid nitrogen for later analysis. The tissue sample was homogenized using a Qiagen Tissuelyser 2 (Qiagen) for 2 minutes at 30 Hz. Lysates from mice and cultured cells were centrifuged at 20,000 *g* for 20 minutes. The resulting supernatant was then used to determine the

protein concentration using the Bradford assay, and then 15 μg of protein was separated on 8%–10% SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc) by a tank transfer system (Bio-Rad Laboratories Inc.). The membranes were blocked with 5% nonfat dry milk (for phospho-proteins 5% BSA was utilized) in 50-nM Tris-HCL (pH 7.5), 150-nM NaCl, and 0.05% Tween-20, and then incubated overnight at 4°C with antibodies against phospho-IRAK (Abcam; ab218130) and ABCA1 (Abcam; ab18180). The membranes were washed with TBS-T, incubated with peroxidase-labeled[10] secondary antibodies at room temperature for 1 hour and visualized with Pierce ECL reagent (ThermoFisher) and GE image quant LAS 4000.

2.4 | Immunocytochemistry

Cultured SAEC were fixed with 4% paraformaldehyde followed by permeabilization with 1% Triton-X in PBS. Samples were blocked for 1 hour at room temperature in 5% normal donkey serum in TBST. Cultures were incubated with rabbit anti-Nf-kB (Abcam; ab16502) overnight at 4°C at 1:200 in 1% normal donkey serum in TBST. Samples were washed in PBS and incubated in donkey anti-rabbit Alexa 488 secondary at 1:1000 (Thermofisher; #A32733) 1% NDS TBST. Slides were counterstained with DAPI and visualized by epifluorescence. Nuclear localization of NF-kB was quantified by comparing the ratio of nuclear to cytoplasmic signal integrated over the total cell area measured. Analysis was performed using NIH ImageJ.

2.5 | MMP-1 assay

MMP-1 Fluorokine E kits (R&D Systems F1M00) were used to determine MMP-1 levels and performed per manufacturer's protocol.

2.6 | Inflammatory cell counts and lung injury

The BALF was collected from mice or rabbits by perfusing the lungs with 2 or 60 mL of sterile PBS, respectively. Resultant samples were spun for 10 minutes at 2000 RPM to obtain the cell pellet which was resuspended in 300 μL of PBS. Cells were counted using a hematocytometer for which 10 μL of the sample was utilized. To obtain the differential cell count, the slides were prepared using a cytopsin and stained using DifQuik. Histological evaluation of lung destruction was performed on the left lung using sections fixed and pressure perfused to 25-cm H_2O with 10% formalin. Sections were stained with H&E and tissue damage was assessed using mean linear intercept as previously described.[11]

2.7 | Real-time qPCR

Total RNA was extracted from specimens of lung tissue 0.3 cm^3 in size and from cell culture with the use of the RNeasy kit (Qiagen). The RNA was then converted into cDNA, which was used for real-time (RT) PCR analysis. Reaction were performed using TaqPath PCR master mix with TaqMan gene expression probes for MMP-1 and ABCA1, Hs0089965_m1 and Hs01059137_m1, respectively (ThermoFisher). Reactions were performed and analyzed using a QuantStudio 3 RT PCR machine (ThermoFisher).

2.8 | Analysis of screening assay results

The compounds in this clinical set are all known and have been tested and utilized in humans for various indications. These molecules, dissolved in DMSO, were tested at the concentration of 10 μmol/L for their capacity to modulate MMP-1 smoke induced transcriptional activation. The percentage inhibition of the CSE/MMP-1 induction was calculated for each tested compound on a per-plate basis, using the equation: %inhibition of compound = 100 × [1 − (test well − median high-signal control)/(median high-signal control − median low-signal control)]. We consider compounds that block more than 80% and no more than 120% of CSE-induced MMP-1 expression as initial hit compounds (inhibition greater than 120% would indicate baseline inhibition of MMP1 expression unrelated to CSE), as assayed by the CellTiter-Glo (Promega Corp).

2.9 | Statistical analysis

Statistical analysis of the data obtained was performed using the unpaired two-tailed Student's *t*-test when two groups were being compared. ANOVA was used when comparing multiple groups with post hoc Tukey test where appropriate. All analysis was performed using GraphPad Prism software.

3 | RESULTS

3.1 | Screening of the NIH molecular libraries small molecule repository and assay development

A mammalian cell line-based transfection assay was developed in a 96-well format that can easily be implemented

in a high throughput screen. As schematized in Figure 1, the method is based on transfection of a human cell line (HEK 293T) with a vector containing a luciferase reporter gene, which is under the control of the MMP-1 promoter. Previously, our group demonstrated that the cigarette smoke response element of the MMP-1 promoter lies in the distal 1 kb of the promoter region. Although this element is sufficient to induce MMP-1 transcription, gene activation with cigarette smoke was only seen with the full length wild-type sequence.[7] This full 4.4-kb promoter sequence was cloned into a pGL-3 plasmid to create the luciferase reporter construct. MMP1/pGL3 and transfected into HEK293 cells. The ability of CSE to reliably induce luciferase expression over several runs was first examined to determine the interassay variability. As shown in Figure 2A, the assay produces stable results across several days of experimentation. The Z' factor of the assay as calculated by the method described in the Assay Guidance at National Institutes of Health Center for Translational Therapeutics (NCTT) (http://assay.nih.gov/assay/index.php/Table_of_contents) was 0.71 ± 0.05 SEM after 5 days of repetition, consistent with the characteristic of an excellent high throughput assay. Positive controls utilizing the ERK inhibitor PD98059 demonstrated that activation of the smoke responsive element of the MMP-1 requires ERK-mediated signaling via TLR-4 (Figure 2B) and dose-dependent inhibition of luciferase expression was observed consistent with published data on the promoter.[7,12] Furthermore, no response was observed to DMSO which is the standard (28.2Al) solvent for compounds (27.84R) in the high throughput screen. Following validation of the assay, the ability of 1400 compounds from the NMLSMR to inhibit luciferase production in response to 5% CSE was assessed. As shown in Figure 2C, 15 compounds reduced luciferase production by ≥100% in the primary screen. An independent compound batch for each of these was obtained from NMLSMR and 10 dilution points of 1:3 serial dilutions were tested in triplicate to determine the IC_{50} values for each compound. As shown in Table 1, nine compounds achieved

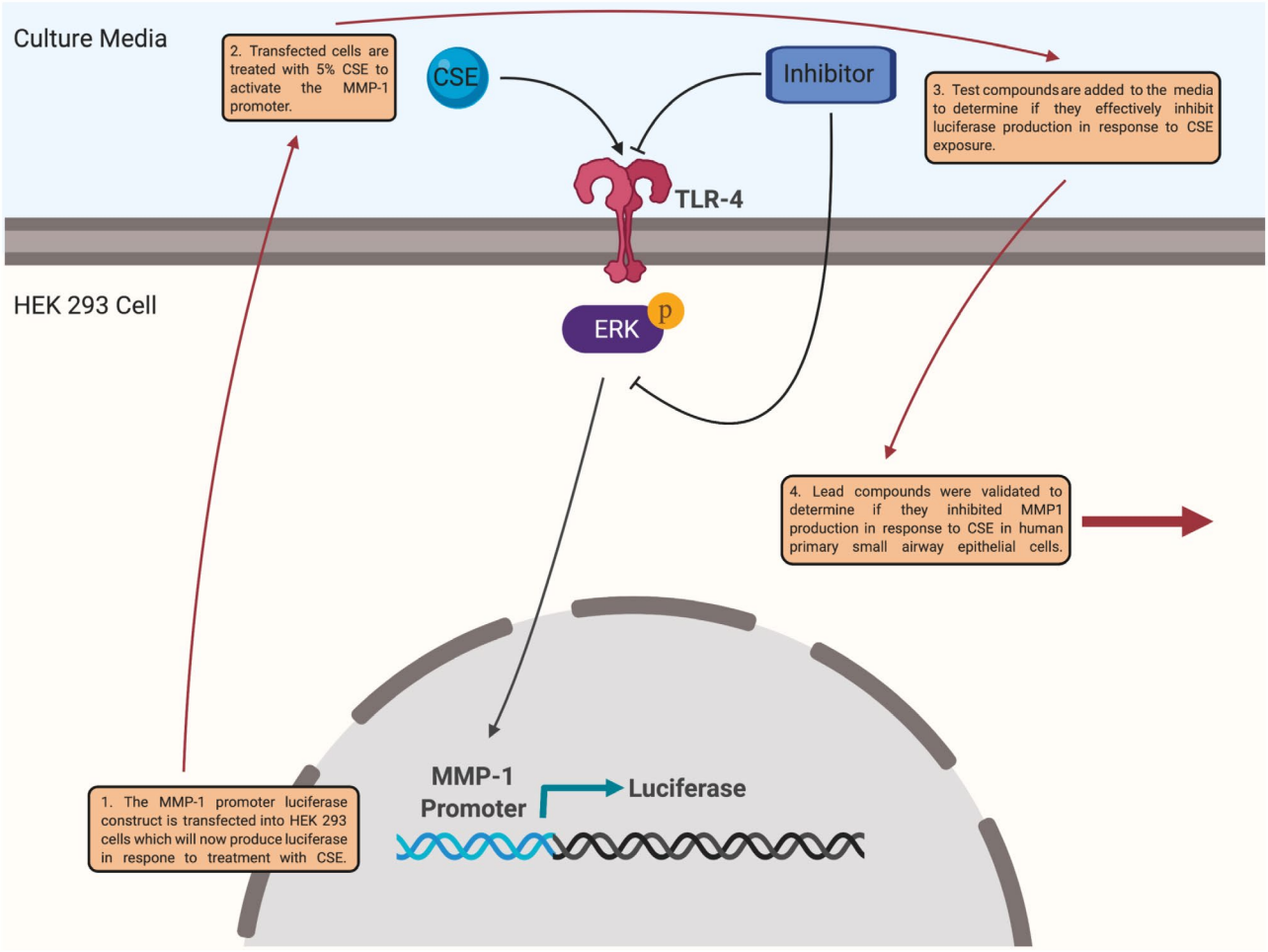

FIGURE 1 Representative diagram of NMLSMR screening assay using the cigarette smoke responsive MMP-1 promoter region. Schematic of concept behind the MMP1/pGL3 screening assay in HEK293 cells. Cigarette smoke extract activates the TLR4 signaling axis leading to the subsequent activation of the MMP-1 promoter which has been fused to the luciferase gene in the absence of inhibitory molecules. A positive hit in the NMLSMR for inhibition is observed as a decrease in luciferase expression (Created with BioRender.com)

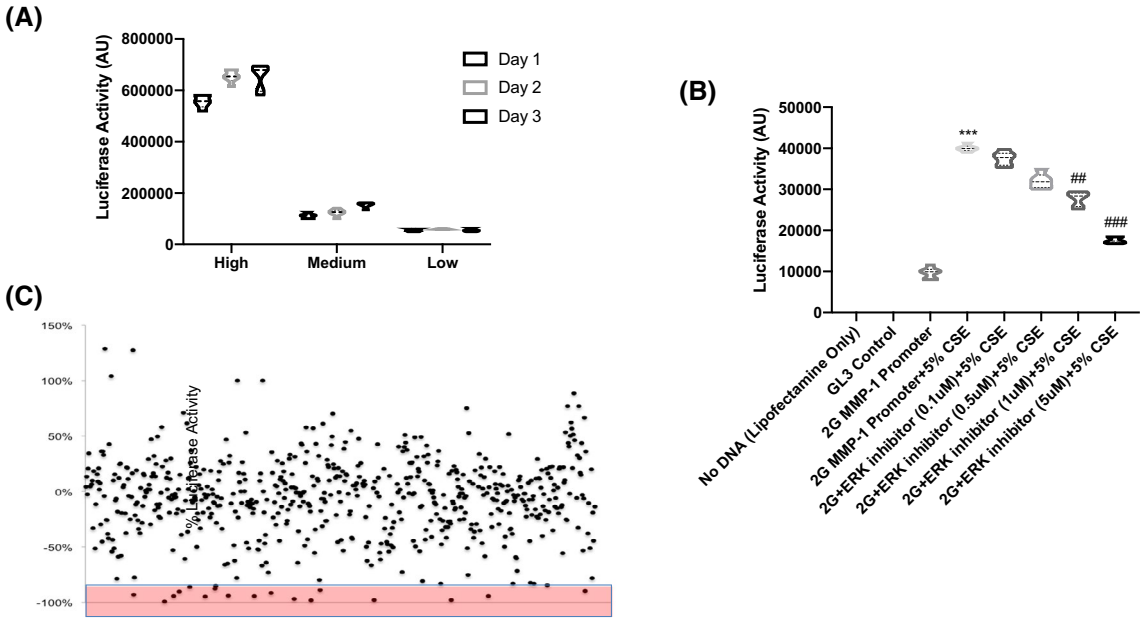

FIGURE 2 Validation of and NMLSMR targets identified by the high throughput assay. A, To test for reproducibility of the assay, 293T cells were transfected with the MMP-1/pGL3 reporter plasmid. Cells were seeded in three 96-well plates using an interleaved format and treated with 5% CSE media (high-signal) 2% CSE (medium-signal) no CSE (low-signal). After a 24-h incubation, the luciferase activity was measured in each well. Representative data from days 1–3 is shown. The calculated Z' factor was 0.71 ± 0.05 SEM, $n = 5$. B, As expected, the ERK inhibitor PD98059 inhibited luciferase expression in a dose-dependent fashion in positive controls exposed to 5% CSE ($^{***}p \leq 0.001$, significantly different from control; $^{\#\#\#}p \leq 0.001$, significantly different from promoter + CSE; $^{\#\#}p \leq 0.01$, significantly different from promoter + CSE). C, Data from the high throughout screen displaying each compound tested as a block dot. The y-axis illustrates the change in MMP1 expression compared with expression levels in CSE exposed in HEK293 cells. The drugs in the −100% range (red shaded area) are capable of nullifying all CSE induced MMP1 transcriptional activity

TABLE 1 Targets from the NMLSMR identified by the MMP1/pGL3 screening system

Compound name	% MMP-1 inhibition	Compound designation
Simvastatin	105.68	Statin
Mevastatin	115.36	Statin
Triclosan	138.35	Ant-fungal
Ketoconazole	131.91	Anti-fungal
Duloxetine	125.32	Anti-depressant
Nefazodone	110.10	Anti-depressant
Fluoxetine	130.44	Anti-depressant
Sertraline	149.38	Anti-depressant

Note: Identities of drug targets shown to significantly inhibit luciferase in response to cigarette smoke extract.

nearly 100% inhibition of CSE driven expression of luciferase by the MMP-1 promoter with IC_{50} values of ≤1 μmol/L which included (a) selective serotonin reuptake inhibitors (SSRIs; two compounds), (b) statins (two compounds), (c) antifungal azoles (three compounds). Examining the IC_{50} values in conjunction with the potential side effect profile of these compounds, the SSRIs fluoxetine and duloxetine were identified as lead candidates for further study. As HEK 293 cells are derived from kidney, we proceeded to confirm and extend these findings in human small airway epithelial cells and the lungs of rabbits and mice in vivo.

3.2 | SSRI's inhibit CSE induced upregulation of TLR-4 signaling and inhibited the expression and secretion of downstream proteolytic and pro-inflammatory mediators in vitro

Prior data from our laboratory establishes MMP-1 as a critical agent in proteolytic lung destruction in emphysema.[3] Importantly, its production is linked to activation of TLR-4 by cigarette smoke. Given these data, to validate the lead candidates, we next investigated if SSRI treatment induced a significant reduction in MMP-1 secretion in response to CSE in lung epithelial cell cultures. Cells were treated with 5% CSE ±10 μmol/L fluoxetine followed by RT-qPCR analysis of MMP-1 expression and assessment of secreted MMP-1 in culture medium by ELISA assay. As shown in Figure 3A,B, treatment of 5% CSE exposed SAEC cultures with 10-μmol/L fluoxetine reduced both MMP-1 mRNA expression as well as MMP-1 secretion into the culture media. Importantly, fluoxetine addition decreased the CSE induced

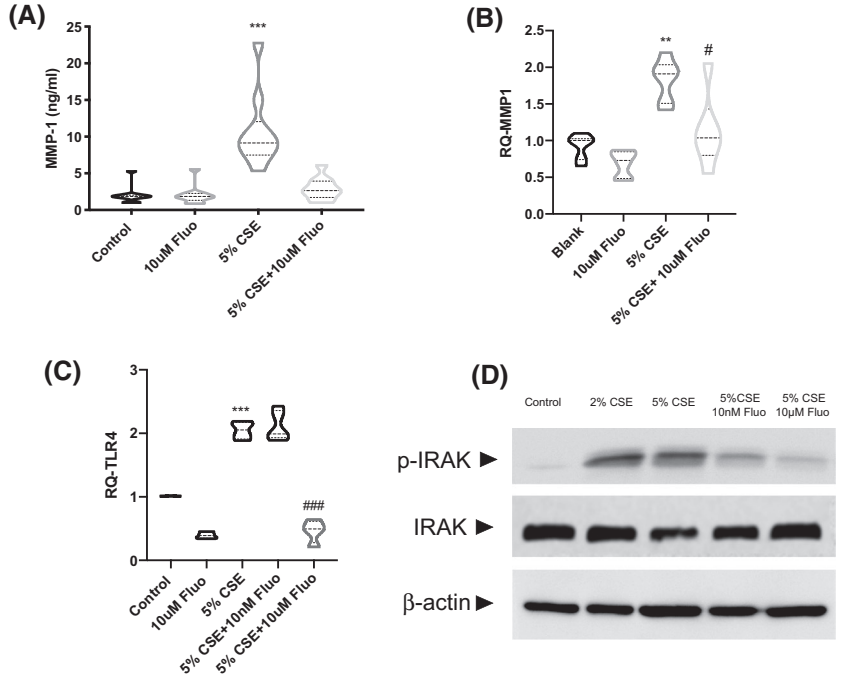

FIGURE 3 Fluoxetine treatment reduces MMP-1 expression in CSE treated SAEC via inhibition of TLR-4 signaling. A, SAEC cultures were treated with 5% CSE ±10-μmol/L fluoxetine for 24 h. Media was harvested, and MMP-1 levels were assessed by ELISA. CSE treated cultured secreted significantly more MMP-1 as compared to controls (5.2 ± 1.3 vs. 2.1 ±0.3 ng/mL, respectively; $^{***}p \leq 0.001$, $n = 12$). Treatment with 10-μmol/L fluoxetine reduced CSE stimulated MMP-1 secretion to control levels (2.6 ± 0.3 ng/mL). B, Real-time qPCR was performed on SAEC cultures treated with 5% CSE ±10-μmol/L fluoxetine for 24 h. CSE treatment upregulated MMP-1 mRNA expression as compared to controls (1.82 ± 0.11 vs. 0.92 ± 0.06 RQ, respectively; $^{**}p \leq 0.01$, $n = 6$). This increase was significantly inhibited by treatment with fluoxetine (Fluo) (1.82 ± 0.11 vs. 1.11 ± 0.20 RQ, respectively; $^{\#}p \leq 0.05$, $n = 6$). C, Treatment with CSE significantly increased TLR-4 expression (0.23 ± 0.11 vs. 2.11 ± 0.42 RQ, respectively; $^{***}p \leq 0.001$, $n = 6$). This increase was inhibited by addition of 10-μmol/L fluoxetine to CSE stimulated cultures (2.11 ± 0.42 RQ vs. 0.51 ± 0.09 RQ, respectively; $^{\#\#\#}p \leq 0.001$, $n = 6$). D, Western blot demonstrating fluoxetine mediated inhibition of the CSE induced increase in IRAK phosphorylation

upregulation of TLR-4 mRNA (Figure 3C). Consistent with this, SSRI treatment resulted in inhibition of both IRAK phosphorylation, a downstream mediator of TLR-4 signaling (Figure 3D). SSRI treatment also significantly reduced the nuclear translocation of Nf-kappa beta, a transcriptional effector of TLR-4 activation, in response to treatment with 5% CSE (Figure 4A,B). When examined together, these data indicate that SSRIs act to inhibit CSE induced activation of TLR-4 and its associated downstream signaling elements, indicating TLR-4 stimulated secretion of injurious agents is downregulated by SSRI treatment.

3.3 | Duloxetine treatment decreases MMP-1 expression in rabbit macrophages in vitro and prevents smoke induced emphysema in vivo

To determine if SSRI treatment significantly affects cigarette smoke induced proteolytic lung destruction, an established rabbit model of emphysema was utilized.[8] Rabbits were used in lieu of mice because, unlike mice, rabbits produce MMP-1, an enzyme integral to proteolytic lung destruction and emphysema progression,[13] and therefore, they better replicate the protease biology observed in emphysematous human lung. To confirm SSRIs were effective agonists in rabbits, BALF was collected from room air exposed rabbits and alveolar macrophages were cultured for 24 hours and treated with CSE ±10 μmol/L fluoxetine or duloxetine. Consistent with observations in SAEC cultures, SSRI treatment significantly reduced the expression of MMP-1 (Figure 5A,B). It was next assessed if SSRI treatment could effectively reduce MMP-1 expression in smoke exposed rabbits as well as prevent the development of emphysema. Rabbits were exposed to cigarette smoke for 12 weeks in order to establish emphysema followed by an additional 4 weeks of smoke ± treatment with oral duloxetine. Duloxetine was chosen because of its longer half-life that permitted once daily dosing which decreased handling needs for the rabbits allowing for more humane treatment.[14] As demonstrated with our observations in vitro, duloxetine decreased the production of MMP-1 in the BAL of smoke exposed rabbits as assessed by ELISA

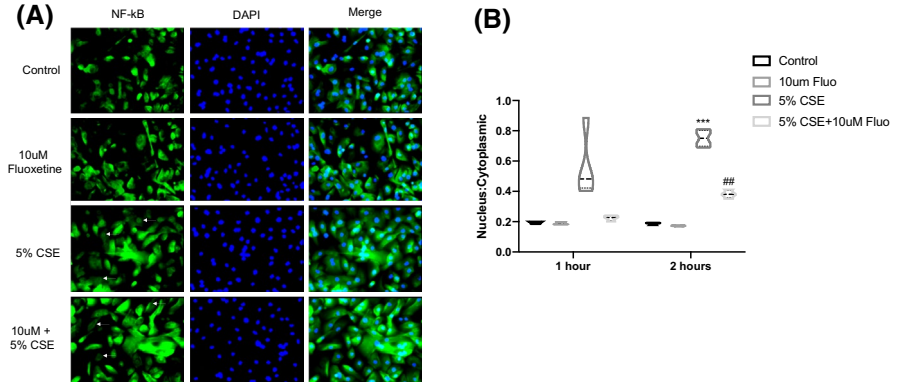

FIGURE 4 SSRI treatment prevents nuclear translocation of NF-kB in response to CSE treatment in SAEC. SAEC cultures were treated with 5% CSE ±10-μmol/L fluoxetine (Fluo) for 24 h. At 1 and 2 h, following treatment cultures were fixed, and immunocytochemistry was performed to assess the extent of nuclear localization of NF-kB. A, Representative images demonstrating inhibition of CSE induced NF-kB translocation by fluoxetine at the examined time points; NF-kB is in green and nuclei are stained with DAPI (blue). Arrows show examples. B, NIH ImageJ was used to measure the nuclear:cytoplasmic fluorescence ratio to quantify the degree of nuclear translocation in each condition. As shown, CSE treatment for 2 h significantly increased the nuclear:cytoplasmic ratio compared to controls (0.71 ± 0.05 RU vs. 0.18 ± 0.09 RU, respectively; $^{**}p \leq 0.01$, $n = 3$). This increase was inhibited by treatment of cultures with 10-μmol/L fluoxetine (0.71 ± 0.05 RU vs. 0.41 ± 0.08 RU, respectively; $^{\#\#}p \leq 0.01$, $n = 3$)

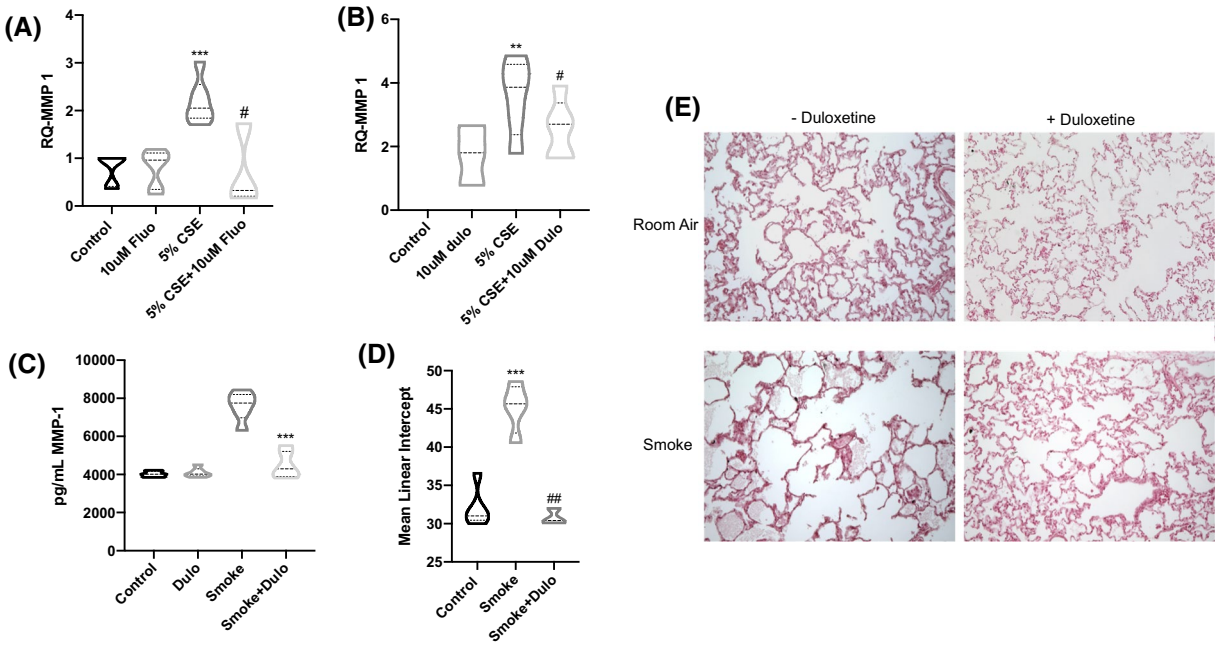

FIGURE 5 SSRI treatment reduced MMP-1 expression in smoke exposed rabbits and ameliorates the development of smoke-induced lung injury. A and B, Treatment with either 10-μmol/L fluoxetine or duloxetine decreased the expression of MMP-1 in cultured rabbit alveolar macrophages as assessed by real-time qPCR. Rabbits were exposed to smoke or room air for 10 weeks. Following this, a cohort of animals from each group received oral duloxetine daily for the remaining 10 weeks of smoke exposure. Bronchoalveolar lavage fluid and tissue sections were then obtained for further analysis. C, Treatment with oral fluoxetine significantly decreased the presence of MMP-1 in BALF from smoke exposed mice as assessed by ELISA. D. Mean linear intercepts were calculated from H&E sections for each experimental group. Duloxetine treatment returned MLI values to control levels. (32.1 ± 1.1 μm vs. 45.1 ± 1.6 μm vs. 30.8 ± 0.5 μm; $^{***}p \leq 0.001$, significantly different from control; $^{\#\#}p \leq 0.01$, significantly different from smoke, $n \geq 3$). (D) Representative H&E sections demonstrating the attenuation of smoke-induced lung injury by duloxetine treatment

assay (Figure 5C). Morphometric analysis was performed on H&E stained sections to determine mean linear intercept (MLI) values for each experimental group. As expected, rabbits exposed to cigarette smoke for 16 weeks without drug treatment displayed increased MLI values as compared to room air controls. In contrast, animals that received 4 weeks

of oral duloxetine treatment were characterized by MLI values that were not significantly different from control values (Figure 5D). This finding is evident in H&E section with the presence of cystic lung changes associated with emphysema being attenuated by treatment with duloxetine in smoke exposed rabbits (Figure 5E). Taken together, these data clearly indicate that suppression of MMP-1 production by an SSRI prevents proteolytic destruction of lung parenchyma following exposure to cigarette smoke.

3.4 | SSRI treatment reduces lung inflammation in a mouse model of acute smoke exposure

Emphysema and cigarette smoke induced lung injury are associated with initiation of an inflammatory response within the lung. As observed in our in vitro experiments with SAEC culture, SSRI treatment inhibits the activity of the proinflammatory TLR-4 signaling axis following CSE exposure. We next expanded this finding by testing the effectiveness of SSRI treatment on reducing the inflammatory arm of cigarette smoke induced lung injury in vivo using an acute model of smoke exposure in mice. In this case, mice were utilized as we were only examining the initiating inflammatory response and not assessing MMP1 production or lung destruction. Mice were exposed to cigarette smoke for 10 days and received treatment with the oral SSRI fluoxetine twice daily for the final 4 days of smoke exposure. As shown in Figure 6A, animals that that received SSRI treatment had significantly decreased inflammatory cell counts in their BALF, characterized by decreased infiltration by macrophages as assessed on differential cell count (Figure 6C).

3.5 | SSRI treatment restores ABCA1 expression in smoke exposed lung

Lipid regulation has been implicated in the establishment and progression of inflammatory disease and alterations in lipid species and transport within the lung appears to be critical to the development of inflammation.[15] The ATP Binding Cassette Antiporter (ABCA) family proteins are critical to this process, and their expression is significantly affected by inflammation[16] which results in downregulation of their expression and the loss their potent anti-inflammatory effects. Importantly, prior work by our laboratory has demonstrated that reestablishing ABCA1 expression via liver receptor X (LXR) agonism following smoke exposure effectively reduces inflammation and abrogates the development of emphysema in smoke exposed mice.[11] Given that TLR-4 is an established negative regulator of both LXR activity and ABCA1 expression,[17] it was hypothesized that if SSRI treatment was abrogating the inflammatory response, it should have similar effects on ABCA1 expression. This hypothesis was first tested in SAEC cultures treated with 5% CSE for 20 hours followed by 4 hours of SSRI treatment. As predicted, these cells showed significant upregulation of ABCA1 expression as indicated by RT-qPCR (Figure 7A) when compared to non-SSRI-treated cells. We next examined the effects of SSRI treatment on mice undergoing acute smoke exposure. Mice were treated as previously described and lung tissue was collected for analysis of ABCA1 expression by RT-qPCR. As shown in Figure 7B, treatment with fluoxetine prevented the downregulation of ABCA1 expression in response to cigarette smoke exposure. This change was accompanied by a significant increase in ABCA1 protein expression as assessed by western blot (Figure 7C). Taken

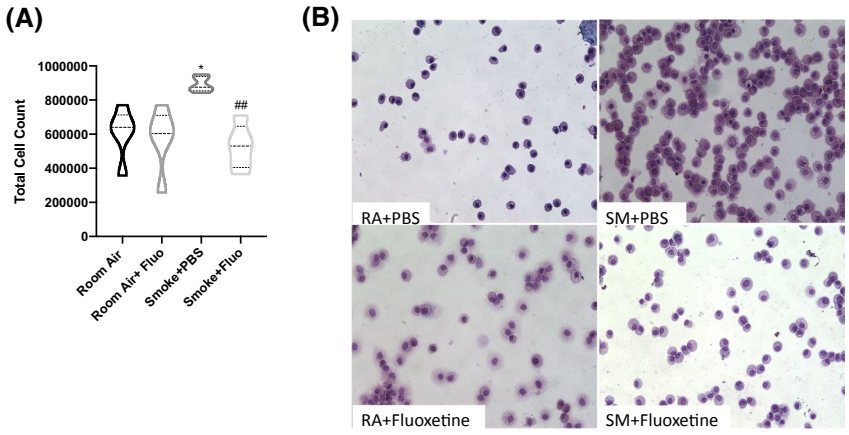

FIGURE 6 Fluoxetine treatment reduces acute inflammation in smoke-exposed mice. Mice were exposed to room air or smoke for 6 days. For the final 4 days of smoke exposure, mice received oral fluoxetine. Bronchoalveolar lavage fluid was collected for analysis of inflammatory cell infiltration via cytospin and Difquik staining. (A) Treatment with fluoxetine significantly reduced the total cell counts in the BAL of smoked exposed animals as compared to PBS controls. (B) Representative DifQuik stains of cytospins demonstrating decreased inflammatory cell counts in smoke-exposed animas receiving treatment with fluoxetine

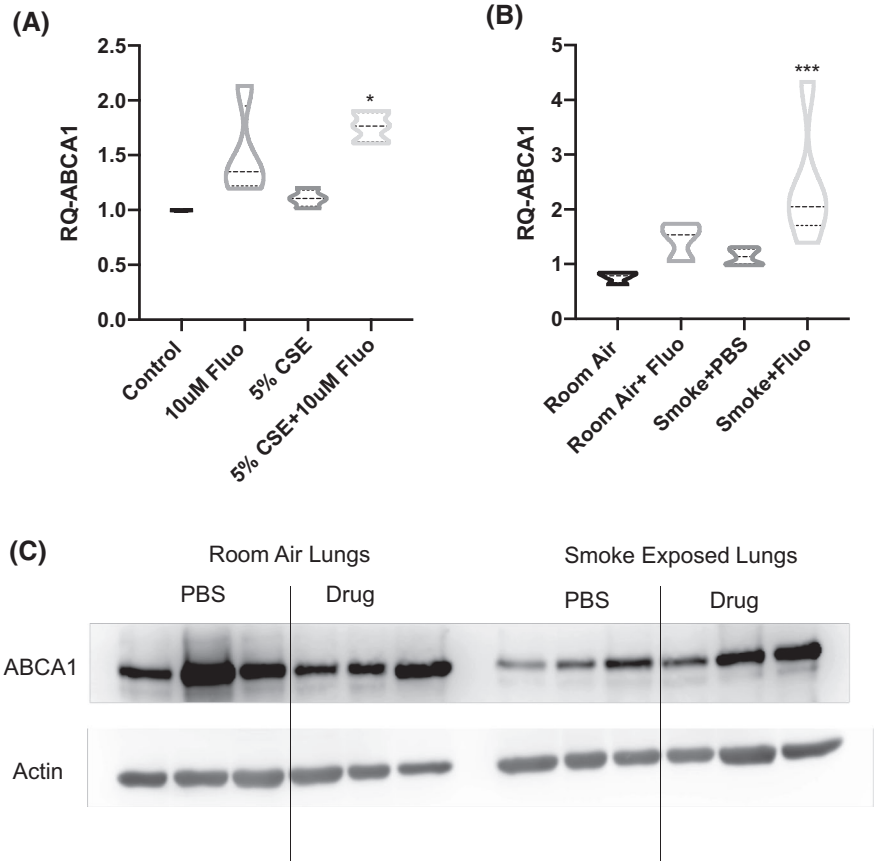

FIGURE 7 Fluoxetine treatment upregulates the expression of the anti-inflammatory ABCA1 lipid transporter in SAEC in vitro and acutely smoke exposed mice in vivo. A, Cultured SAEC were exposed to 5% CSE ±10-μmol/L fluoxetine. Cells were harvested and mRNA collected for analysis by qPCR. Fluoxetine resulted in a significant upregulation of ABCA1 by cultured epithelial cells as compared to smoked exposed cells (1.7 ± 0.05 RQ vs. 1.0 ± 0.03 RQ, respectively; $^{*}p \leq 0.05$, $n \geq 3$). Mice received 6 days of exposure to room air or cigarette smoke followed by 4 days of treatment with fluoxetine. Mice were sacrificed, and mRNA and protein were harvested for analysis. B, Fluoxetine treatment abrogated the CSE induced decrease in ABCA1 mRNA (1.1 ± 0.05 RQ vs. 2.4±0.5 RQ, respectively; $^{***}p \leq 0.001$, $n \geq 4$) in smoke exposed mice. C, Western blot demonstrating a smoke induced decrease in the presence of ABCA1 protein that is prevented by treatment of smoke exposed mice with fluoxetine (each lane represents and individual animal)

together, these data suggest that SSRI treatment may be a safe and effective method to alter ABCA1 expression and reduce inflammation and lung destruction in response to injury.

4 | DISCUSSION

The ability to effectively and safely modulate the activity of proinflammatory pathways is of central importance to the future treatment of chronic disease. This is especially relevant in COPD where chronic injury to the pulmonary epithelium is hypothesized to result in phenotypic cellular changes that cause constitutive secretion of proinflammatory and proteolytic agents causing continuous damage to the lungs.[18,19] Current treatment regimens for COPD that utilize inhaled beta-adrenergic agonists and steroids focus only on the alleviation of symptoms and do not address underlying disease pathophysiology.[20] With the high prevalence of COPD, it is critical to develop new therapies that address the underlying mechanisms of disease to potentially protect the lung from ongoing destruction and prevent morbidity. Central to this goal will be the development of a therapy that reverses the conversion of the pulmonary epithelium to a senescent secretory phenotype by exposure to cigarette smoke and other pollutants. It is for this reason that we developed a novel screening tool utilizing a reporter linked to the promoter of a major effector of cigarette smoke induced damage, MMP1, to screen libraries for compounds that alter secretion of damaging compounds at the transcriptional level.[21] Several classes of compounds were seen in our screen that demonstrated significant inhibition of luciferase expression induced by the MMP-1 promoter; both simvastatin and mevastatin inhibited luciferase expression induced by the MMP-1 promotor following CSE exposure. Prior studies examining statins have has mixed results concerning benefits for COPD patients. Statin treatment is demonstrated to decrease inflammatory markers including CRP, Il-6, and TNF-alpha in COPD patients.[22,23] A recent meta-analysis revealed increased FEV_1

and FEV_1/FVC in addition to reduced rates of acute COPD exacerbations, suggesting that tangible clinical improvements occur with statin use.[24] However, while encouraging, these results are contradictory to a prospective study of statin use by COPD patients conducted by Criner et al.[25] Given these conflicting data, we made the decision not to further validate statins. However, it is encouraging that our screen picked up a compound that has received attention as a potential therapeutic and been utilized in clinical trials thereby lending support to the potential effectiveness of targets uncovered by our screening system. We ultimately chose to examine the potential of SSRIs despite the fact that triclosan and ketoconazole were more potent given their more widespread use and more favorable side effect profile in patients.

Proteolytic lung destruction is a defining feature of cigarette smoke related lung injury and emphysema. Importantly, lung destruction progresses in spite of smoking cessation supporting the theory chronic inflammatory injury induces a senescent inflammatory epithelial cell phenotype.[26,27] It has been established that MMP-1 expression and secretion is upregulated in emphysema patients and is a major player in progressive lung destruction and disease development.[13] Its expression has been shown to be TLR-4 responsive as well as driven by the activation of Nf-kB, a downstream effector of TLR4 activity.[12] Chronic activation of the TLR-4/NF-kB signaling axis also mediates the transition of cells into a secretory phenotype associated with senescence and chronic injury.[27] Therefore, based on its TLR-4 antagonist activity, it was predicted that SSRIs would mitigate smoked-induced lung destruction in rabbits. Consistent with this notion, SSRIs reduced MMP-1 expression and secretion in rabbit alveolar macrophages as well as in the BALF of smoked-exposed animals. These findings predicted that SSRI treatment should halt the progression of lung destruction in injured animals. Consistent with this, treatment of smoke exposed rabbits with SSRI resulted in reduced MLI as compared to those only receiving smoke. The observation that SSRI treatment appears to not only halt but reverse smoke induced damaged, as shown by the reversion of MLI to control levels in cigarette smoked exposed rabbits, supports the notion inhibition of TLR-4 signaling may help reverse the conversion of lung epithelia and immune cells into a phenotype characterized by chronic protease secretion. Reduction of inflammation is another critical feature of any medication required to treat the progression of COPD. Previously, our laboratory has shown that agonism of the liver X receptor transcription factor decreases cigarette smoke induced inflammation and lung damage by upregulating the anti-inflammatory lipid transporter ABCA1.[11] ABCA1 surface expression is known to decrease cytokine release, enhance macrophage phagocytic activity, and generally promote an anti-inflammatory chemical mileu.[16] Importantly, ABCA1 upregulation is inversely influenced by TLR4 activation. Given these data, it is not surprising treatment with SSRI similarly upregulates ABCA1 in smoke exposed epithelial cells and supports the notion SSRI treatment may normalize their phenotype. Importantly, SSRI treatment may represent another means of leveraging the ABCA1 pathway to treat COPD since LXR agonists have been associated with unacceptable side effects.[28]

Prior work by Waiskopf et al.[10] indicated that both in silico and in vitro that SSRIs interact via the TLR-4 receptor at the LPS binding pocket to assert inhibitory activity. Consistent with this, studies focused on central nervous system inflammation have demonstrated a profound anti-inflammatory effect of SSRIs, aside from their effects on serotonin reuptake. SSRIs have been shown to alter secretion of multiple cytokines from neurons and microglia as well as nuclear translocation of NF-kB and IKKB phosphorylation in response to inflammatory stimuli.[29,30] Critically, all these pathways are known downstream effectors of TLR-4 activity. Paralleling these changes in mice, serum analysis of depressed patients indicates significant increases in the presence of inflammatory biomarkers such as C-reactive protein, IL-6 TNF-alpha, IL-1B, and the IL-2 and IL-1 soluble receptors, all of which are known to be produced in response to TLR-4 activation.[31] Our laboratory has shown that cigarette smoke exerts its pro-inflammatory effects on the lungs via the TLR-4 receptor.[12] In agreement with this and the observed inhibitory effect of SSRIs on TLR-4 we observed decreased expression and secretion of MMP-1 in cultured SAECs exposed to CSE. Furthermore, SSRI treatment prevented the activation of and nuclear localization of IRAK and Nf-kB, respectively, which are both well-demonstrated downstream effectors of TLR-4 activity in response to cigarette smoke.[12] These findings are in agreement with studies demonstrating that treatment of SAEC with CSE dramatically alters signaling pathways downstream of TLR-4 activation. This includes secondary messengers such as ERK and IRAK and importantly transcription factors such as Nf-kB and AP-1 that alter the transcriptional machinery of a cell and by default its phenotype.[32]

Although primarily known for their activity as modulators of serotonin reuptake in the brain, SSRIs also have potent anti-inflammatory effects.[33,34] Based on in silico data, SSRIs are predicted to strongly interact with TLR-4.[10] This is supported by experimental data in several models.[29] For instance, modulation of TLR-4 signaling by SSRIs in subarachnoid hemorrhage models is demonstrated to downregulate TLR-4, MyD88, and NF-kB expression.[30] In macrophages, similar SSRI-mediated downregulation of IL-6 and TNF-alpha in response to LPS stimulation is observed.[35] Inhibition of inflammatory signaling following SSRI treatment has also been seen in diabetic rats.[36] Importantly, these pathways demonstrate similar patterns of activation in COPD related inflammatory lung injury. It is well documented that in response to cigarette smoke TLR4 signaling via MyD88 and ERK is dramatically upregulated.[37] This in turn results in the activation and expression of multiple

inflammatory cytokines resulting in immune cell recruitment to the lung. Furthermore, the activation of the TLR4-MyD88 axis in the lung is responsible for increased expression of MMPs, in particular MMP-1, directly causing lung destruction leading to emphysema.[12,21] Given the similarities between pulmonary TLR4 signaling in COPD and inflammatory process mitigated by SSRIs in other cell types and tissues, SSRIs may present a novel therapeutic opportunity for the treatment of COPD.

Although these data are promising, it cannot be ignored that in a large retrospective trial by Vozoris et al.[38] small but significant increases in hospitalization for respiratory complaints and mortality were observed in new SSRI/SNRI users. Notably, this study only examined new SSRI/SNRI users where a new diagnosis of depression is likely. Since depression itself is associated with increased morbidity and is thought to be a pro-inflammatory state, its presence potentially confounds these findings.[39] Furthermore, Vozoris et al.[38] consider both SSRIs and SNRIs together and did not stratify by drug type which calls into question the generalizability of these data to all SSRIs. Interestingly, in the outpatient population, seemingly contradictory data demonstrated significantly decreased rates of COPD exacerbation after starting therapy, supporting a potential beneficial effect of these medications in agreement with the current report. Altogether, our data provide evidence arguing for the potential repurposing of SSRIs as therapy for the treatment of COPD. As indicated by our screen SSRIs appear to exert their effects by modifying TLR-4 pro-inflammatory signaling. This ultimately results in decreased protease secretion and the upregulation of anti-inflammatory pathways such as ABCA1. This mechanism is novel amongst COPD treatments as it may provide a therapy that has the ability to alter the course of COPD development rather than treat the symptoms in patients. While it appears that SSRIs work via the TLR-4 pathway it is unclear if this is at the receptor level or its downstream targets, further work will be required to determine its exact effects and develop potentially new agents to increase efficacy and reduce adverse side effects.

AUTHOR CONTRIBUTIONS

Adam Gerber, Shiomi Takayuki, Moinca Goldklang, Jarrod Sonett, Vincent Anguiano, Becky Mercer, and Tina Zelonina all performed experimental work discussed in the paper. Adam Gerber, Jarrod Sonett, and Shiomi Takayuki are responsible for preparation of the manuscript. Monica Goldklang and Jeanine D'Armiento provided critical feedback on the manuscript. Jeanine D'Armiento is responsible for oversight of the project.

CONFLICT OF INTEREST

The authors have no conflicts of interests to declare.

REFERENCES

1. Centers for Disease C, et al. Prevalence of current cigarette smoking among adults and changes in prevalence of current and some day smoking–United States 1996-2001. *MMWR Morb Mortal Wkly Rep*. 2003;52(14):303-307.
2. Snider GL. Emphysema: the first two centuries—and beyond. A historical overview, with suggestions for future research: Part 2. *Am Rev Respir Dis*. 1992;146(6):1615–1622.
3. D'Armiento J, Dalal SS, Okada Y, et al. Collagenase expression in the lungs of transgenic mice causes pulmonary emphysema. *Cell*. 1992;71(6):955–961.
4. Imai K. Correlation of lung surface area to apoptosis and proliferation in human emphysema. *Eur Respir J*. 2005;25(2):250–258.
5. Mercer BA, Kolesnikova N, Sonett J, et al. Extracellular regulated kinase/mitogen activated protein kinase is up-regulated in pulmonary emphysema and mediates matrix metalloproteinase-1 induction by cigarette smoke. *J Biol Chem*. 2004;279(17):17690–17696.
6. Seagrave J, Barr EB, March TH, et al. Effects of cigarette smoke exposure and cessation on inflammatory cells and matrix metalloproteinase activity in mice. *Exp Lung Res*. 2004;30(1):1–15.
7. Mercer BA, Wallace AM, Brinckerhoff CE, et al. Identification of a cigarette smoke-responsive region in the distal MMP-1 promoter. *Am J Respir Cell Mol Biol*. 2009;40(1):4–12.
8. Goldklang MP, Tekabe T, Zelonina T, et al. Single-photon emission computed tomography/computed tomography imaging in a rabbit model of emphysema reveals ongoing apoptosis in vivo. *Am J Respir Cell Mol Biol*. 2016;55(6):848–857.
9. Laurent P, Janoff A, Kagan HM. Cigarette smoke blocks cross-linking of elastin in vitro. *Chest*. 1983;83(5):63S–65S.
10. Waiskopf N, Ofek K, Gilboa-Geffen A, et al. AChE and RACK1 promote the anti-inflammatory properties of fluoxetine. *J Mol Neurosci*. 2014;53(3):306–315.
11. Sonett J, Goldklang M, Sklepkiewicz P, et al. A critical role for ABC transporters in persistent lung inflammation in the development of emphysema after smoke exposure. *FASEB J*. 2018;32(12):6724–6736.
12. Geraghty P, Dabo AJ, D'Armiento J. TLR4 protein contributes to cigarette smoke-induced matrix metalloproteinase-1 (MMP-1) expression in chronic obstructive pulmonary disease. *J Biol Chem*. 2011;286(34):30211–30218.
13. Wallace AM, Loy Leanna B, Abboud Raja T, et al. Expression of matrix metalloproteinase-1 in alveolar macrophages, type II pneumocytes, and airways in smokers: relationship to lung function and emphysema. *Lung*. 2014;192(4):467–472.
14. Knadler MP, et al. Duloxetine: clinical pharmacokinetics and drug interactions. *Clin Pharmacokinet*. 2011;50(5).
15. Chen H, Lobo E, Chappell J, et al. Lipid metabolism in chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis*. 2019;14:1009–1018.
16. Chai AB, Ammit AJ, Gelissen IC. Examining the role of ABC lipid transporters in pulmonary lipid homeostasis and inflammation. *Respir Res*. 2017;18(1):41.
17. Gu HF, Li N, Xu Z-Q, et al. Chronic unpredictable mild stress promotes atherosclerosis via HMGB1/TLR4-mediated downregulation of PPARgamma/LXRalpha/ABCA1 in ApoE(-/-) Mice. *Front Physiol*. 2019;10:165.
18. Yeo J, Morales DA, Chen T, et al. RNAseq analysis of bronchial epithelial cells to identify COPD-associated genes and SNPs. *BMC Pulm Med*. 2018;18(1):42.

19. Gao W, Li L, Wang Y, et al. Bronchial epithelial cells: the key effector cells in the pathogenesis of chronic obstructive pulmonary disease? *Respirology*. 2015;20(5):722–729.
20. Lareau SC, Yawn B. Improving adherence with inhaler therapy in COPD. *Int J Chron Obstruct Pulmon Dis*. 2010;5:401–406.
21. Wallace AM, Mercer BA, He J, et al. Functional characterization of the matrix metalloproteinase-1 cigarette smoke-responsive region and association with the lung health study. *Respir Res*. 2012;13:79.
22. Zhang W, Zhang Yi, Li C-W, et al. Effect of statins on COPD: A meta-analysis of randomized controlled trials. *Chest*. 2017;152(6):1159–1168.
23. Sanja M, Jozsef P, Sanja PG, et al. Cytokines and statin therapy in chronic obstructive pulmonary disease patients. *Scand J Clin Lab Invest*. 2018;78(7–8):533–538.
24. Janda S, Park K, FitzGerald JM, et al. Statins in COPD: a systematic review. *Chest*. 2009;136(3):734–743.
25. Criner GJ, Connett JE, Aaron SD, et al. Simvastatin for the prevention of exacerbations in moderate-to-severe COPD. *N Engl J Med*. 2014;370(23):2201-2210.
26. Michaeloudes C, Bhavsar PK, Mumby S, et al. Role of metabolic reprogramming in pulmonary innate immunity and its impact on lung diseases. *J Innate Immun*. 2019;12(1):31–46.
27. Hamsanathan S, Alder JK, Sellares J, et al. Cellular senescence: The trojan horse in chronic lung diseases. *Am J Respir Cell Mol Biol*. 2019;61(1):21–30.
28. Fessler MB. The challenges and promise of targeting the liver X receptors for treatment of inflammatory disease. *Pharmacol Ther*. 2018;181:1–12.
29. Caiaffo V, Oliveira BDR, Sá FB, et al. Anti-inflammatory, anti-apoptotic, and antioxidant activity of fluoxetine. *Pharmacol Res Perspect*. 2016;4(3):231.
30. Liu FY, Cai J, Wang C, et al. Fluoxetine attenuates neuroinflammation in early brain injury after subarachnoid hemorrhage: a possible role for the regulation of TLR4/MyD88/NF-kappaB signaling pathway. *J Neuroinflammation*. 2018;15(1):347.
31. Rawdin BJ, Mellon SH, Dhabhar FS, et al. Dysregulated relationship of inflammation and oxidative stress in major depression. *Brain Behav Immun*. 2013;31:143–152.
32. Sekine T, Hirata T, Ishikawa S, et al. Regulation of NRF2, AP-1 and NF-kappaB by cigarette smoke exposure in three-dimensional human bronchial epithelial cells. *J Appl Toxicol*. 2019;39(5):717-725.
33. Wang L, Wang R, Liu L, et al. Effects of SSRIs on peripheral inflammatory markers in patients with major depressive disorder: a systematic review and meta-analysis. *Brain Behav Immun*. 2019;79:24–38.
34. Kohler CA, Freitas TH, Stubbs B, et al. Peripheral alterations in cytokine and chemokine levels after antidepressant drug treatment for major depressive disorder: Systematic review and meta-analysis. *Mol Neurobiol*. 2018;55(5):4195–4206.
35. Durairaj H, Steury MD, Parameswaran N. Paroxetine differentially modulates LPS-induced TNFalpha and IL-6 production in mouse macrophages. *Int Immunopharmacol*. 2015;25(2):485-492.
36. Habib M, Shaker S, El-Gayar N, et al. The effects of antidepressants "fluoxetine and imipramine" on vascular abnormalities and Toll like receptor-4 expression in diabetic and non-diabetic rats exposed to chronic stress. *PLoS One*. 2015;10(3):e0120559.
37. Doz E, Noulin N, Boichot E, et al. Cigarette smoke-induced pulmonary inflammation is TLR4/MyD88 and IL-1R1/MyD88 signaling dependent. *J Immunol*. 2008;180(2):1169–1178.
38. Vozoris NT, Wang X, Austin PC, et al. Serotonergic antidepressant use and morbidity and mortality among older adults with COPD. *Eur Respir J*. 2018;52(1):1800475.
39. Kiecolt-Glaser JK, Derry HM, Fagundes CP. Inflammation: depression fans the flames and feasts on the heat. *Am J Psychiatry*. 2015;172(11):1075–1091.

How to cite this article: Adam G, Shiomi T, Monica G, et al. Suppression of cigarette smoke induced MMP1 expression by selective serotonin re-uptake inhibitors. *The FASEB Journal*. 2021;35:e21519. https://doi.org/10.1096/fj.202001966RR