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Cigarette smoke inhibits efferocytosis via deregulation of sphingosine kinase signaling: reversal with exogenous S1P and the S1P analogue FTY720

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ABSTRACT

Alveolar macrophages from chronic obstructive pulmonary disease patients and cigarette smokers are deficient in their ability to phagocytose apoptotic bronchial epithelial cells (efferocytosis). We hypothesized that the defect is mediated via inhibition of sphingosine kinases and/or their subcellular mislocalization in response to cigarette smoke and can be normalized with exogenous sphingosine-1-phosphate or FTY720 (fingolimod), a modulator of sphingosine-1-phosphate signaling, which has been shown to be clinically useful in multiple sclerosis. Measurement of sphingosine kinase 1/2 activities by [^{32}P]-labeled sphingosine-1-phosphate revealed a 30% reduction of sphingosine kinase 1 ($P < 0.05$) and a nonsignificant decrease of sphingosine kinase 2 in THP-1 macrophages after 1 h cigarette smoke extract exposure. By confocal analysis macrophage sphingosine kinase 1 protein was normally localized to the plasma membrane and cytoplasm and sphingosine kinase 2 to the nucleus and cytoplasm but absent at the cell surface. Cigarette smoke extract exposure (24 h) led to a retraction of sphingosine kinase 1 from the plasma membrane and sphingosine kinase 1/2 clumping in the Golgi domain. Selective inhibition of sphingosine kinase 2 with 25 μM ABC294640 led to 36% inhibition of efferocytosis ($P < 0.05$); 10 μM sphingosine kinase inhibitor/5C (sphingosine kinase 1-selective inhibitor) induced a nonsignificant inhibition of efferocytosis, but its combination with ABC294640 led to 56% inhibition ($P < 0.01$ vs. control and < 0.05 vs. single inhibitors). Cigarette smoke-inhibited efferocytosis was significantly ($P < 0.05$) reversed to near-control levels in the presence of 10–100 nM exogenous sphingosine-1-phosphate or FTY720, and FTY720 reduced cigarette smoke-induced clumping of sphingosine kinase 1/2 in the Golgi domain. These data strongly support a role of sphingosine kinase 1/2 in efferocytosis and as novel therapeutic targets in chronic obstructive pulmonary disease. *J. Leukoc. Biol.* 100: 195–202; 2016.

Introduction

COPD is a predominantly cigarette-smoke related, chronic inflammatory airways disease that ranks highly among the common causes of death worldwide. Once COPD is established, smoking cessation alone will not completely reverse the chronic inflammation in lungs, improve lung function, or reduce the developed susceptibility to new aggravations, such as infection. COPD is currently incurable, existing treatments are largely symptomatic, and there is an urgent need for identification of new therapeutic targets.

We [1] and others [2] have previously reported an abnormal accumulation of apoptotic airway epithelial cells in COPD. Effective clearance of apoptotic cells ("efferocytosis") is important to prevent loss of the cell membrane integrity and leakage of cell contents into the surrounding tissue. This prevents exposure of tissue to toxic enzymes, oxidants, and other intracellular components, such as proteases and caspases [3]. We have shown that the efferocytosis ability of alveolar macrophages is defective in the airways of subjects with COPD and that the nonengulfed apoptotic cells may undergo secondary necrosis with ensuing

Abbreviations: 7-AAD = 7-aminoactinomycin D, COPD = chronic obstructive pulmonary disease, HDAC = histone deacylase, MeOH = methanol, S1P = sphingosine-1-phosphate, SKI = sphingosine kinase inhibitor, SPHK = sphingosine kinase, TLC = thin-layer chromatography

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inflammation [1, 4]. We have also shown that smoking per se impairs the efferocytosis capacity of alveolar macrophages from COPD patients [5, 6] and that the capacity of these macrophages to phagocytose bacteria is also impaired [7].

Sphingolipid metabolites, such as ceramide, sphingosine, and S1P, are signaling molecules involved in diverse cellular processes, including those in response to cellular stress. These molecules are interconvertible; for example, sphingosine is generated from ceramide by ceramidases and converted to S1P by SPHKs (SPHK1 or SPHK2). Importantly, their effects are typically opposing to each other: whereas ceramide is associated with apoptosis and growth arrest, S1P may promote survival and proliferation [8]. The control of the dynamic balance between such opposing effects, called the "sphingolipid rheostat," is essential for the cellular response to cell stress or other stimuli [8]. Although the exact mechanisms driving the effects of these mediators on efferocytosis are unclear, accumulating evidence suggests that perturbations in the sphingolipid signaling system may play a role in cigarette smoke-induced macrophage dysfunction. Previous studies have shown that alveolar macrophages exposed to cigarette smoke exhibit increased ceramide levels and that ceramides or sphingosine can impair efferocytosis [9]. McQuiston et al. [10] showed in mice that S1P can improve the ability of alveolar macrophages to phagocytose *Cryptococcus neoformans*. We previously identified a role for S1P in actin cytoskeletal dynamics required for macrophage phagocytic function [11]. Further studies showed that S1P, by primarily but not exclusively binding to S1PRs, induces the rapid and complex relay of signaling and externalization events that precede and control actin assembly in macrophages [11, 12].

The formation of S1P and its possible contribution to optimal macrophage function are reliant on effective SPHK activity. We previously investigated the expression profile of the sphingosine signaling system in the lung of patients with COPD [13]. We showed that alveolar macrophages from COPD patients exhibit increased relative mRNA expression of SPHK1 and SPHK2, in addition to S1PR2 and S1PR5, when compared with alveolar macrophages from control subjects [14]. We further showed that these increases are mirrored by treatment of THP-1 macrophages with cigarette smoke [14]. Effects of cigarette smoke on SPHKs at the protein level have, however, not been investigated. We hypothesized that SPHK1/2 signaling is required for regulation of efferocytosis and that the suppression effect of cigarette smoke on efferocytosis in macrophages may be mediated via inhibition of SPHK enzyme activities and/or dislocation of these molecules from their normal subcellular localization. We further asked whether such deregulations induced by cigarette smoke could be negated by exogenous S1P and FTY720 (fingolimod), a non-selective agonist for multiple S1PRs that has been shown to be clinically useful in diseases, such as multiple sclerosis [15–17].

MATERIALS AND METHODS

Macrophage differentiation

A THP-1 monocytic cell line (American Type Culture Collection, Manassas, VA, USA) was differentiated into macrophages by seeding at a density of 4×10^5 cells/ml in 24-well plastic plates and then stimulating with 100 nM PMA for 72 h, as described previously [18–20]. Experiments were carried out between passages 6 and 20.

SPHK activity assay

SPHK1 and SPHK2 activities were measured in THP-1 macrophages using an isoform-selective assay, as reported previously [21]. In brief, the assay measures the production of [^{32}P]-labeled S1P following the addition of exogenous sphingosine and [γ^{32}P] ATP. The S1P product is purified by Bligh-Dyer solvent extraction, separated by TLC, and the radiolabeled S1P quantified by exposing the TLC plate to a storage phosphor screen.

Efferocytosis of apoptotic bronchial epithelial cells

Efferocytosis was measured as reported previously [1, 20]. In brief, 16-human bronchial epithelial cell targets were maintained in continuous culture, induced to apoptosis by use of UV radiation, and then labeled with 250 nM sytox orange (Molecular Probes, Eugene OR, USA). We have previously optimized the induction of apoptosis in target epithelial cells and showed that UV radiation for 20 min produced the maximum, most consistent rates of total apoptosis with minimal necrosis, with apoptosis consistently detectable in >80% of treated cells [4] The apoptotic cells were then incubated immediately with macrophages at a ratio of 10:1 and at 37°C/5% CO_2 for 1.5 h. Following a 5 min incubation with 500 μl ice-cold PBS, cells were lifted by gentle, continuous pipetting, then transferred to FACS tubes, pelleted by centrifugation, and then stained with 3 μl CD13 PE-Cy7 (BD Biosciences, San Jose, CA, USA) for 10 min. Autofluorescence was quenched using 1 mg/ml Trypan blue, and 30,000 total events per tube were acquired immediately using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). CD13-stained macrophages and sytox orange-labeled apoptotic cells alone served to determine cutoffs for phagocytosis, which was recorded as the percentage of dual-staining CD13/sytox orange events. Representative flow plots for the efferocytosis assay have been reported previously [4].

Apoptosis of macrophages

Flow cytometric analysis of apoptosis following treatments was performed using Annexin V and 7-AAD, as reported previously [1].

Effect of cigarette smoke exposure on efferocytosis

Cigarette smoke extract (10%) was prepared, and stimulation of THP-1 macrophages, with and without cigarette smoke extract, was performed as described previously [5, 19, 20]. The effect of cigarette smoke exposure on THP-1 efferocytosis was determined.

Effect of cigarette smoke exposure on SPHK activity in macrophage cell cultures

The activity of SPHK1 and SPHK2 was assessed under control conditions and in the presence of 10% cigarette smoke extract for 1 h.

Effect of inhibiting SPHK1 and -2 activities on efferocytosis

Differentiated THP-1 macrophages were cultured in 24-well plates (4×10^5 cells/well) and treated for 24 h ± inhibitors of SPHK1 (10 μM SKI/5C; Sigma-Aldrich, St. Louis, MO, USA) and/or SPHK2 (25 μM ABC294640; MedKoo Biosciences, Chapel Hill, NC, USA). After SPHK inhibition, nonadherent cells were discarded and adherent cells harvested by gentle scraping and centrifugation as described [20] before measurement of efferocytosis by flow cytometry.

Immunofluorescence

SPHK1 and SPHK2 protein expression and subcellular localization in THP-1 macrophages were analyzed at 24 h postexposure to 10% cigarette smoke extract. Immunofluorescence of THP-1 macrophages grown on chamber slides was carried out according to a previously described protocol that included cell fixation with 2.5% formalin in PBS, cell permeabilization with 1% SDS in PBS, blocking with serum-free protein blocker (Dako Australia, Campbellfield, VIC, Australia), incubation with primary antibodies for 18 h at 4°C and with

secondary antibodies for 1 h at ambient temperature, and counterstaining for 15 min with 200 ng/ml DAPI (Sigma-Aldrich, St. Louis, MO, USA) [20]. The primary antibodies included rabbit polyclonal, SPHK1 and -2 (Bioss, Woburn, MA, USA) and mouse monoclonal, β-actin (Sigma-Aldrich, St. Louis, MO, USA) and giantin (Abcam, Cambridge, United Kingdom). The secondary antibodies were donkey IgG F(ab)2 fragments and Alexa-Fluo 594-conjugated anti-rabbit IgG and Alexa-Fluo 647-conjugated anti-mouse IgG (both from Jackson ImmunoResearch, West Grove, PA, USA). Imaging was carried out on a laser confocal microscopy (LSM700; Carl Zeiss Australia, North Ryde, NSW, Australia). From each well of 8-well chamberslides, 4–6 optical fields under a 40× water-immersed objective were captured for analysis. For quantitative analysis, multiple optical fields were photographed using a fluorescence microscope (IX73; Olympus Australia, Notting Hill, VIC, Australia) in a blind manner to prevent bias, as described previously [19], and analyzed using ImageJ software (NIH, Bethesda, MD, USA).

Effect of S1P and FTY720 on efferocytosis

S1P and FTY720 were obtained from Cayman Chemical (Ann Arbor, MI, USA). Stock solutions of S1P (solubilized in 0.3 M NaOH) and FTY720 were prepared in absolute MeOH and DMSO (Sigma-Aldrich, St. Louis, MO, USA), respectively. Differentiated THP-1 macrophages were cultured in 24-well plates (4×10^5 cells/well) and treated for 24 h ± 10% cigarette smoke extract, with or without the presence of S1P (final concentrations 0.1–100 nM) or FTY720 (final concentrations 0.1 nM–1 μM). After stimulation, nonadherent cells were discarded and adherent macrophages harvested by gentle scraping and centrifugation, as described [20]. Macrophage counts were adjusted to 4×10^5/ml before measurement of efferocytosis by flow cytometry.

Statistical analysis

Analysis was performed using SPSS statistic software (SPSS/IBM, Chicago, IL, USA) using the 2-sample Wilcoxon rank sum test or the Kruskal-Wallis test for analyses of >2 groups and post hoc testing using Dunn's test.

RESULTS

Cigarette smoke extract reduced SPHK1 activity in macrophages

SPHK1 and SPHK2 activity was measured in THP-1 macrophages, with or without 10% cigarette smoke extract for 1 h. The SPHK1 activity was reduced significantly by exposure to cigarette smoke for 1 h compared with control cells (**Fig. 1**). There was no significant change in SPHK2 activity in the presence of cigarette smoke at this time point (Fig. 1).

Cigarette smoke altered subcellular localization of SPHK1 and SPHK2 in macrophages

By confocal microscopy analysis, immunoreactivity for SPHK1 and SPHK2 was present in THP-1 macrophages but showed distinct subcellular localization. Thus, SPHK1 was normally detected in THP-1 macrophages in the cytoplasm and near the cell surface, often forming aggregates on tips of cell projections; SPHK2 localized to perinuclear cytoplasm and the nucleus but absent at the cell surface of THP-1 macrophages (**Fig. 2**). Exposure to cigarette smoke resulted in the disappearance of SPHK1 particles near the cell surface. Of note, SPHK1 and SPHK2 in cigarette smoke-treated cells were seen clumping in a juxtanuclear, often asymmetric domain (Fig. 2). Costaining experiments revealed that this domain was partially colocalized with the Golgi marker giant (see Supplemental Fig. 1 and Supplemental Material). Binding of all primary antibodies was specific, as no binding of the fluorescent conjugates was detected in the negative controls, whereby the primary antibodies were replaced with normal rabbit and mouse IgG (data not shown).

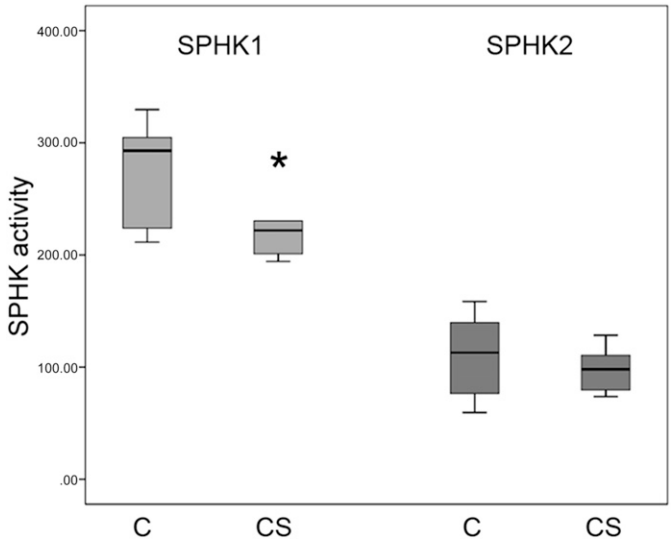

Figure 1. Cigarette smoke extract reduced SPHK activity in cigarette smoke-exposed THP-1 macrophages. SPHK activity was measured in unstimulated THP-1 macrophages and those exposed 10% cigarette smoke extract (CS) for 1 h. SPHK1 activity was reduced significantly by cigarette smoke compared with control (C). There was a small, nonsignificant decrease in SPHK2 activity compared with control in the presence of cigarette smoke after 1 h. Box plots present median ± 25th and 75th percentiles (solid box), with the 10th and 90th percentiles shown by whiskers outside of the box; $n = 3$ samples assayed in duplicate. Two-sample Wilcoxon rank sum test; significantly ($*P < 0.05$) decreased activity compared with controls.

Efferocytosis in macrophages is SPHK dependent

We next used synthetic inhibitors selective to SPHK1 or SPHK2 to confirm that these enzymes are required for maintaining efferocytosis function. A significant (36%; $P < 0.05$) suppression of efferocytosis was demonstrated following inhibition of SPHK2 with 25 μM ABC294640. Inhibition of SPHK1 with 10 μM SKI/5C caused a nonsignificant decrease in efferocytosis; however, a combination of both inhibitors led to a stronger (56%) suppression of efferocytosis, which is statistically significant compared with the control ($P < 0.01$) and single inhibitors ($P < 0.05$; **Fig. 3**).

Exogenous S1P reduced the suppressive effects of cigarette smoke on efferocytosis

Retraction of SPHK1 from the plasma membrane and inhibition of its activity in cigarette smoke-exposed THP-1 macrophages prompted us to hypothesize that extracellular S1P signaling was reduced in these cells. S1P, at concentrations of 0.1–100 nM, was exogenously added to cultured THP-1 macrophages to test whether it can reverse the suppressive effects of cigarette smoke on efferocytosis. Treatment with 10 or 100 nM S1P for 24 h significantly ($P < 0.01$ and < 0.001,

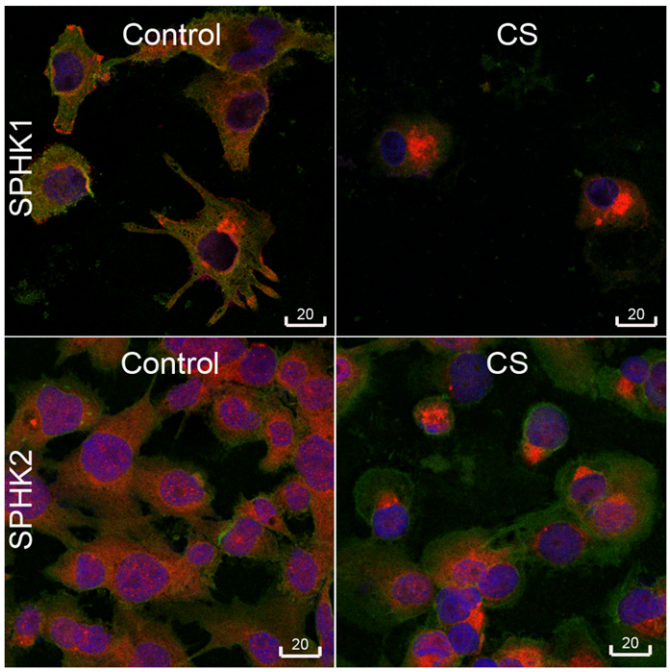

Figure 2. Exposure to cigarette smoke led to altered subcellular localization of SPHK1 and SPHK2. SPHK1 and SPHK2 protein expression and subcellular localization in THP-1 macrophages were analyzed at 24 h in unstimulated cells or those postexposure to 10% cigarette smoke extract (CS) using immunofluorescence. SPHK1 and SPHK2 were stained in red (Alexa-Fluo 594). Green and blue were pseudo-colors for β-actin (Alexa-Fluo 647) and DAPI, respectively. Imaging was carried out on a laser confocal microscopy: from each well of 8-well chamberslides, 4–6 optical fields under a 40× water-immersed objective were captured for analysis. Images were representative of 3 experiments, carried out on different batches of cells showing similar results. Scale bars, μM.

respectively) improved efferocytosis in THP-1 macrophages and reversed efferocytosis in cigarette smoke-treated cells to near-control level ($P < 0.001$ and < 0.05, respectively; 4 experiments; **Fig. 4**). At the applied concentrations of S1P, no negative effects on cell viability were observed (Annexin V staining <2.6%; 7-AAD staining <4.8%).

FTY720 reduced the suppressive effects of cigarette smoke on efferocytosis and prevented SPHK1/2 from clumping in the Golgi domain in cigarette smoke-treated macrophages

FTY720 (fingolimod) is a nonselective S1PR agonist that is clinically applied in multiple sclerosis for its immune-regulatory properties. From preliminary experiments on the 0.1 nM to 10 μM range (data not shown), the optimal concentration of 10 nM FTY720 was chosen for studying its effects on cigarette smoke-exposed THP-1 macrophages. Compared with untreated controls, cigarette smoke-exposed THP-1 macrophages showed significantly decreased efferocytosis function ($P < 0.01$), which was restored significantly in the presence of 10 nM FTY720 ($P < 0.05$; 3 experiments; **Fig. 5**). Qualitative analysis of 3 further experiments showed that FTY720 alone at this concentration did not cause changes in SPHK1/2 immunolocalization (data not shown) and that macrophage exposure to cigarette smoke extract for 24 h induced intracellular clumping of SPHK1 and SPHK2 in the Golgi domain (**Fig. 6B and E** and Supplemental Fig. 1), which was prevented in the presence of FTY720 (Fig. 6C and F); of note, plasma membrane localization of SPHK1 was restored in this condition (Fig. 6C).

DISCUSSION

Suppressed efferocytosis in alveolar macrophages is a well-established factor in COPD, potentially contributing to chronic inflammation of the airway. This concept opens a novel avenue to management of the disease [2–7, 18, 19]. Sphingolipids are integral components of the cell membrane and are present in virtually all body cells. Sphingosine participates in sphingolipid synthesis by phosphorylation to produce S1P by SPHKs or acylation to produce ceramide. The complex balance between these mediators is likely to play an important role in alveolar macrophage efferocytosis in COPD, with ceramide/sphingosine and S1P impairing or increasing macrophage phagocytic function, respectively [9]. We previously showed increased mRNA expression of SPHK1 and SPHK2 in alveolar macrophages from COPD subjects and in cigarette smoke-exposed

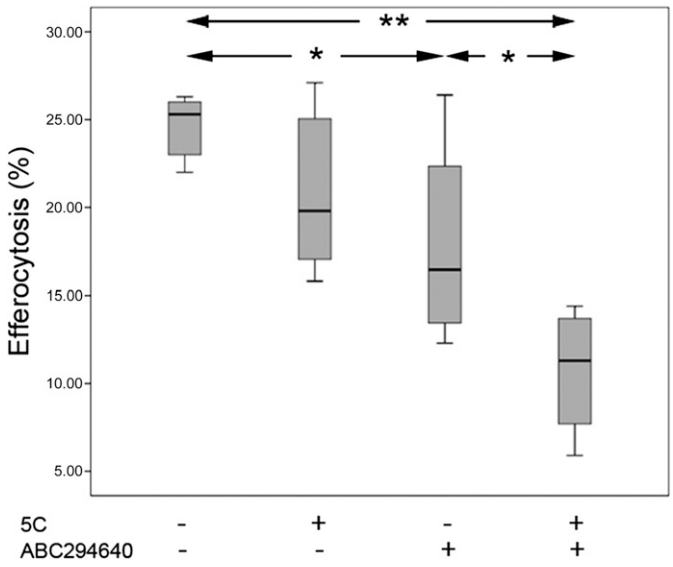

Figure 3. Inhibition of SPHK1/2 led to defective efferocytosis. THP-1 macrophages were treated for 24 h with 25 μM ABC294640 (SPHK2-specific inhibitor), 10 μM SKI/5C (SPHK1-specific inhibitor), or a combination of both of the above or control vehicle before being assayed for efferocytosis capacity. Similar to the effects of cigarette smoke exposure, ABC294640 induced a statistically significant decrease in efferocytosis function in THP-1 macrophages compared with controls ($*P < 0.05$). The application of 5C alone induced a reduction of efferocytosis that was not statistically significant, but its combination with ABC294640 induced a significant decrease of efferocytosis compared with controls ($**P < 0.01$) or individual inhibitors ($P < 0.05$). Data presented as box plots as described for Fig. 1 and analyzed using the Kruskal-Wallis test for analyses of >2 groups and post hoc testing using Dunn's test; $n = 3$ samples assayed in duplicate.

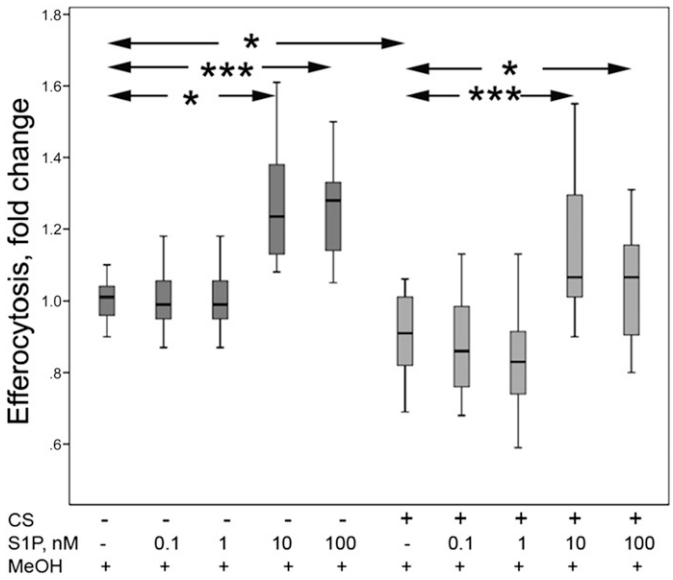

Figure 4. Exogenous S1P reduced the suppressive effects of cigarette smoke on efferocytosis. S1P (stock solution in MeOH) was applied to THP-1 macrophages at final concentrations 0.1–100 nM during 24 h exposure to 10% cigarette smoke extract (CS) or vehicle control. Addition of 10 or 100 nM S1P significantly improved efferocytosis in THP-1 macrophages in an absence of cigarette smoke extract ($*P < 0.05$ or $***P < 0.001$, respectively). Macrophage exposure to 10 or 100 nM S1P significantly reduced the inhibitory effects of cigarette smoke on efferocytosis ($P < 0.001$ or $P < 0.05$, respectively). Data presented as box plots as described for Fig. 1 and analyzed using the Kruskal-Wallis test for analyses of >2 groups and post hoc testing using Dunn's test. Data from 4 separate experiments performed in triplicate are presented.

macrophages in vitro [14]. In the present study, we showed for the first time that SPHK activity, in contrast to mRNA expression, was reduced significantly in cigarette smoke-treated THP-1 macrophages. The blocking of the activity of SPHK1 and SPHK2 with synthetic inhibitors confirmed their role in efferocytosis. Of note, the efferocytosis function in THP-1 macrophages not exposed to cigarette smoke was more sensitive to inhibition of SPHK2 than SPHK1, whereas the SPHK1 activity was more sensitive to cigarette smoke suppressive effects than SPHK2. Interestingly, the combination of both inhibitors led to a suppression of efferocytosis exceeding the effects by individual inhibitors, supporting a notion that SPHK1 and SPHK2 signaling, although sharing common mechanisms, may be involved in regulation of efferocytosis via independent pathways.

The discordance between the up-regulated gene expression of SPHKs shown in our previous study in response to cigarette smoke and our current findings of down-regulated protein functional activity emphasizes the complexity of pleiotropic signaling pathways exemplified by the SPHKs. Nevertheless, the reduced enzyme activity in response to cigarette smoke is likely to suppress efferocytosis for several reasons, including deficient S1P signaling and a reduction in the conversion of sphingosine into S1P, leading to an accumulation of sphingosine, which has been implicated in efferocytosis inhibition [9].

Current data support differential functions of the 2 SPHK enzymes and that depending on the site of S1P generation, this same small lipid signaling molecule may have opposing effects on key cellular processes [22]. With the use of SPHK1 over-expression systems, we [23, 24] and others [25] have shown that this protein has intracellular and plasma membrane localization, the latter specific for the phosphorylation-activated form. Endogenous SPHK1 in THP-1 macrophages in this study was found normally localized to both intracellular cytoplasm and structures near the cell surface. It can be hypothesized that when tethered to the plasma membrane, the phosphorylation-activated form of SPHK1 may up-regulate efferocytosis via multiple mechanisms, e.g., increasing the S1P/dihydroS1P ratio [26], facilitating membrane inward curvatures in negatively charged domains [27], or favoring the so-called "inside-out" signaling, whereby S1P is exported to the extracellular space for targeting the membrane-bound S1PR1–5 [28, 29]. Both a remarkable reduction of the SPHK1 activity and its retraction from structures at the cell surface observed in cigarette smoke-exposed macrophages were in line with data on the proefferocytosis effect of exogenous S1P.

Our data on the localization of SPHK2 to perinuclear structures and the nucleus of THP-1 macrophages are in accordance with the hypothesis that SPHK2-generated S1P targets organelles, such as mitochondria [30] and the nucleus [31, 32]. The precise mechanism(s) of regulation of the phagocytic machinery by SPHK2 are unknown at this stage. In the nucleus, S1P generated by histone H3-associated SPHK2 may bind and inhibit HDAC1/2 [31], and inhibition of the

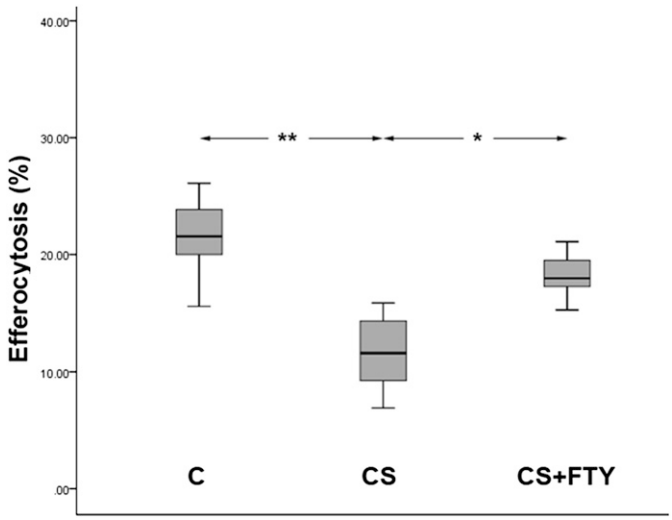

Figure 5. FTY720 negated the suppressive effects of cigarette smoke on efferocytosis. FTY720 (FTY) at final concentrations 10 nM was applied to THP-1 macrophages during 24 h exposure to 10% cigarette smoke extract (CS). Cigarette smoke extract induced significant reduction of efferocytosis compared with controls (C; $**P < 0.01$). A significant improvement in efferocytosis in cigarette smoke-exposed cells was noted in the presence of FTY720 ($*P < 0.05$). Data presented as box plots as described for Fig. 1 and analyzed using the Kruskal-Wallis test for analyses of >2 groups and post hoc testing using Dunn's test; $n = 3$ samples assayed in duplicate.

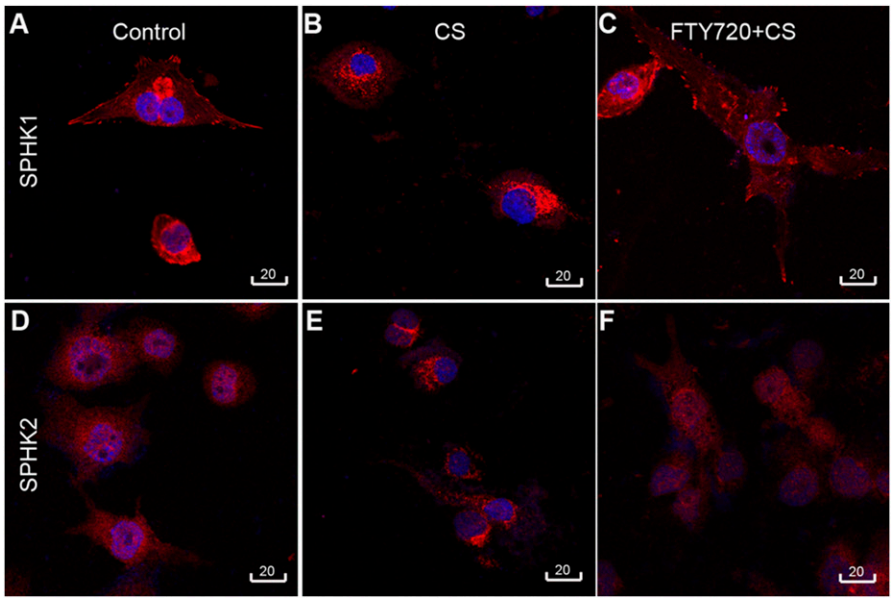

Figure 6. FTY720 prevented SPHK1 and SPHK2 in cigarette smoke-exposed macrophages from intracellular clumping. Cigarette smoke exposure induced SPHK1 and SPHK2 clumping in a juxtanuclear domain, which was prevented in the presence of 10 nM FTY720. Representative confocal images of 3 experiments showing similar results. Red, SPHK1 or SPHK2 (Alexa-Fluo 594). Blue was a pseudocolor of DAPI. Scale bars, μM.

HDAC has recently been implicated in the suppressive effect of cigarette smoke on efferocytosis in alveolar macrophages [33]. An anti-efferocytosis effect of SPHK2 (or at least its nuclear fraction) could be deduced from the above findings, which contradicted our data, suggesting a proefferocytosis effect of SPHK2. We hypothesize that SPHK2 may participate in regulation of efferocytosis via both inhibitory pathways, such as HDAC, and unknown, yet stimulatory pathways, in particular, by its phosphorylation. Thus, the protein kinase D pathway, which was shown in HeLa cells to control the SPHK2 exit from the nucleus [32], could also mediate uridine disphosphate-stimulated phagocytosis in microglia [34]. Interestingly, our further finding that SPHK1/2 partially colocalized with the Golgi marker giantin and exhibited increased clumping in the Golgi domain in cells exposed to cigarette smoke in this study suggested the following: (1) that Golgi may mediate SPHK1/2 trafficking to their sites of function and (2) that the latter could be deranged by effects of cigarette smoke. The mechanism of cigarette smoke-induced mislocalization of SPHK1/2 is unknown at this stage: it could be that chemically modified SPHK1/2 proteins failed to engage with the Golgi apparatus, that the Golgi function itself was affected by cigarette smoke, or both.

From a therapeutic perspective, our finding of a stimulative effect of FTY720 on efferocytosis in macrophages is of particular interest. FTY720 is a potent immunoregulator and has already been approved for clinical applications in multiple sclerosis [15, 17]. It has also been studied intensively in other diseases, including respiratory (e.g., bronchiolitis obliterans syndrome following lung transplantation) [35, 36]. FTY720 has complex mechanisms of action. As a pro-drug, upon uptake into the cell via the spinster homolog 2 transporter, it is phosphorylated by SPHK2 and can act as a nonselective agonist for S1PRs [15].With chronic exposure, however, it displays antagonistic activity by inducing internalization and degradation of S1PR1 that results in prolonged receptor down-regulation [37]. Furthermore, an anti-cancer activity of FTY720 has been shown to be independent of S1PRs. In human pulmonary artery smooth muscle cells, MCF-7 breast cancer cells, and androgen-independent LNCaP-AI prostate cancer cells, FTY720 applied at a micromolar range was shown to reduce the SPHK1 activity by proteasomal degradation of the enzyme [38]. The proefferocytosis effect of FTY720 on THP-1 macrophages described in this study was, however, revealed at the nanomolar range of concentrations; this could possibly be associated with FTY720 agonist effects on S1P receptors or other reported effects, e.g., activation of the SPHK2 [39] or inhibition of ceramide synthases [16]. Although the preliminary studies presented herein support the potential for FTY720 as a macrophage-targeted therapeutic option for COPD, further work is clearly indicated to understand more completely its action on SPHK activity and subsequent phagocytic function in macrophages, especially in the context of COPD and in the presence of cigarette smoke. We also acknowledge the limitations of THP-1 macrophages as surrogates for primary macrophages, although we have recently published work showing that these cells do indeed show changes in sphingosine signaling that are consistent with primary macrophages. With the use of PCR, we found significant increases in *SPHK1/2* (3.4- and 2-fold increases, respectively) and *S1PR2* and *-5* (4.3- and 12.2-fold increases, respectively)in alveolar macrophages from COPD subjects vs. controls [14]. In THP-1 macrophages, exposure to cigarette smoke also significantly increased mRNA expression of *SPHK1/2*, *S1PR2*, and *S1PR5*, confirming the results in human macrophages.

In conclusion, we showed that SPHK1 and SPHK2 are involved in regulation of efferocytosis in macrophages and that the suppressive effect of cigarette smoke on efferocytosis function in macrophages could be mediated by subcellular

redistribution of SPHKs and inhibition of their activity. This may offer a novel approach for macrophage-targeted therapy of COPD.

AUTHORSHIP

H.B.T. designed the study and performed experiments on immunofluorescence, analyzed data, and wrote the manuscript. J.B. designed and performed experiments on SPHK enzyme activity, analyzed data, and revised the manuscript. M.W. designed and performed experiments on effects of exogenous S1P, analyzed data, and revised the manuscript. R. Hamon performed the cell culture, analyzed the effects of cigarette smoke and FTY720, and revised the manuscript. E.R. performed the cell culture, analyzed the effects of cigarette smoke, and revised the manuscript. G.H. supervised experiments of efferocytosis and revised the manuscript. P.N.R. and R. Haberberger conceived of the study and revised the manuscript. S.M.P. conceived of the study, analyzed data, and revised the manuscript. L.T.D. performed experiments on SPHK enzyme activity, analyzed data, and revised the manuscript. S.H. conceived of the study, analyzed data, and wrote the manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

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