

Restoring cigarette smoke-induced impairment of efferocytosis in alveolar macrophages

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Cigarette smoke has been associated with susceptibility to different pulmonary and airway diseases. Impaired alveolar macrophages (AMs) that are major phagocytes in the lung have been associated with patients with airway diseases and active smokers. In the current report, we show that exposure to second-hand cigarette smoke (SHS) significantly reduced efferocytosis *in vivo*. More importantly, delivery of recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF) to the alveolar space restored and refurbished the efferocytosis capability of AMs. Exposure to SHS significantly reduced expression of CD16/32 on AMs, and treatment with GM-CSF not only restored but also significantly increased the expression of CD16/32 on AMs. GM-CSF treatment increased uptake and digestion/removal of apoptotic cells by AMs. The latter was attributed to increased expression of Rab5 and Rab7. Increased efferocytosis of AMs was also tested in a disease condition. AMs from GM-CSF-treated, influenza-infected, SHS-exposed mice showed significantly better efferocytosis activity, and mice had significantly less morbidity compared with phosphate-buffered saline-treated group. GM-CSF-treated mice had increased amphiregulin levels in the lungs, which in addition to efferocytosis of AMs may have attributed to their protection against influenza. These results will have great implications for developing therapeutic approaches by harnessing mucosal innate immunity to treat lung and airway diseases and protect against pneumonia.

INTRODUCTION

Exposure to cigarette smoke has been associated with susceptibility to different pulmonary diseases and several epidemiological studies have shown that pneumonia is more common in smokers than nonsmokers.^{1–3} However, mechanisms by which cigarette smoke exposure increase susceptibility to pulmonary infections and diseases are not well understood. We have shown that exposure to second-hand cigarette smoke (SHS) increases susceptibility to tuberculosis and influenza by affecting T-cell responses.⁴ Others have shown that exposure to cigarette smoke activates Nrf2 in macrophages and reduces neutrophil recruitment, reduces alveolar macrophages' (AMs') phagocytic ability and expression of several important recognition molecules, and impairs clearance of apoptotic cells through oxidant-dependent activation of RhoA.^{5–8}

Several mechanisms are involved in maintaining the lung homeostasis, including mucociliary clearance and phagocytosis

of apoptotic cells, inhaled particulate matters, and invading organisms. AMs are the most prominent phagocytes in the lung and the ingestion of apoptotic cells by phagocytes, termed “efferocytosis”, is known to have an important role in reducing inflammation in damaged tissues.^{9–11} Efferocytosis by AMs is pivotal in the maintenance of lung homeostasis and reduces oxidant damage, and initiates antiprotease activity, and growth factors.^{7,12,13} Interaction of various apoptotic cell ligands, bridging molecules, phagocytes cell receptors, and signaling pathways determine the levels of efferocytosis and regulate efferocytosis by controlling the balance of several Rho GTPases, including Rac1 and RhoA.^{14–17} Exposure to cigarette smoke changes the phenotype and function of AMs⁸ and suppresses AMs' efferocytosis activity.^{6,7,18,19}

Defective efferocytosis results in chronic inflammation and significantly increases the likelihood of developing chronic obstructive pulmonary disease, lung injury, and cancer. Despite

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several antismoking campaigns, tobacco use remains the single largest preventable cause of death and disease, cigarette smoking remains responsible for >480,000 deaths annually in the United States, and >41,000 of these deaths are from exposure to SHS (<http://www.cdc.gov/tobacco/campaign/tips/resources/data/cigarette-smoking-in-united-states.html>). More importantly, many victims of cigarette smoke suffer from permanent damage, and even after cessation of smoking they need ongoing medical care. Hence, there is an urgent need for a better understanding of mechanisms by which cigarette smoke affects the lung homeostasis and consequently its innate immunity, as well as novel therapeutic agents and strategies.

In the current report, we demonstrate that exposure to second-hand cigarette smoke (SHS) reduces efferocytosis of apoptotic cells by AMs through reduced expression of CD16/32 receptor. Moreover, treating SHS-exposed animals with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) increases uptake and removal of apoptotic cells in SHS-exposed AMs *in vivo*, and consequently reduces inflammation and protects SHS-exposed animals against pneumonia.

RESULTS

Reversing deleterious effects of SHS on efferocytosis of AMs

Exposure to cigarette smoke reduces phagocytic activities of AMs including efferocytosis.^{5–8,18–20} We used a novel *in vivo* system, and mice were either exposed to SHS for 6 weeks or to ambient air (control mice). SHS-exposed mice were treated with either mouse recombinant GM-CSF ($1 \mu\text{g g}^{-1}$ body weight) or phosphate-buffered saline (PBS) once daily for 1 week. Mouse lung epithelial (MLE) cells were treated with staurosporine to induce apoptosis. On day 8, all the mice received 1×10^6 carboxyfluorescein succinimidyl ester (CFSE)-labeled apoptotic MLE cells intranasally. Three hours after transferring CFSE-stained apoptotic MLE cells, bronchoalveolar lavage (BAL) samples were collected and the clearance of apoptotic cells was studied using flow cytometry analyses. BAL cells from naïve mice consisted of 99% AMs, as determined by morphometric analysis. BAL samples were stained for F4/80 as a marker for AMs.

Mice exposed to SHS showed a significantly higher percent of free uningested CFSE-stained apoptotic MLE cells in BALs when compared with nonexposed controls (average percent of 41.1 ± 6.1 vs. 22.5 ± 8.5 , respectively; **Figure 1a,b**). Moreover, the efferocytosis efficiency of AMs in nonexposed control mice was 46.35 ± 11.54 vs. 17.57 ± 3.93 in SHS-exposed mice (**Figure 1a,c**).

Treating SHS-exposed mice with GM-CSF significantly increased the uptake and clearance of apoptotic MLE cells by AMs. The average percent of uningested CFSE-stained apoptotic MLE cells in BALs of GM-CSF-treated, SHS-exposed mice radically reduced compared with PBS-treated, SHS-exposed mice (1.1 ± 0.7 vs. 41.1 ± 6.1 , respectively; **Figure 1a,b**). This indicated the increased uptake of apoptotic cells in GM-CSF-treated group. Interestingly, the

efferocytosis efficiency of AMs in SHS-exposed GM-CSF-treated mice was significantly higher compared with that of PBS-treated, SHS-exposed mice (91.78 ± 4.3 vs. 17.59 ± 3.93) (**Figure 1a,c**).

Fate of apoptotic cells in the AMs

To examine the effects of SHS on the AMs and the fate of ingested apoptotic cells by AMs, we stained apoptotic MLE cells with red pHrodo dye before delivery to alveolar space of SHS-exposed and control mice and used confocal microscopy to visualize the location of apoptotic MLE cells inside the AMs. pHrodo dyes are nonfluorescent at neutral pH and fluoresce brightly in acidic environment such as lysosome. Apoptotic MLE cells were stained with red pHrodo dye and delivered to naïve control, SHS-exposed PBS-treated, and SHS-exposed GM-CSF-treated mice. AMs were collected and subjected to cytospin and confocal microscopy. Expression of CD11c as AM marker in SHS-exposed mice was comparable to those of naïve control mice (**Figure 2a3–4,b3–4**). Treatment with GM-CSF, significantly increased expression of CD11c (**Figure 2c3–4**). pHrodo-stained apoptotic MLE cells were fluorescing in AMs from both SHS-exposed and control mice in comparable levels (**Figure 2a5,b5**). Treating SHS-exposed mice with GM-CSF radically increased levels of red pHrodo fluorescence in AMs (**Figure 2c5**), indicating the presence of large amounts of red pHrodo-labeled apoptotic cells in low pH environment. In all cases, pHrodo red fluorescence colocalized with CD11c marker (**Figure 2a6,b6,c6**), depicting the intracellular pH-rodo-stained cells from extracellular ones.

Increased efferocytosis reduces inflammation

Efferocytosis is of critical importance in tissue homeostasis and in resolution of inflammation.^{17,21} We evaluated the effects of pulmonary delivery of GM-CSF on the levels of inflammation in the lungs, and measured levels of several cytokines (including proinflammatory cytokines interleukin-12 (IL-12) and tumor necrosis factor- α) in the BALs of SHS-exposed, wild-type mice after delivery of apoptotic MLE cells. Treatment with GM-CSF significantly reduced the IL-12 and tumor necrosis factor- α levels in the lungs (**Figure 3a,b**). We also measured the levels of the anti-inflammatory cytokines IL-4 and IL-13, which have been studied in relation to enhanced efferocytic capacity of macrophages.²² BALs from GM-CSF-treated, SHS-exposed mice that were challenged with apoptotic MLE cells had significantly higher levels of IL-4 and IL-13 when compared with their PBS-treated, SHS-exposed counterparts (**Figure 3c,d**).

SHS exposure impairs expression of CD16/32 on AMs

AMs are the resident professional phagocytes that provide a first line of host defense by internalizing and degrading different pathogens, apoptotic cells, and inhaled particles. Different mechanisms have been identified by which SHS exposure can affect phagocytosis and efferocytosis by AMs.^{5–8,18} We next studied the surface molecules on AMs that bind to apoptotic cells and initiate their uptake. AMs from

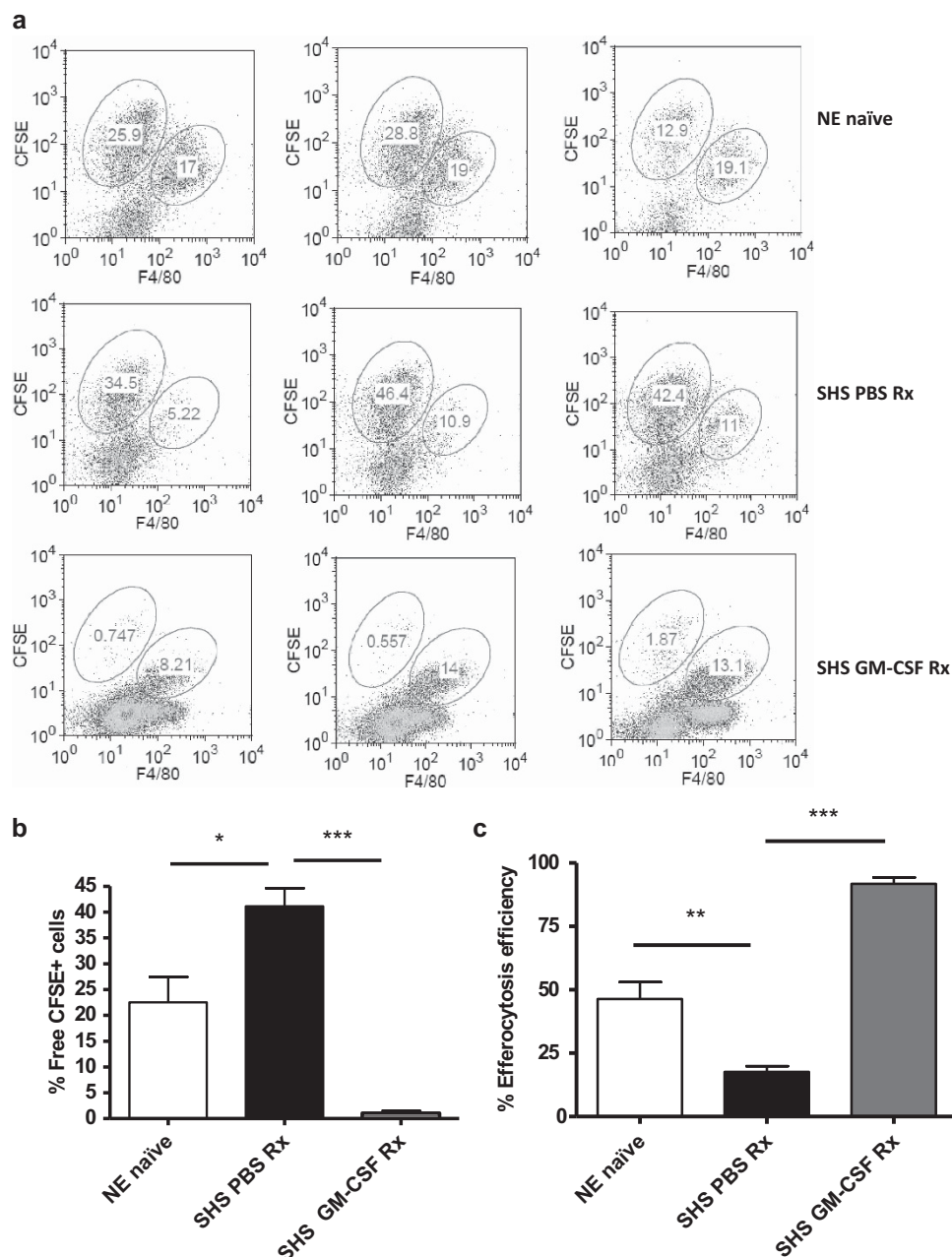


Figure 1 Exposure to second-hand cigarette smoke (SHS) reduces efferocytosis by alveolar macrophages. Staurosporine-induced apoptotic mouse lung epithelial (MLE) cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and intranasally transferred to naïve nonexposed control, wild-type mice (NE) and naïve SHS-exposed mice treated with either phosphate-buffered saline (PBS) or $1 \mu\text{g g}^{-1}$ body weight recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF) for 7 days intranasally. Bronchoalveolar lavage (BAL) fluid was collected 3 h later and stained with APC-F4/80, as a marker for alveolar macrophages. (a) Flow cytometry analysis of individual mice is shown. (b) Average of free CFSE + apoptotic MLE cells from panel a. (c) Efferocytosis efficiency was calculated as percent of efferocytosed CFSE + apoptotic cells (CFSE + and F4/80 + double-positive cells)/total percent of CFSE + cells. A representative of two to four independent experiments with 3–5 mice per group are shown. Error bars show s.e.m. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

SHS-exposed mice and their nonexposed counterparts were stained for expression of CD51, CD36, MERTK, MARCO, and CD16/32. Exposure to SHS only reduced the expression of CD16/32 that is known as Fc γ III/II receptors (**Supplementary Figure 1** online). SHS-exposed AMs showed significant reduction in expression of CD16/32 compared with AMs from nonexposed control mice (**Figure 4**). Delivery of GM-CSF to alveolar space significantly improved expression of CD16/32

in SHS-exposed mice and its expression increased $>2,700\%$ (27-fold) compared with AMs from PBS-treated, SHS-exposed and $>500\%$ (5.2-fold) compared with nonexposed control mice (**Figure 4**).

GM-CSF enhances expression of Rab5 and Rab7

Small GTPases such as Arf, Rab, Ras, Ran, and Rho regulate endomembrane trafficking in eukaryotic cells, and Rab

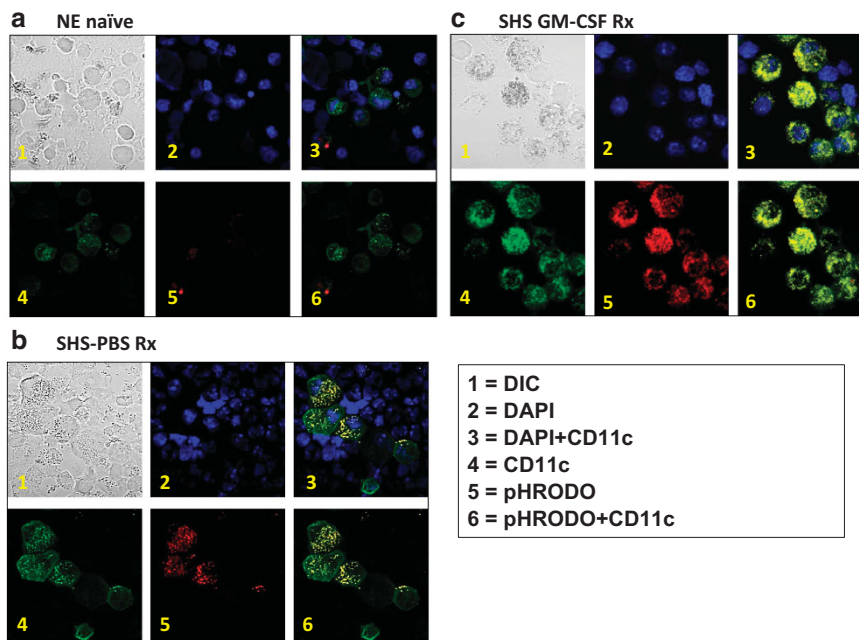


Figure 2 Localization of apoptotic cells in the alveolar macrophages (AMs). Apoptotic mouse lung epithelial (MLE) cells were stained with red pHrodo and administered intranasally to (a) control naïve, (b) second-hand cigarette smoke (SHS)-exposed phosphate-buffered saline (PBS)-treated, and (c) SHS-exposed granulocyte–macrophage colony-stimulating factor (GM-CSF)-treated mice. BALs were collected and subjected to confocal microscopy. (1) DIC; (2) DAPI; (3) DAPI + CD11c; (4) CD11c; (5) pHRODO; (6) pHRODO + CD11c. 60 × Magnification. A representative of two independent experiments with 3–4 mice per group is depicted.

GTPases have pivotal roles in phagocytic trafficking by mediating vesicle formation, maturation, and transport in eukaryotic cells.^{23–25} For instance, in the phagocytosis/efferoctosis pathway, cargo is sequentially transported from Rab5 to Rab4 and Rab11 domains for recycling purposes, but from Rab5 to Rab7 domains for degrading purposes. In the latter case, Rab7 has a key regulating role in phagolysosome fusion transport of lysosome-destined enzymes and internalized surface proteins to the lysosome through the endocytic pathway.^{23,26–29} We measured mRNA expression levels of Rab5 and Rab7 in the AMs of SHS-exposed mice treated with GM-CSF and PBS, and from their nonexposed naïve counterparts. Exposure to SHS did not have any significant effects on the expression of Rab5 and Rab7. However, GM-CSF treatment significantly increased the expression of Rab5 and Rab7 in AMs (Figure 5a,b). Similarly, exposure to SHS did not affect the activity of Rac1 and RhoA; however, GM-CSF increased the activity of Rac1 in AMs of SHS-exposed mice but did not show significant effects on RhoA activity (Figure 5c,d).

Efferoctosis of AMs in pulmonary infection

Exposure to SHS and smokers are more prone to community-acquired pneumonia such as influenza. We and others have shown the pivotal roles of AMs in influenza. Hence, to determine the role of treatment with GM-CSF on apoptotic cell clearance by AMs in SHS-exposed mice in a disease condition, we used influenza A virus (IAV) infection as a model. SHS-exposed mice were infected with PR8 influenza virus after 7 days of GM-CSF or PBS treatment. All mice received

CFSE-labeled apoptotic MLE cells intranasally, and the apoptotic cell clearance was measured by flow cytometry 24 h after infection as described above. Infection with IAV increased the uptake of CFSE-labeled apoptotic MLE cells by AMs of SHS-exposed, PBS-treated mice as the average percent of uningested free CFSE-labeled apoptotic MLE cells was $26.7 \pm 2.07\%$ (Figure 6a,b). GM-CSF-treated, SHS-exposed mice had significantly lower uningested free apoptotic MLE cells in the BALs ($2.7 \pm 0.5\%$) compared with PBS-treated, SHS-exposed mice ($26.7 \pm 2.07\%$). GM-CSF treatment of SHS-exposed mice also resulted in significant increase in efferoctosis efficiency by F4/80 AMs ($89.42 \pm 1.51\%$) compared with PBS-treated group ($50.79 \pm 9.46\%$), indicating the supremacy of GM-CSF-treated AMs in the uptake and clearance of CFSE + apoptotic cells even in IAV infection (Figure 6a,c). These experiments clearly show that AMs of GM-CSF-treated, SHS-exposed mice can uptake, digest, and remove the apoptotic cells much more effectively compared with that of their PBS-treated counterparts.

Delivery of GM-CSF to the lungs protects SHS-exposed mice against deleterious effects of SHS

To assess the role of AMs' efferoctosis in a disease condition, we exposed C57/Bl6 mice to SHS for 6 weeks and during the last week of exposure a group of mice were treated with $1 \mu\text{g g}^{-1}$ GM-CSF or PBS for 7 days ($n = 6–8$). On day 8, all mice were infected with sublethal dose of IAV (0.2 LD50 (lethal dose, 50%)) to mimic the clinical conditions in humans. Infected mice were monitored daily for morbidity and clinical signs of

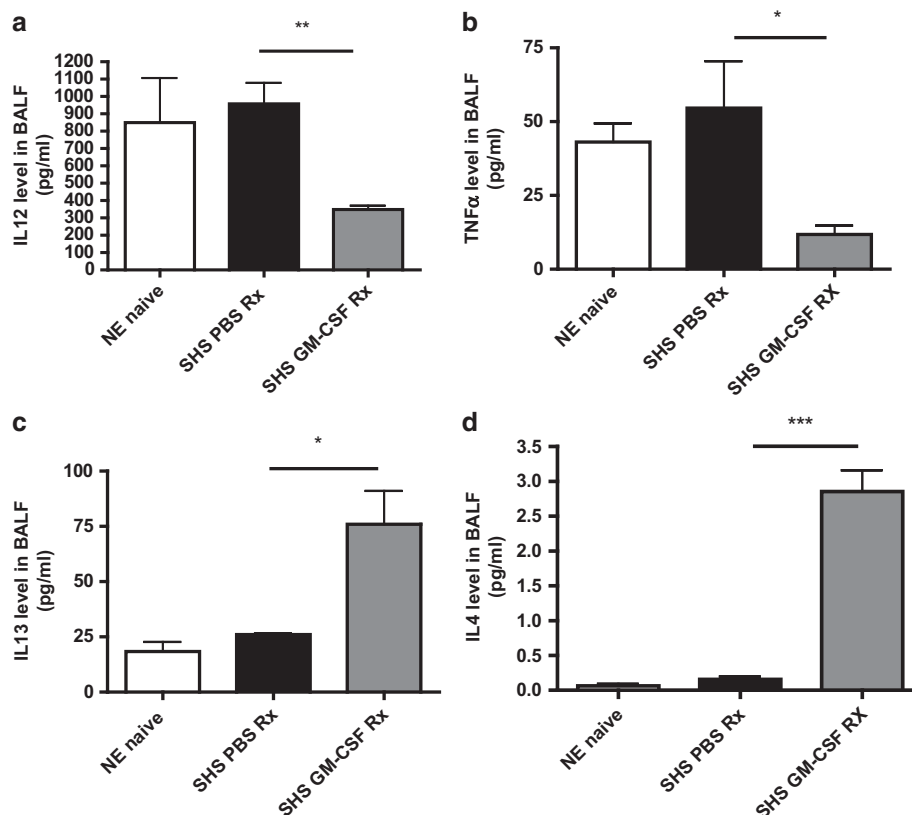


Figure 3 Effects of second-hand cigarette smoke (SHS) exposure on pro- and anti-inflammatory cytokines in the lungs. Wild-type C57Bl/6 mice were treated as in **Figure 1**. Levels of IL-12 (**a**), TNF α (**b**), IL-13 (**c**) and IL-4 (**d**) in the bronchoalveolar lavage (BAL) samples were measured using enzyme-linked immunosorbent assay (ELISA) as described in the Methods section. Mean of 3–5 mice per group is shown, with error bars showing s.e.m. * $P < 0.05$, ** $P < 0.007$, and *** $P < 0.001$.

influenza disease. All PBS-treated, SHS-exposed mice lost weight (~25%) by day 10 after infection, and it took ~3 weeks to regain their preinfection weight. However, GM-CSF-treated, SHS-exposed mice showed no significant weight loss, and by 14 days after infection, they started to pass their preinfection weight (**Figure 7a**). We also used micro-CT scanning (GE eXplore Locus) to evaluate anatomical changes and disease severity in live animals, and the Flexivent ventilator (Scireq, Pittsburgh, PA) to measure lung volumes. On day 21 after IAV infection, the lung volumes of mice were measured and then scanned by micro-CT scanning. This showed extensive consolidation of the lung in PBS-treated, SHS-exposed mice (**Figure 7b**, lower panels) when compared with GM-CSF-treated, SHS-exposed animals (**Figure 7b**, upper panels). The mean lung volumes at peak inspiration were markedly reduced in PBS-treated, SHS-exposed mice (522 mm³), compared with GM-CSF-treated, SHS-exposed mice (979 mm³).

Tissue homeostasis is an important factor in the outcome of any pathology in the lung. Amphiregulin promotes the growth of normal epithelial cells and has recently been described as an important molecule in host defense against influenza and bacterial coinfection.^{30–32} The levels of amphiregulin in the lungs of naïve GM-CSF-treated, SHS-exposed mice were

significantly higher compared with those of naïve PBS-treated, SHS-exposed mice. Amphiregulin levels were also higher in GM-CSF-treated, SHS-exposed mice 1 and 2 days after IAV infection (**Figure 7c**).

DISCUSSION

Homeostasis of the airways and alveolar space is of paramount importance for maintaining the function of the respiratory system. AMs have a pivotal role in uptake, digestion, and removal of dead and apoptotic cells, cell debris, bacteria, and inhaled particles. AMs from patients with airway diseases such as chronic obstructive pulmonary disease, asthma, and cystic fibrosis have impaired phagocytic function.^{33,34} Exposure to cigarette smoke affects expression of several important recognition molecules on AMs and expression of CD31, CD91, CD44, and CD71 are reduced on AMs from current smokers with chronic obstructive pulmonary disease and healthy smokers compared with healthy never-smoker control subjects.⁶ Cigarette smoke exposure also impairs efferocytosis of apoptotic cells through oxidant-dependent activation of RhoA.⁷

In the current report, for the first time we have shown that exposure to SHS reduces uptake and removal of apoptotic cells *in vivo* and that treating SHS-exposed mice with recombinant

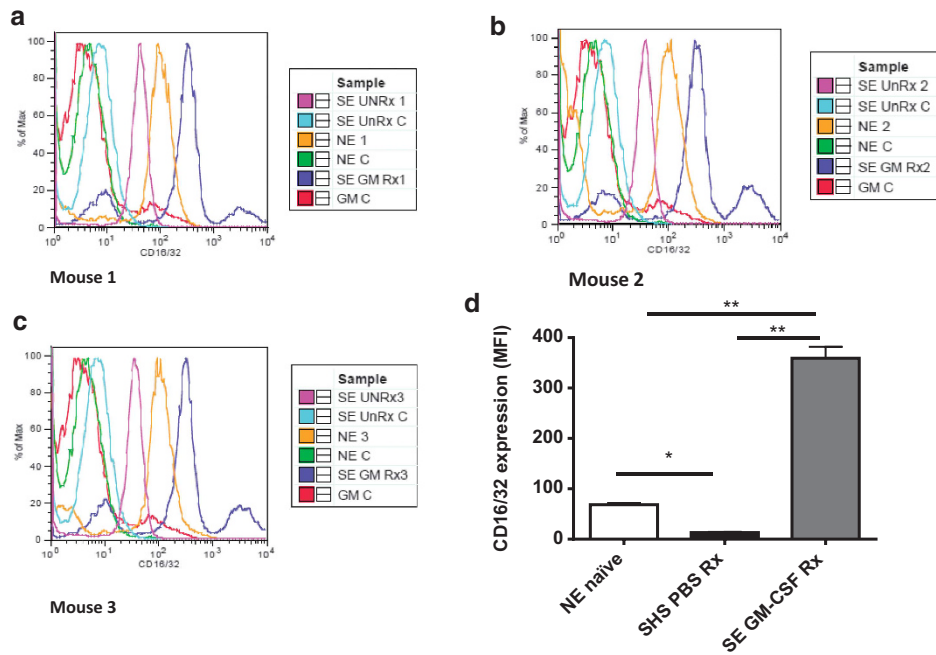


Figure 4 Exposure to second-hand cigarette smoke (SHS) impairs expression of CD16/32 on alveolar macrophages. Naïve C57Bl/6 mice were exposed to SHS and nonexposed wild-type mice (NE) were used as control. SHS-exposed mice were treated with either phosphate-buffered saline (PBS) or recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF), $1 \mu\text{g g}^{-1}$ body weight for 7 days. Alveolar macrophages (AMs) from all three groups were collected and stained for expression of CD16/32. (**a–c**) Histograms of flow cytometry analysis of three individual mice are shown. (**d**) Average of mean fluorescent intensity (MFI) of each group is shown. Error bars depict s.e.m. * $P < 0.05$ and ** $P < 0.001$. C, control isotype antibody; NE, nonexposed; SE-GMRx, SHS-exposed treated with GM-CSF; SE UNRx, SHS-exposed untreated.

GM-CSF fully reverses deleterious effects of SHS exposure on efferocytosis of AMs (**Figure 1**). Others have shown that macrophages from procysteine-treated mice have improved phagocytic activity.⁸ However, recombinant GM-CSF in the lungs had striking effects on treating defective AMs from naïve SHS-exposed mice and reducing lung inflammation. Considering that the severity of lung diseases depends on the severity of the inflammatory responses, GM-CSF therapy had pleotropic effects in SHS-exposed mice by increasing efferocytosis of AMs, reducing proinflammatory cytokines (IL-12 and tumor necrosis factor- α), and increasing anti-inflammatory cytokines (IL-13 and IL-4) (**Figure 3**).

We also showed that treating SHS-exposed mice with recombinant GM-CSF not only increases homeostasis of the lung but also increases lung innate immunity against influenza. Treating with GM-CSF increased efferocytosis of apoptotic cells by AMs, reduced weight loss of infected mice, and significantly improved lung volume and anatomical changes in SHS-exposed mice infected with influenza virus (**Figures 6** and **7**). Moreover, GM-CSF treatment significantly increased expression of amphiregulin, which is important in tissue homeostasis in the lung, promotes the growth of normal epithelial cells, and is an important molecule in host defense against influenza and bacterial coinfection.^{30–32} We and others have shown that lung innate lymphoid cells produce amphiregulin that is essential for restoring epithelial integrity and tissue repair, as well as for preventing lung damage after secondary bacterial infections.^{30–32}

We now provide evidence for the first time that GM-CSF in the lung of SHS-exposed mice increases the expression of amphiregulin. This provides an additional potential mechanism to protect lungs against severity of pneumonia in SHS-exposed mice.

Defective efferocytosis in smokers and patients with airway diseases has been shown in several studies.^{6–8,17} A variety of membrane-associated receptors on AMs are involved in recognition of apoptotic cells by AMs such as CD36, CD51/CD61, cystic fibrosis transmembrane conductance regulator, phosphatidylserine receptor, class A scavenger receptor, CD31, and lung collectin-associated receptors, CD91 and CD44.^{17,33–39} Reduced expression of recognition receptors on the cell surface is one of the mechanisms that is involved in ineffective efferocytosis. Hodge *et al.*⁶ have shown that AMs from smokers with chronic obstructive pulmonary disease and healthy smokers exhibited reduced CD31, CD91, CD44, and CD71, and enhanced Ki-67 compared with healthy never-smoker control subjects.⁶ Ritchens *et al.*⁷ have shown that exposure to cigarette smoke inhibits efferocytosis through oxidant-dependent activation of the RhoA-Rho kinase pathway.⁷ In the current report, we have shown that exposure to SHS reduces expression of CD16/32 receptor on AMs but does not affect phagolysosome fusion as indicated with low pH in the AMs (**Figures 4** and **2**, respectively). We also have shown that treating defective AMs with GM-CSF substantially increased expression of CD16/32 and the amount of ingested apoptotic MLE cells even beyond its expression levels in healthy

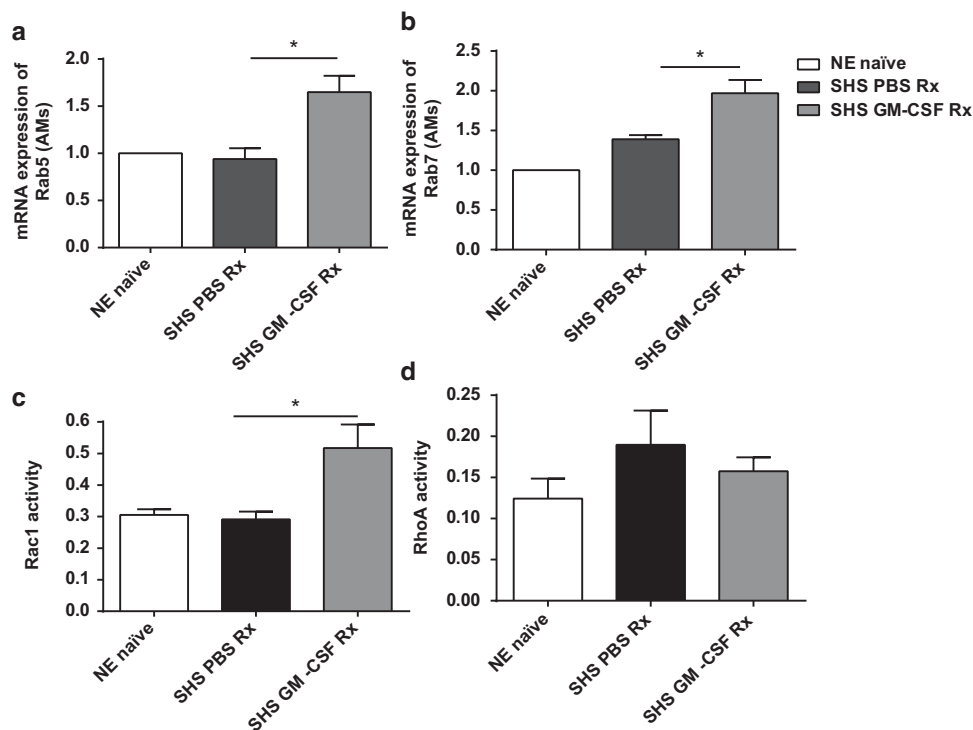


Figure 5 Effects of second-hand cigarette smoke (SHS) on expression of small GTPases in alveolar macrophages (AMs). AMs from naïve nonexposed control, wild-type mice (NE) and SHS-exposed mice treated with either $1 \times$ PBS (phosphate-buffered saline) or recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF) ($1 \mu\text{g g}^{-1}$ body weight for 7 days) were collected. Total RNA was extracted, cDNA was synthesized, and quantitative reverse transcription-PCR was carried out. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene and expression levels of (a) Rab5 and (b) Rab7 were normalized against the *GAPDH* mRNA level. (c) Rac1 and (d) RhoA activity of AMs from NE and GM-CSF- or PBS-treated SHS-exposed mice was measured using G-LISA RhoA activation assay protocol. A representative of two independent experiments with 3–5 mice per group are shown with error bars as s.e.m. * $P < 0.05$.

nonexposed AMs (Figures 2 and 4). Although there is no direct evidence for involvement of $\text{Fc}\gamma$ receptors in efferocytosis of apoptotic cells, autoantibodies from patients with systemic lupus erythematosus are able to opsonize apoptotic cells and inhibit their uptake by macrophages via an $\text{Fc}\gamma\text{R}$ -dependent mechanism.⁴⁰ Our results are also in agreement with previous report that CD16^+ monocytes are much more efficient in the phagocytosis of apoptotic PMN compared with CD16^- cells.⁴¹ In this study, we describe a novel role for $\text{Fc}\gamma$ receptors in efferocytosis function of SHS-exposed AMs and the mechanisms through which GM-CSF increases efferocytosis of SHS-exposed AMs. This is in line with the findings of Berclaz *et al.*⁴² that GM-CSF regulates AM $\text{Fc}\gamma\text{R}$ -mediated phagocytosis through PU.1 transcription factor. These results present novel therapeutic opportunities for the treatment of airways diseases due to exposure to SHS and could improve patient morbidity and mortality.

Treating with recombinant GM-CSF not only increased efferocytosis of apoptotic cells but it also increased the removal of apoptotic cells by AMs; as average of total CFSE + apoptotic MLE cells in PBS-treated, SHS-exposed mice was 50.1 ± 9.23 compared with 12.8 ± 3.36 in GM-CSF-treated group (Figure 1). Considering that $>90\%$ of apoptotic CFSE + MLE cells were internalized by GM-CSF-treated AMs (Figure 1, lower panels, $\text{F4/80} + \text{CFSE}^+$ cells), the result

signifies the superior capacity of GM-CSF-treated macrophages in efferocytosis of apoptotic MLE cells. GM-CSF also increased the number of activated AMs that express F4/80 and the expression of F4/80 on AMs. F4/80 molecule is a pan-macrophage marker of tissue macrophages, and an activation marker for resident macrophages.^{43,44} O'Reilly *et al.*⁴⁵ have shown that the Ets transcription factor PU.1 binds to proximal promoter region of F4/80 gene in macrophages and that this transcription–promotor association has a vital role in the expression of F4/80 in macrophages. Considering that GM-CSF regulates terminal differentiation and innate immune functions of AMs through PU.1,⁴⁶ we see a distinct population of F4/80^+ AMs in GM-CSF-treated mice (Figures 1a and 6a, lower rows).

We have shown defective efferocytosis in SHS-exposed mice *in vivo* and that treating SHS-exposed mice with intranasal recombinant GM-CSF significantly increases uptake and clearance of apoptotic cells from the lungs. In GM-CSF-treated naïve SHS-exposed mice, only $12.83 \pm 3.36\%$ of total BAL cells were CFSE + compared with $50.14 \pm 9.23\%$ in PBS-treated, SHS-exposed mice (Figure 1a). This may be due to different factors. First, AMs from GM-CSF-treated group may have increased capacity to digest and remove apoptotic cells from alveolar space and airways, as confirmed by increased uptake of apoptotic MLEs in Figure 2. Second,

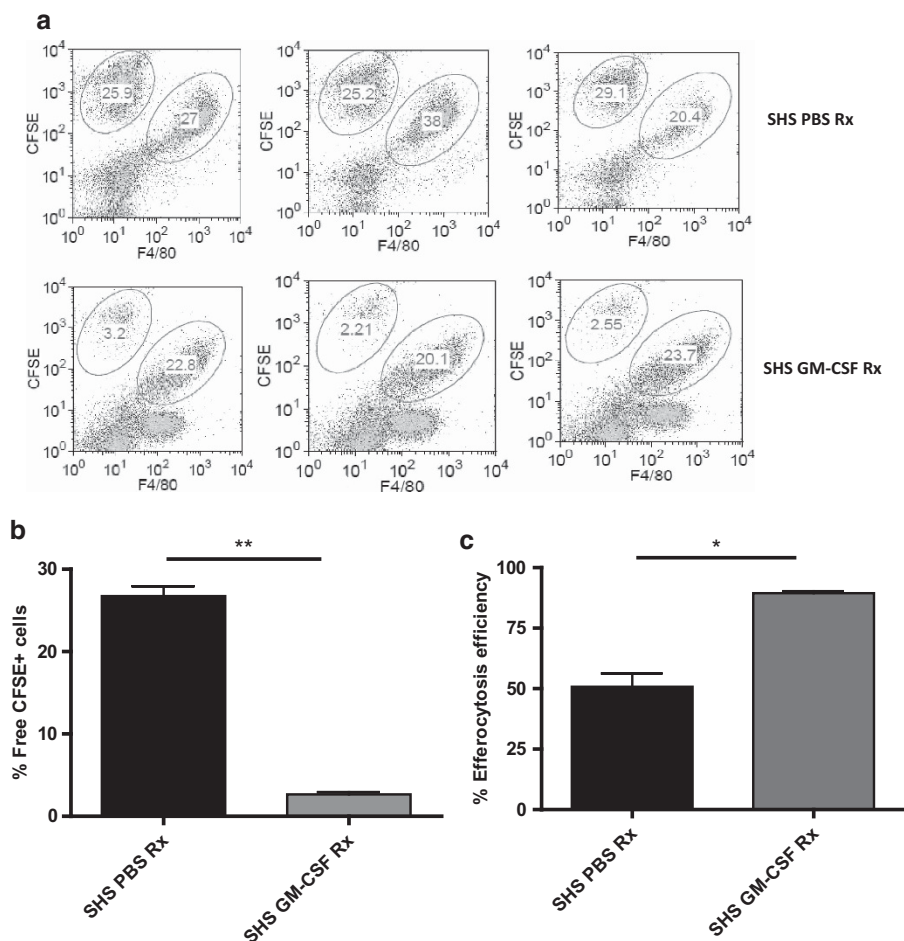


Figure 6 Granulocyte-macrophage colony-stimulating factor (GM-CSF) corrects impaired efferocytosis of alveolar macrophages (AMs) in influenza infection. Wild-type C57Bl/6 mice were exposed to second-hand cigarette smoke (SHS), and treated intranasally with recombinant GM-CSF ($1 \mu\text{g g}^{-1}$ body weight) or phosphate-buffered saline (PBS) for 7 days. On day 8, all mice were infected with PR8 influenza virus. Twenty-four hours later, all mice received carboxyfluorescein succinimidyl ester (CFSE)-labeled apoptotic mouse lung epithelial (MLE) cells intranasally and the apoptotic cell clearance was measured by flow cytometry, as described in the legend of **Figure 1**. (a) Flow cytometry analysis of each individual SHS-exposed mouse either treated with PBS or GM-CSF is shown. (b) Average percent of free CFSE+ apoptotic MLE cells from panel a is shown. (c) Depicted efferocytosis efficiency of AMs was calculated using the following formula: percent of efferocytosed CFSE+ apoptotic cells (CFSE+ and F4/80+ double-positive cells)/total percent of CFSE+ cells. A representative of three to four independent experiments are shown with 3–5 mice per group. Error bars show s.e.m. * $P < 0.002$ and ** $P < 0.0001$.

GM-CSF treatment resulted in increased numbers of (F4/80+) AMs. Third, the most likely case is combination of both increased digestion and removal capacity of AMs and increased number of F4/80 AMs.

Clearance of apoptotic cells depends on the digestion of engulfed cells that will result in limiting inflammation and will facilitate lung tissue repair. Rab5 and Rab7 are known to contribute in phagosome maturation and phagosome-lysosome fusion,⁴⁷ and hence we analyzed the expression of Rab5 and Rab7 in AMs. We did not see any significant changes in the expression of Rab5 and Rab7 between naïve SHS-exposed and their nonexposed control counterparts. However, GM-CSF treatment increased the expression of both Rab5 and Rab7 in AMs of SHS-exposed mice. GM-CSF treatment also increased the activity of Rac1, but not the activity of RhoA in AMs of SHS-exposed mice. The effect of GM-CSF

on GTPases fits well with increased efferocytosis as treating with GM-CSF increased the levels of Rac1, which is activated by phosphatidylserine and lipoprotein-related receptors and positively regulates efferocytosis but did not affect the RhoA that its active form negatively regulates efferocytosis (**Figure 5c,d**). Carey *et al.*⁴⁸ have shown that lack of GM-CSF and PU.1 reduces the expression and function of Rab5 and Rab7 and affects the intracellular tropism of adenovirus, but to the best of our knowledge, this is the first report on increasing digesting and degrading apoptotic cells by elevating Rab5 and Rab7 expression through treating AMs with recombinant GM-CSF. We do not know the mechanisms by which GM-CSF affects overexpression of Rab5 and Rab7 in AMs, but speculate that it can be through direct or indirect effects of GM-CSF. For instance, GM-CSF has a significant role in regulating alveolar levels of SP-A and

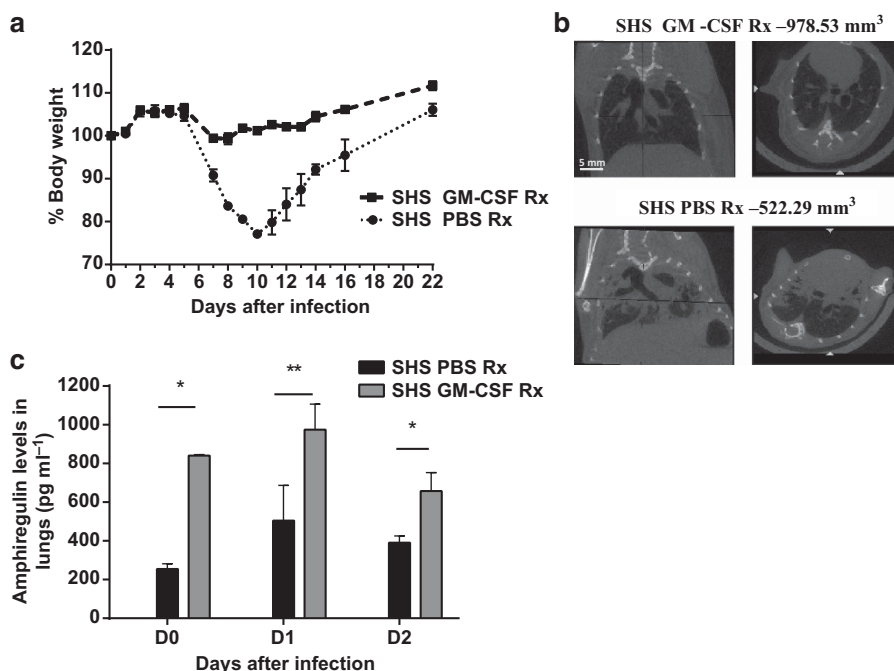


Figure 7 Effect of granulocyte–macrophage colony-stimulating factor (GM-CSF) treatment on second-hand cigarette smoke (SHS)-exposed influenza A virus (IAV)-infected mice. (a) SHS-exposed C57Bl/6 wild-type (WT) mice (6 per group) were treated with either phosphate-buffered saline (PBS) or GM-CSF, and then infected with a sublethal dose (0.2 LD₅₀ (lethal dose, 50%)) of influenza PR8 virus. Weight loss and clinical signs were recorded daily. (b) Anatomical changes in the lungs of (top panels) GM-CSF-treated and (lower panels) PBS-treated mice 21 days after infection with influenza virus. CT scanning was carried out, using a GE eXplore Locus micro-CT scan. (Left panels) Coronal and (right panels) transverse sections of the thorax are depicted. Lung volumes of pertinent mice were measured before scanning using FlexiVent. (c) Lung homogenates from SHS-exposed mice treated with either GM-CSF or PBS as in panel a were generated and levels of amphiregulin was measured using enzyme-linked immunosorbent assay (ELISA), prior, 1, and 2 days after infection with influenza virus. Depicted data are average of 5 mice per group and error bars show s.e.m. * $P < 0.05$ and ** $P < 0.01$.

interaction between AM and SP enhances phagocytosis and endocytosis, suggesting that an SP-A mediates modulation of small Rab GTPases.^{24,49}

In conclusion, we have shown that SHS exposure reduces efferocytosis of AMs and we provide the first evidence that delivery of GM-CSF to the lungs restores and enhances function of AMs in SHS-exposed animals, as well as confers extraordinary protection in disease conditions such as pneumonia. This novel approach can facilitate the development of novel strategies for boosting innate immunity and homeostasis of the lung. Hence, it can be used in different airway diseases and prevent exacerbations due to community-acquired pneumonias or pulmonary infections with unknown origins.

METHODS

Mice. Six- to eight-week-old wild-type C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). All the experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at Tyler.

Cigarette smoke exposure. Mice were exposed to second-hand cigarette smoke for 2 h twice per day for 6 weeks in a smoke exposure chamber (TE-10z; Teague Enterprises, Woodland, CA), which exposes animals to a combination of fresh air and a defined level of cigarette smoke from Kentucky 3R4F reference cigarettes (<http://www.ca.uky.edu/refcig/>). The exposure chamber was calibrated for total suspended particles. In our studies, we exposed mice to $50 \mu\text{g m}^{-3}$

total suspended particles. The levels of total suspended particles in night clubs and bars have been studied and ranged from 110 to $1,714 \mu\text{g m}^{-3}$ (<http://www.cdc.gov/niosh/nioshtic-2/00233383.html>).

GM-CSF treatment. During the last week of SHS exposure, mice were treated daily intranasally with $1 \mu\text{g g}^{-1}$ body weight mouse recombinant GM-CSF or PBS once daily for 1 week under light anesthesia induced by intraperitoneal injection of xylazine and ketamine.

IAV inoculation. Mice were inoculated with mouse adapted influenza virus A/Puerto Rico/8/34 (PR8) (H1N1) strain in all the experiments. The LD₅₀ of the virus strain was determined by limiting dilution. All infected mice were monitored for morbidity and mortality daily.

Efferocytosis assay. MLE cells were treated with staurosporine ($2 \mu\text{M}$) to induce apoptosis. Apoptotic MLE cells were labeled with CFSE according to the manufacturer's recommended protocol (Life Technologies, Carlsbad, CA). Under deep general anesthesia, CFSE-labeled apoptotic MLE cells were intranasally transferred to naïve SHS-exposed, GM-CSF-treated SHS-exposed, and nonexposed control, wild-type mice. In a pilot study, intranasal and intratracheal inoculation of MLE cells were compared with each other and showed similar results. BAL fluids were collected 3 h later and cells were stained with APC-F4/80, as a marker for AMs. Flow cytometry was used to quantify removal and phagocytosis of CFSE + apoptotic MLE cells.

Confocal microscopy. MLE cells were treated with $2 \mu\text{M}$ staurosporine (Fisher Scientific, Pittsburgh, PA) for 16 h to induce apoptosis. The apoptosis was confirmed by flow cytometry using Annexin V-PI staining. Apoptotic cells were washed two times with $1 \times$ PBS and labeled with Annexin V-Biotin (Miltenyi Biotec, San Diego, CA). The labeled apoptotic cells were incubated with pHrodo

Red Avidin (Life Technologies) to allow the coupling of pHrodo-avidin with biotinylated Annexin V on apoptotic cells. pHrodo-stained apoptotic cells were washed thoroughly and administered intranasally. BAL fluids were collected and AMs were stained with DAPI (4',6-diamidino-2-phenylindole) and CD11c. BAL cells were fixed and subjected to confocal microscopy for engulfed pHrodo-labeled apoptotic cells using Zeiss confocal system (LSM 510 META, Zeiss, Jena, Germany) equipped with an inverted microscope (Axio Observer Z1). The images were processed using LSM Zen 2009 software and compiled using Adobe photoshop software.

Micro-CT scanning. Micro-CT scanning (GE eXplore Locus, GE Healthcare, Pittsburgh, PA) was used to evaluate anatomic changes and disease severity in live animals. Mice were anesthetized, intubated, and connected to the ventilator (Flexivent from Scireq, Montreal, Canada). Fifteen minutes after induction of anesthesia intraperitoneally as delineated above, induced mice started to receive isoflurane (0.41 min⁻¹ of O₂ and 0.5% of isoflurane). To help anesthetized animal to maintain their body temperature, a heating pad was used. During CT scanning, the ventilator performed the following series of respiratory manipulations: normal breathing for 4–5 breaths, a deep inhale, and then a breath hold during which the CT images were taken. Deep anesthesia was maintained during CT scanning to prevent spontaneous breaths from interfering with image quality.

Cytokines and amphiregulin. BAL fluid supernatants were collected 3 h after inoculating apoptotic MLE cells and stored at –80 °C. Multiplex ELISA (enzyme-linked immunosorbent assay) (23 plex) was used to measure 23 cytokines in the BAL fluid supernatant (Bio-Rad, Hercules, CA). IL-12 (p70) was measured by using standard ELISA (BioLegend, San Diego, CA). Supernatants from lung homogenates prior (D0, uninfected), 1 (D1), and 2 days (D2) after influenza infection were collected and stored at –80 °C. Amphiregulin levels in lung homogenate supernatants were measured by ELISA (R&D Systems, Minneapolis, MN).

Detecting expression of CD16/32. BAL cells from naïve C57BL/6 nonexposed control mice and SHS-exposed mice treated with either PBS or recombinant GM-CSF were collected. BAL cells were stained with F4/80 and CD16/32 (eBioscience, San Diego, CA) and subjected to flow cytometry analyses. Results were analyzed using the FlowJo Software (Ashland, OR) and depicted as mean fluorescence intensity.

Quantification of Rab5 and Rab7. BAL cells were collected from naïve C57BL/6 nonexposed control mice, and SHS-exposed mice were treated with either PBS or recombinant GM-CSF. Total RNA was extracted from BAL cells using Trizol (Life Technologies). cDNA was synthesized using iScript reverse transcription supermix (Bio-Rad). iTaq universal SYBR Green Supermix was used for quantitative reverse transcription-PCR. *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) was used as a house keeping gene.

RhoA and Rac1 activity. BAL cells were collected from naïve C57BL/6 mice, including nonexposed control mice and SHS-exposed mice treated with either PBS or recombinant GM-CSF. BAL cell lysates were prepared and Rac1 and RhoA activity was measured using G-LISA RhoA activation assay protocol as described by the manufacturer (Cytoskeleton, Denver, CO).

Statistical analysis. A two-way analysis of variance, one-way analysis of variance, or unpaired Student's *t*-test was used for the analysis. A Bonferroni *post hoc* test was used to compare between groups after the two-way analysis of variance analysis. All measures of variance are presented as standard error of means.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

H.S. is the inventor of two pending patents filed by the Board of Regents, The University of Texas System, for use of GM-CSF to prevent influenza and its secondary bacterial pneumonia.

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REFERENCES

- Finklea, J.F., Sandifer, S.H. & Smith, D.D. Cigarette smoking and epidemic influenza. *Am. J. Epidemiol.* **90**, 390–399 (1969).
- Kark, J.D. & Lebiush, M. Smoking and epidemic influenza-like illness in female military recruits: a brief survey. *Am. J. Public Health* **71**, 530–532 (1981).
- Kark, J.D., Lebiush, M. & Rannon, L. Cigarette smoking as a risk factor for epidemic a(h1n1) influenza in young men. *N. Engl. J. Med.* **307**, 1042–1046 (1982).
- Feng, Y. *et al.* Exposure to cigarette smoke inhibits the pulmonary T-cell response to influenza virus and *Mycobacterium tuberculosis*. *Infect. Immun.* **79**, 229–237 (2011).
- Yageta, Y. *et al.* Role of Nrf2 in host defense against influenza virus in cigarette smoke-exposed mice. *J. Virol.* **85**, 4679–4690 (2011).
- Hodge, S., Hodge, G., Ahern, J., Jersmann, H., Holmes, M. & Reynolds, P.N. Smoking alters alveolar macrophage recognition and phagocytic ability: implications in chronic obstructive pulmonary disease. *Am. J. Respir. Cell Mol. Biol.* **37**, 748–755 (2007).
- Richens, T.R. *et al.* Cigarette smoke impairs clearance of apoptotic cells through oxidant-dependent activation of RhoA. *Am. J. Respir. Crit. Care Med.* **179**, 1011–1021 (2009).
- Hodge, S. *et al.* Cigarette smoke-induced changes to alveolar macrophage phenotype and function are improved by treatment with procysteine. *Am. J. Respir. Cell Mol. Biol.* **44**, 673–681 (2011).
- McDonald, P.P., Fadok, V.A., Bratton, D. & Henson, P.M. Transcriptional and translational regulation of inflammatory mediator production by endogenous TGF-beta in macrophages that have ingested apoptotic cells. *J. Immunol.* **163**, 6164–6172 (1999).
- Huynh, M.L., Fadok, V.A. & Henson, P.M. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. *J. Clin. Invest.* **109**, 41–50 (2002).
- Fadok, V.A., Bratton, D.L., Konowal, A., Freed, P.W., Westcott, J.Y. & Henson, P.M. Macrophages that have ingested apoptotic cells *in vitro* inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J. Clin. Invest.* **101**, 890–898 (1998).
- Golpon, H.A. *et al.* Life after corpse engulfment: phagocytosis of apoptotic cells leads to VEGF secretion and cell growth. *FASEB J.* **18**, 1716–1718 (2004).
- Odaka, C., Mizuochi, T., Yang, J. & Ding, A. Murine macrophages produce secretory leukocyte protease inhibitor during clearance of apoptotic cells: implications for resolution of the inflammatory response. *J. Immunol.* **171**, 1507–1514 (2003).
- Burridge, K. & Wennerberg, K. Rho and Rac take center stage. *Cell* **116**, 167–179 (2004).
- Tosello-Trampont, A.C., Nakada-Tsukui, K. & Ravichandran, K.S. Engulfment of apoptotic cells is negatively regulated by Rho-mediated signaling. *J. Biol. Chem.* **278**, 49911–49919 (2003).
- Leverrier, Y. & Ridley, A.J. Requirement for Rho GTPases and PI 3-kinases during apoptotic cell phagocytosis by macrophages. *Curr. Biol.* **11**, 195–199 (2001).
- Vandivier, R.W., Henson, P.M. & Douglas, I.S. Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. *Chest* **129**, 1673–1682 (2006).

18. Noda, N. *et al.* Cigarette smoke impairs phagocytosis of apoptotic neutrophils by alveolar macrophages via inhibition of the histone deacetylase/Rac/CD9 pathways. *Int. Immunol.* **25**, 643–650 (2013).
19. Hodge, S., Hodge, G., Scicchitano, R., Reynolds, P.N. & Holmes, M. Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells. *Immunol. Cell Biol.* **81**, 289–296 (2003).
20. Nouri-Shirazi, M., Tinajero, R. & Guinet, E. Nicotine alters the biological activities of developing mouse bone marrow-derived dendritic cells (DCs). *Immunol. Lett.* **109**, 155–164 (2007).
21. Inoue, M., Ishibashi, Y., Nogawa, H. & Yasue, T. Carbocysteine promotes phagocytosis of apoptotic cells by alveolar macrophages. *Eur. J. Pharmacol.* **677**, 173–179 (2012).
22. Kasaian, M.T. *et al.* An IL-4/IL-13 dual antagonist reduces lung inflammation, airway hyperresponsiveness, and IgE production in mice. *Am. J. Respir. Cell Mol. Biol.* **49**, 37–46 (2013).
23. Zerial, M. & McBride, H. Rab proteins as membrane organizers. *Nat. Rev. Mol. Cell Biol.* **2**, 107–117 (2001).
24. Sender, V., Moulakakis, C. & Stamme, C. Pulmonary surfactant protein A enhances endolysosomal trafficking in alveolar macrophages through regulation of Rab7. *J. Immunol.* **186**, 2397–2411 (2011).
25. Somsel, R.J. & Wandinger-Ness, A. Rab GTPases coordinate endocytosis. *J. Cell Sci.* **113** (Part 2), 183–192 (2000).
26. Sonnichsen, B., De, R.S., Nielsen, E., Rietdorf, J. & Zerial, M. Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *J. Cell Biol.* **149**, 901–914 (2000).
27. Rink, J., Ghigo, E., Kalaidzidis, Y. & Zerial, M. Rab conversion as a mechanism of progression from early to late endosomes. *Cell* **122**, 735–749 (2005).
28. Lakadamyali, M., Rust, M.J. & Zhuang, X. Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. *Cell* **124**, 997–1009 (2006).
29. Bucci, C., Thomsen, P., Nicoziani, P., McCarthy, J. & van, D.B. Rab7: a key to lysosome biogenesis. *Mol. Biol. Cell* **11**, 467–480 (2000).
30. Subramaniam, R. *et al.* Protecting against post-influenza bacterial pneumonia by increasing phagocyte recruitment and ROS production. *J. Infect. Dis.* **209**, 1827–1836 (2014).
31. Jamieson, A.M. *et al.* Role of tissue protection in lethal respiratory viral-bacterial coinfection. *Science* **340**, 1230–1234 (2013).
32. Monticelli, L.A. *et al.* Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat. Immunol.* **12**, 1045–1054 (2011).
33. Vandivier, R.W. *et al.* Dysfunctional cystic fibrosis transmembrane conductance regulator inhibits phagocytosis of apoptotic cells with proinflammatory consequences. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **297**, L677–L686 (2009).
34. Donnelly, L.E. & Barnes, P.J. Defective phagocytosis in airways disease. *Chest* **141**, 1055–1062 (2012).
35. Teder, P. *et al.* Resolution of lung inflammation by CD44. *Science* **296**, 155–158 (2002).
36. Gardai, S.J. *et al.* By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. *Cell* **115**, 13–23 (2003).
37. Brown, S., Heinisch, I., Ross, E., Shaw, K., Buckley, C.D. & Savill, J. Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment. *Nature* **418**, 200–203 (2002).
38. Savill, J., Hogg, N., Ren, Y. & Haslett, C. Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. *J. Clin. Invest.* **90**, 1513–1522 (1992).
39. Li, M.O., Sarkisian, M.R., Mehal, W.Z., Rakic, P. & Flavell, R.A. Phosphatidylserine receptor is required for clearance of apoptotic cells. *Science* **302**, 1560–1563 (2003).
40. Reefman, E., Horst, G., Nijk, M.T., Limburg, P.C., Kallenberg, C.G. & Bijl, M. Opsonization of late apoptotic cells by systemic lupus erythematosus autoantibodies inhibits their uptake via an Fcgamma receptor-dependent mechanism. *Arthritis Rheum.* **56**, 3399–3411 (2007).
41. Mikolajczyk, T.P. *et al.* Interaction of human peripheral blood monocytes with apoptotic polymorphonuclear cells. *Immunology* **128**, 103–113 (2009).
42. Berclaz, P.Y., Shibata, Y., Whitsett, J.A. & Trapnell, B.C. GM-CSF, via PU.1, regulates alveolar macrophage Fcgamma R-mediated phagocytosis and the IL-18/IFN-gamma-mediated molecular connection between innate and adaptive immunity in the lung. *Blood* **100**, 4193–4200 (2002).
43. Lin, H.H., Stacey, M., Stein-Streilein, J. & Gordon, S. F4/80: the macrophage-specific adhesion-GPCR and its role in immunoregulation. *Adv. Exp. Med. Biol.* **706**, 149–156 (2010).
44. Lin, H.H. *et al.* The macrophage F4/80 receptor is required for the induction of antigen-specific efferent regulatory T cells in peripheral tolerance. *J. Exp. Med.* **201**, 1615–1625 (2005).
45. O'Reilly, D. *et al.* Functional analysis of the murine Emr1 promoter identifies a novel purine-rich regulatory motif required for high-level gene expression in macrophages. *Genomics* **84**, 1030–1040 (2004).
46. Shibata, Y., Berclaz, P.Y., Chronoes, Z.C., Yoshida, M., Whitsett, J.A. & Trapnell, B.C. GM-CSF regulates alveolar macrophage differentiation and innate immunity in the lung through PU.1. *Immunity* **15**, 557–567 (2001).
47. Hochreiter-Hufford, A. & Ravichandran, K.S. Clearing the dead: apoptotic cell sensing, recognition, engulfment, and digestion 1. *Cold Spring Harb. Perspect. Biol.* **5**, a008748 (2013).
48. Carey, B., Staudt, M.K., Bonaminio, D., van der Loo, J.C. & Trapnell, B.C. PU.1 redirects adenovirus to lysosomes in alveolar macrophages, uncoupling internalization from infection. *J. Immunol.* **178**, 2440–2447 (2007).
49. Yoshida, M., Ikegami, M., Reed, J.A., Chronoes, Z.C. & Whitsett, J.A. GM-CSF regulates protein and lipid catabolism by alveolar macrophages. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **280**, L379–L386 (2001).