Agonistic induction of PPARγ reverses cigarette smoke–induced emphysema

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The development of emphysema in humans and mice exposed to cigarette smoke is promoted by activation of an adaptive immune response. Lung myeloid dendritic cells (mDCs) derived from cigarette smokers activate autoreactive Th1 and Th17 cells. mDC-dependent activation of T cell subsets requires expression of the *SPP1* gene, which encodes osteopontin (OPN), a pleiotropic cytokine implicated in autoimmune responses. The upstream molecular events that promote SPP1 expression and activate mDCs in response to smoke remain unknown. Here, we show that peroxisome proliferator–activated receptor γ (*PPARG/Pparg*) expression was downregulated in mDCs of smokers with emphysema and mice exposed to chronic smoke. Conditional knockout of PPARγ in APCs using *Cd11c-Cre Pparg*$^{flox/flox}$ mice led to spontaneous lung inflammation and emphysema that resembled the phenotype of smoke-exposed mice. The inflammatory phenotype of *Cd11c-Cre Pparg*$^{flox/flox}$ mice required OPN, suggesting an antiinflammatory mechanism in which PPARγ negatively regulates *Spp1* expression in the lung. A 2-month treatment with a PPARγ agonist reversed emphysema in WT mice despite continual smoke exposure. Furthermore, endogenous PPARγ agonists were reduced in the plasma of smokers with emphysema. These findings reveal a proinflammatory pathway, in which reduced PPARγ activity promotes emphysema, and suggest that targeting this pathway in smokers could prevent and reverse emphysema.

Introduction

Despite increased awareness of smoking hazards, the incidence of smoking-related lung diseases continues to rise worldwide. The immense economic and societal burden of smoking-related lung diseases, including emphysema, chronic bronchitis, and lung cancer, is compounded by the lack of effective treatment options (1, 2). Following acute exposure to smoke, lung macrophages increase expression of the matrix metalloproteinases (MMPs) MMP9 and MMP12, which inhibit endogenous antiproteinases (e.g., α1-antitrypsin), and degrade lung matrix molecules (e.g., elastin, collagen) that are essential for maintenance of lung integrity (3, 4). This acute, innate immune-activating effect of smoke inhalation alone could induce lung damage, but enigmatically, a subset of susceptible former smokers show rapid and sustained loss of lung function that becomes physiologically significant (5–7). The genetic and additional environmental factors other than smoke exposure that together induce severe, progressive lung disease remain largely unknown, although recurrent lung infections potentially hasten lung function decline (8–10).

Recent studies have, however, elucidated a role for acquired immunity in the development of emphysema in humans and mice, including the importance of Th1 and Th17 cells (11–16). We recently cloned elastin-specific T cells from the peripheral blood of smokers with emphysema and showed that this autoimmune phenomenon predicts clinically relevant outcomes (17). In an experimental model of emphysema, we further showed that lung myeloid DCs (mDCs) induce the differentiation of CD4 T cells into Th1 and Th17 cells, a process that requires expression of the osteopontin (OPN) gene *SPP1* (18). The increased prevalence of circulating antinuclear and antitissue antibodies in smokers with lung disease further points to the potential autoimmune nature of emphysema (19, 20). These observational and experimental findings underscore the dysregulated nature of immunity in emphysema, but the mechanisms that trigger these responses remain unclear.

A potential contributor to the immune dysregulation observed in emphysema is peroxisome proliferator–activated receptor γ (PPARγ). PPARγ is expressed in APCs such as mDCs and negatively regulates their function, altering antigen uptake, cell maturation, activation, migration, and cytokine production (21–23). Further, PPARγ agonist treatment of mDCs induces T cell anergy (24) and promotes Treg differentiation (25). Macrophages deficient in PPARγ contribute to spontaneous lung inflammation and increased Th1 polarization (26).

In this study, we show that lung mDCs from human smokers show reduced expression of PPARγ and demonstrate parallel findings from mouse lung APCs in an experimental model of emphysema. Further, specific deletion of PPARγ in CD11c$^+$ cells results in spontaneous lung parenchymal destruction analogous to smoke-induced emphysema. Treatment of mice with ciglitazone, a PPARγ agonist in a model of emphysema, or ablation of the *Spp1* gene in mice with CD11c-specific deletion of PPARγ reversed emphysema, suggesting that targeting the OPN/PPARγ axis even in established lung disease provides new therapeutic options.

Results

Reduced expression of PPARγ in pathologic lung mDCs in human emphysema. To identify the genetic factors that govern the activation

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of lung mDCs in smokers with emphysema, we performed a whole-genome-wide mRNA expression analysis using CD1a$^+$/MHC-II$^+$ lung mDCs from smokers with and without emphysema (GSE26296). This analysis revealed a distinct gene expression profile marked by upregulation of *MMP12*, *MMP9*, CD1 family members *CD1A*, *CD1B*, *CD1C*, and *CD1E*, and other DC-related genes such as *CD207*, *NDRG2*, *CLEC5A*, and *SPP1* in lung mDCs of smokers with emphysema (Figure 1A). Comparison of these data with published (23) and publicly available gene expression profiles (GSE8658) of human monocyte–derived DCs (MDDCs) treated with rosiglitazone, a PPARγ agonist, revealed an opposite pattern of gene expression. Specifically, expression of a large number of candidate genes that were significantly increased in lung mDCs in emphysema were significantly decreased in MDDCs in response to rosiglitazone (e.g., *MMP12*, *MMP9*, CD1 family members, *CD207*, *NDRG2*, *CLEC5A*, and *SPP1*) (ref. 23 and Figure 1A). Similarly, the PPARγ agonist induced genes (e.g., *FABP4*, *ABCG2*, *HRASLS3*, *OAS1*, and *C1QG*) in MDDCs that were significantly reduced in emphysematous lung mDCs (Figure 1B). Together, these findings suggest an important negative regulatory role for PPARγ in mDC activation and chronic lung inflammation.

To further determine a potential role for PPARγ in emphysema, we next examined the relative expression of PPARγ in the same microarray dataset (GSE26296). As expected, we found significantly decreased expression of *PPARG* (Figure 1C) in mDCs from emphysematous lung when compared with mDCs from controls (smokers without emphysema). The microarray findings were confirmed using lung mDCs isolated from a larger cohort of well-characterized smokers with emphysema, showing significantly reduced expression of the *PPARG* gene when compared with controls (Figure 1D). We further found a significant negative correlation between *PPARG* expression and disease severity as assessed by the reduced forced expiratory volume in 1 second (FEV1%), confirming the association between *PPARG* and lung disease (Figure 1D). WT mice exposed to 4 months of smoke showed a decrease in *Pparg* expression in lung alveolar macrophages and CD11b$^+$/CD11c^{hi} DCs when compared with that seen in air-exposed mice (Figure 1E). This finding was specific to lung APCs, because *Pparg* expression assessed in the whole lung tissue of mice exposed to air or smoke was comparable (Figure 1E).

PPARγ deficiency in APCs leads to spontaneous development of emphysema. These clinical and observational data suggest that emphysema is associated with decreased lung mDC–specific expression of PPARγ and that PPARγ could act as a negative regulator of lung inflammation in smokers. To test this, we examined whether PPARγ expression could alter the function of mDCs in mice. We have previously shown that in mice, activation of CD11c$^+$ lung APCs that include CD11c$^+$CD11b^{lo} alveolar macrophages and CD11c$^+$CD11b^{hi} lung mDCs plays a critical role in the development of emphysema (18). Using mice that express the Cre-recombinase gene under the CD11c promoter (*CD11c-Cre*), crossed with mice bearing a floxed PPARγ allele (*Pparg$^{flox/flox}$*), we developed transgenic mice with specific ablation of PPARγ in CD11c$^+$ APCs (hereinafter referred to as *CD11c-Cre Pparg$^{flox/flox}$* mice). Although mice with APC-specific PPARγ deficiency showed increased BAL cellularity as early as 2 months of age (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI70587DS1), we found normal lung histology with no lung developmental abnormalities (Supplemental Figure 2). However, in contrast to control (*Pparg$^{flox/flox}$*) mice, *Cd11c-Cre Pparg$^{flox/flox}$* mice spontaneously developed increased inflammation in the airways by 5 to 6 months of age (Figure 2A), including 8- to 10-fold more CD11c$^+$CD11b^{hi} mDCs in BAL fluid (Supplemental Figure 3). These findings were consistent with the enhanced secretion of chemokines and other proinflammatory molecules detected from lung APCs of these mice following LPS stimulation (Supplemental Figure 4).

Increased recruitment of pathogenic APCs to the lungs in the absence of APC-specific PPARγ was further associated with lung parenchymal destruction in 5-month-old *CD11c-Cre Pparg$^{flox/flox}$* mice (Figure 2B) that was similar to emphysematous changes detected in WT mice exposed to cigarette smoke over a 4-month period (18). Quantification of lung parenchyma changes using micro-CT imaging and unbiased lung morphometry (mean linear intercept; MLI) showed increased volume and decreased lung density, the hallmarks of emphysema, in *CD11c-Cre Pparg$^{flox/flox}$* mice when compared with that detected in control animals (Figure 2C). Together, these findings document the spontaneous onset of bronchoalveolar inflammation and emphysema in *CD11c-Cre Pparg$^{flox/flox}$* mice.

We next examined the expression of *Mmp12*, *Mmp9*, and *Spp1* in BAL fluid cells from *CD11c-Cre Pparg$^{flox/flox}$* mice, given the previously demonstrated importance of these molecules in smoke-induced human and experimental emphysema (18, 27). We found significantly elevated expression of these proinflammatory genes in the BAL fluid of 5- to 6-month-old *CD11c-Cre Pparg$^{flox/flox}$* mice when compared with that found in controls (Figure 2D). Intracellular staining of lung parenchymal CD3$^+$ T cells revealed that CD4 and γδ T cells accounted for the majority of IL-17A–expressing cells in *CD11c-Cre Pparg$^{flox/flox}$* mice, which again phenocopies the acquired T cell responses seen in WT mice chronically exposed to smoke (ref. 18, Supplemental Figure 5, and Figure 2, E and F). Collectively, these findings demonstrate that the selective loss of PPARγ in CD11c$^+$ APCs leads to increased accumulation of proinflammatory CD11c$^+$CD11b^{hi} mDCs in the lungs, enhanced expression of MMPs and *Spp1* in BAL fluid, recruitment and/or differentiation of IL-17A–secreting T cells to lung, and emphysema. These findings are indistinguishable from lung and BAL inflammation found in mice exposed to chronic smoke and in human emphysema and suggest that PPARγ functions as a negative regulator of pulmonary APC activation that may underlie lung inflammation.

Spp1 gene encoding OPN is downstream of PPARγ inhibitory action. We have previously shown that *Spp1$^{-/-}$* mice develop attenuated lung inflammation in response to cigarette smoke and are protected against emphysema (18). Here, we asked whether *Spp1* expression in *CD11c-Cre Pparg$^{flox/flox}$* mice promotes lung and BAL cell inflammation and contributes to the spontaneous emphysema phenotype seen in this genetic background. To address this, we crossed *Spp1$^{-/-}$* mice with *CD11c-Cre Pparg$^{flox/flox}$* mice to generate *CD11c-Cre Pparg$^{flox/flox}$ Spp1$^{-/-}$* mice. Examination of the lungs in 6- to 10-month-old *CD11c-Cre Pparg$^{flox/flox}$ Spp1$^{-/-}$* mice showed normal lung morphology and normal lung volume, confirmed by micro-CT imaging, and unbiased lung morphometry, determined by MLI measurements (Figure 3, A–C). We next examined the BAL fluid cellularity and inflammatory signature from the same groups of mice and found a significant decrease in cell numbers and in *Mmp12* gene expression, while we also detected an insignificant trend for reduced *Mmp9* gene expression (Figure 3, D and E, and data not shown). Intracellular cytokine (ICC) analysis of lung inflammatory cells further showed decreased numbers of Th17 cells in the lungs of *CD11c-Cre Pparg$^{flox/flox}$*

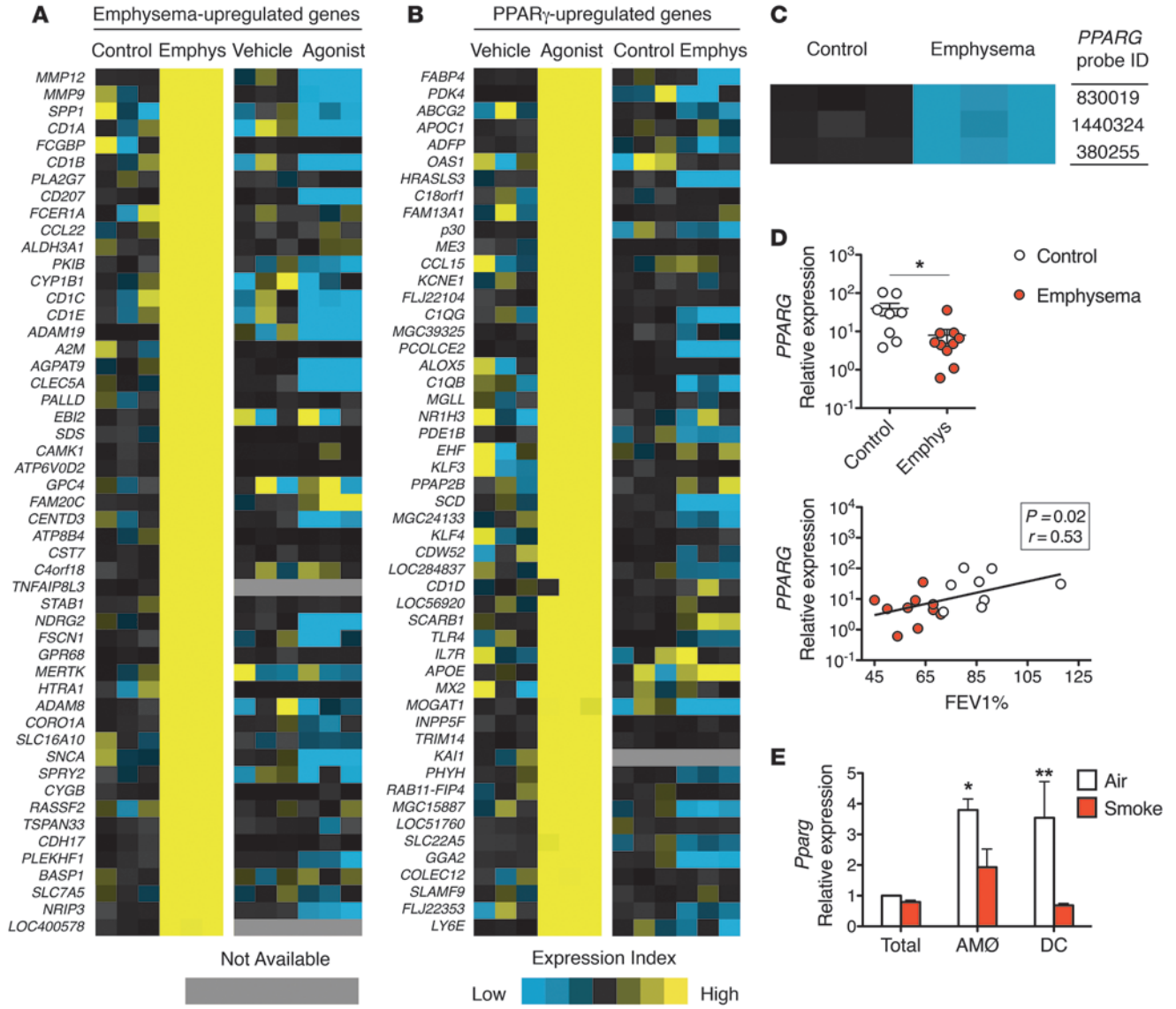

Figure 1

Decreased PPARγ expression in emphysematous lung mDCs. (**A** and **B**) Microarray comparison of genes upregulated in emphysema and following PPARγ agonism. (**A**) Heatmaps depicting gene expression in lung mDCs obtained from former smokers without (control; $n = 3$) and with emphysema (GSE26296) ($n = 3$). Right panel: Expression of the same genes in human MDDCs with and without PPARγ agonist treatment (GSE8658). $n = 3$ per group. (**B**) Left: Heatmap of the top 51 genes upregulated in PPARγ agonist–treated human MDDCs (GSE8658). Right: Heatmap depicting expression of the same genes in control and emphysematous lung DCs (GSE26296). $n = 3$ per group. Heatmaps scales: Bright blue to bright yellow: <–2.0, –1.59, –1.26, 1.0, 1.26, 1.59, >2.0. (**C**) Heatmap showing *PPARG* gene expression by microarray analysis (GSE26296). $P < 0.003$. (**D**) *PPARG* mRNA expression in control lung DCs ($n = 8$) and emphysematous lung DCs ($n = 10$) as determined by qPCR (normalized to 18S expression). $^*P < 0.05$ as determined by a Mann-Whitney *U* test. Lower panel: Correlation between *PPARG* expression and emphysema severity (FEV1%) as assessed by linear regression. (**E**) qPCR analysis of *Pparg* mRNA expression (normalized to 18S expression) in total lung cells, lung DCs, and alveolar macrophages (AMΦ) from air- ($n = 3$) and cigarette smoke–exposed mice ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, by 2-tailed Student's *t* test. Emphys, emphysema.

Spp1$^{-/-}$ mice compared with those in the lungs of *Spp1*-sufficient *CD11c-Cre Pparg*$^{flox/flox}$ mice (Figure 3, F and G). These findings, along with our data showing increased *Spp1* expression in *CD11c-Cre Pparg*$^{flox/flox}$ mice (Figure 2D), indicate the existence of a reciprocal *Spp1-Pparg* gene regulatory loop, whereby inhibition of PPARγ results in increased expression of OPN, thus providing a rationale for targeting this axis in emphysema.

PPARγ agonists inhibit pathogenic lung APCs. The data thus far indicated that PPARγ deficiency in APCs leads to expression of several proinflammatory genes and the development of spontaneous emphysema and, moreover, that cigarette smoke decreases PPARγ expression in the lungs. Therefore, we next asked whether exogenous PPARγ agonists could attenuate the proinflammatory activity of smoke-exposed human lung mDCs. CD1a/MHC-II$^+$ human

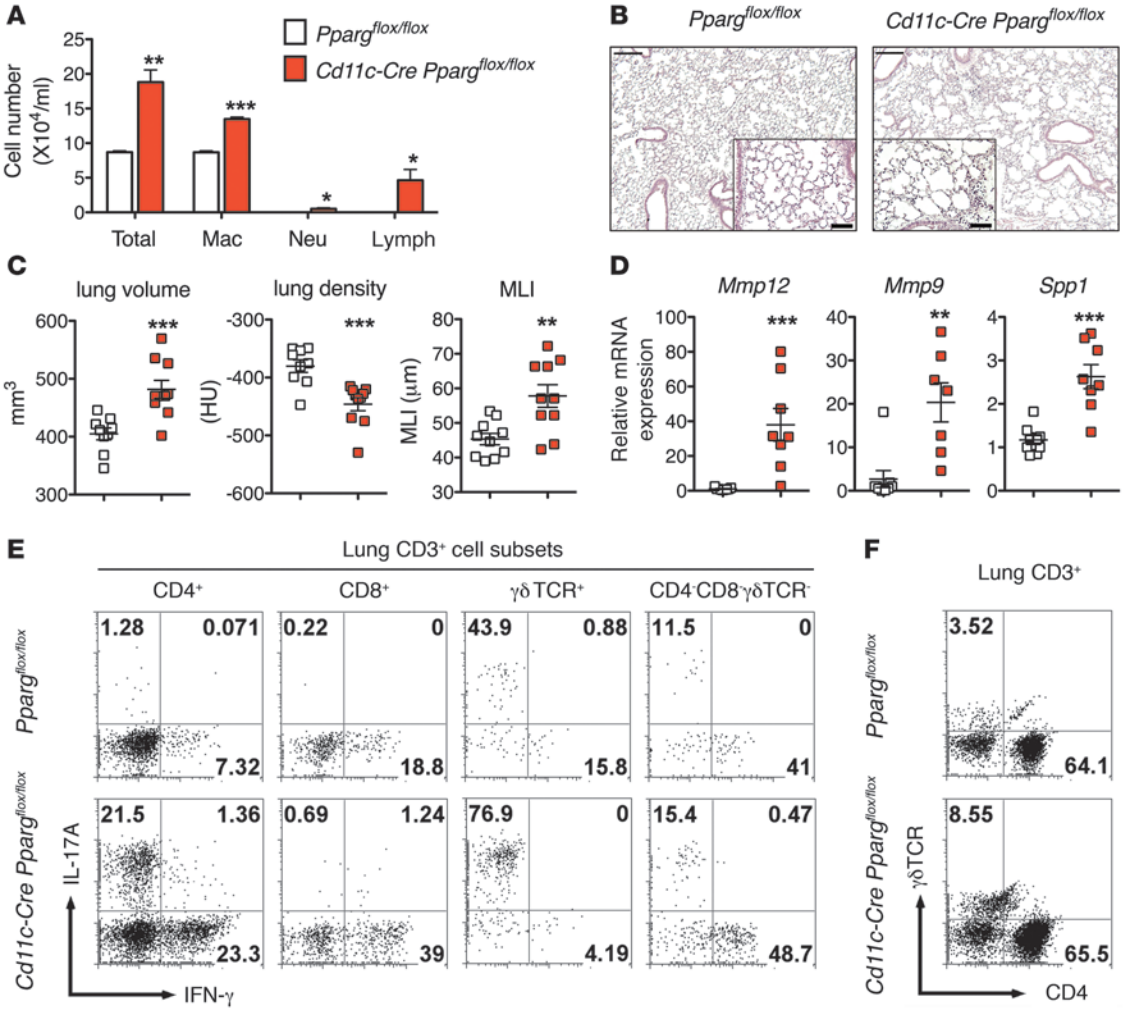

Figure 2

PPARγ deficiency in APCs results in spontaneous emphysema. (**A** and **B**) Increased lung parenchyma and BAL fluid inflammatory cells in *CD11c-Cre Pparg*$^{flox/flox}$ mice. (**A**) Analysis of BAL fluid from 5- to 6-month-old *Pparg*$^{flox/flox}$ and *CD11c-Cre Pparg*$^{flox/flox}$ mice (n = 5 per group) showing total cells, macrophages (Mac), lymphocytes (Lymph), and neutrophils (Neu). ***P < 0.001, **P < 0.01, *P < 0.05, as determined by a Student's t test. (**B**) H&E staining of formalin-fixed, 5-μm lung sections showing airway enlargement and increased inflammation in 5- to 6-month-old mice of the indicated genotypes. Data are representative of two independent studies (n = 5 per group). Scale bars: 400 μm (insets: 100 μm). (**C**–**F**) Spontaneous emphysema in mice with APC-specific PPARγ deficiency. (**C**) Micro-CT quantification of lung volume and density in the indicated groups of mice. MLI was measured in the same groups of mice using unbiased morphometry (n = 8–10). ***P < 0.001, **P < 0.01, as determined by a Mann-Whitney U test. (**D**) PCR-determined expression of *Mmp12*, *Mmp9*, and *Spp1* mRNA in total BAL fluid cells (n = 6–8 per group) (normalized to 18S expression). ***P < 0.001, **P < 0.01, by Mann-Whitney U test. (**E**) Representative ICC staining of IL-17A and IFN-γ from distinct CD3$^+$ T cell subsets. Numbers in each quadrant indicate the percentage of positive cells for the indicated cytokines. Data are representative of two independent studies (n = 5 in each group per study). (**F**) Flow cytometric analysis of γδ T cells and CD4$^+$ T cells from total lung CD3$^+$ lymphocytes, as in **E**.

lung mDCS were first examined for the expression of previously identified proinflammatory genes (GSE26296) (Figure 4A, upper panel) that were elevated when compared with controls. We then treated freshly isolated lung mDCs from smokers with emphysema using vehicle (0.1% DMSO) or ciglitazone, a specific agonist of PPARγ, and found decreased expression of *MMP12*, *MMP9*, *SPP1*, and *CD1A*, while *CXCL10* and *ALDH1A1* expression was increased (Figure 4A, lower panel). Consistently, we found that ciglitazone treatment also reversed gene expression in the lung APCs of mice exposed to smoke (Supplemental Figure 6). We next asked whether treatment with ciglitazone could block Th1/Th17 cell differentiation in vitro. Ciglitazone-treated human lung mDCs showed a decreased ability to induce human Th1 and Th17 cell differentiation in vitro when compared with vehicle, as determined by ICC assay or direct protein measurement (Figure 4, B and C). To examine whether a decrease in Th1/Th17 cell development is specifically induced in response to agonistic induction of ciglitazone, we used the same experimental condition to neutralize the drug with GW9662, a potent and irreversible inhibitor of PPARγ. We found that neutralization of ciglitazone with GW9662 restored the ability of APCs to induce Th1/Th17 cell differentiation (Figure 4, B and C). Similarly, we found that ciglitazone treatment reduced

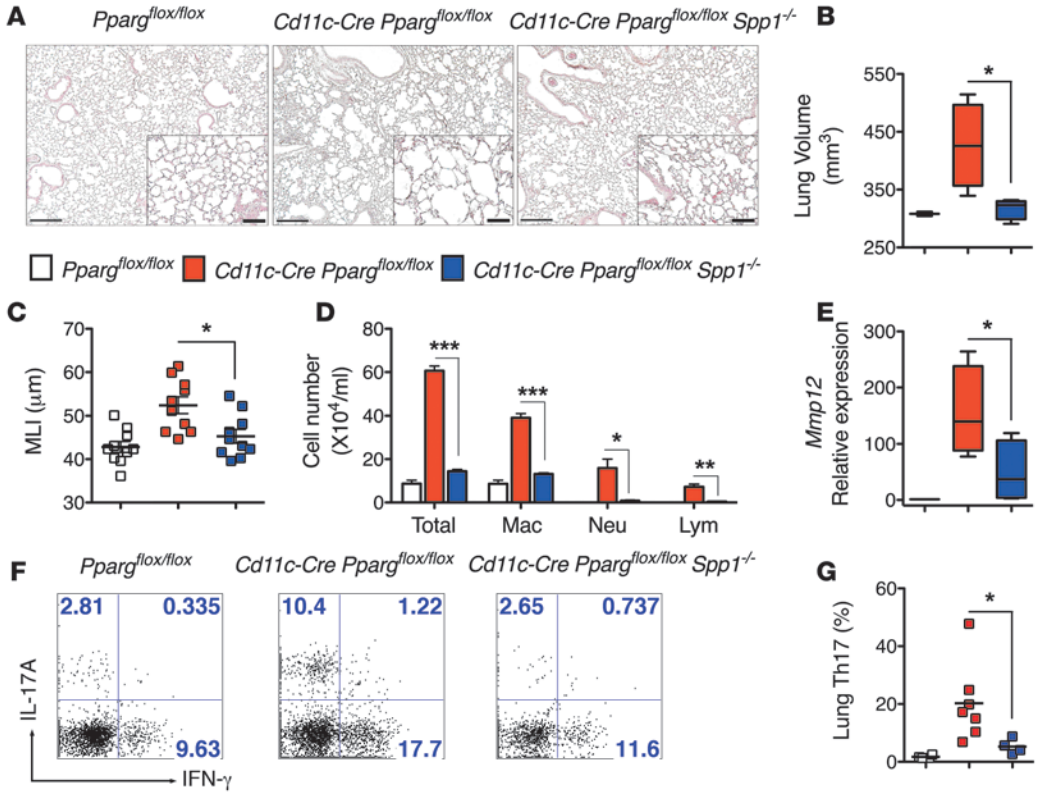

Figure 3

Spp1 deficiency impedes spontaneous emphysema development in the absence of PPARγ. (**A**) H&E staining of lung sections showing airway enlargement and inflammation. Data are representative of three independent studies ($n = 4$ in each group). Scale bars: 400 μm (insets: 100 μm). (**B**) Micro-CT quantification of lung volume in 6-month-old *Pparg*$^{flox/flox}$ (WT), *CD11c-Cre Pparg*$^{flox/flox}$, and *CD11c-Cre Pparg*$^{flox/flox}$ *Spp1*$^{-/-}$ mice ($n = 4$ per group). *$P < 0.02$, by 1-way ANOVA and Bonferroni's multiple comparison test. (**C**) MLI measurements in the same group of mice were obtained using unbiased morphometry. ***$P < 0.001$, *$P < 0.01$, by 1-way ANOVA and Bonferroni's multiple comparison test. (**D**) BAL fluid analysis from the same mice ($n = 4$ in each group). **$P < 0.002$, *$P < 0.01$, as determined by 1-way ANOVA and Bonferroni's multiple comparison test. (**E**) *Mmp12* mRNA expression in total BAL fluid cells ($n = 4$ in each group) (normalized to 18S expression). *$P < 0.04$, by 1-way ANOVA and Bonferroni's multiple comparison test. (**F**) Representative ICC staining of IL-17A and IFN-γ from $CD3^+/CD4^+$ T cells ($n = 4$ in each group). Numbers in each quadrant indicate the percentage of positive cells for the indicated cytokines. (**G**) Cumulative data from two independent studies showing the relative abundance of Th17 cells in the lungs, as determined in **E** ($n = 4$ in the *Pparg*$^{flox/flox}$ and *CD11c-Cre Pparg*$^{flox/flox}$ *Spp1*$^{-/-}$ group and $n = 7$ in the *CD11c-Cre Pparg*$^{flox/flox}$ group). *$P < 0.01$, by 1-way ANOVA and Bonferroni's multiple comparison test.

the ability of smoke-exposed mouse lung APCs to induce IL-17 and IFN-γ expression in T cells; inhibition of ciglitazone with GW9662 also restored T cell cytokine production (Figure 4D).

PPARγ agonist reverses cigarette smoke–induced emphysema. Our data thus far indicated that the overall effect of enhanced endogenous PPARγ expression is to inhibit Th17-dependent lung inflammation in the setting of smoke exposure. We next explored whether in vivo treatment with ciglitazone in a preclinical model of emphysema could prevent or reverse the disease phenotype. Mice were exposed to 3 months of cigarette smoke (Supplemental Figure 7), followed by i.n. treatment with ciglitazone twice a week for 2 months, while continuing to be exposed to smoke. After a total of 5 months of smoke exposure, mice treated with ciglitazone showed reduced emphysema, as quantified by micro-CT, which detected a reduction in lung volume (Figure 5A). Lung histology and unbiased quantitative morphometry using MLI also confirmed less lung destruction in the PPARγ agonist–treated mice when compared with that seen in vehicle-treated control animals (Figure 5, B and C). Consistently, we found that ciglitazone-treated mice showed reduced BAL inflammatory cells (Figure 5D) and significantly attenuated expression of emphysema-related genes, in particular, *Mmp12*, *Mmp9*, and *Spp1* (Figure 5E). The decrease in proinflammatory genes was further accompanied by a reduction in Th17 cells in the lungs of mice treated with ciglitazone (Figure 5F). Serial micro-CT quantification of lung volume over time also showed a significant decrease in lung destruction and reversal of emphysema over the 2-month treatment period (Figure 5G). Reversal of emphysema was not secondary to fibrosis, because we did not detect increased collagen deposition in the lungs of ciglitazone-treated mice (Supplemental Figure 8). Mice treated with smoke and ciglitazone also did not show any evidence for decreased lung volume, further ruling out fibrotic changes (Figure 5A).

Reduced endogenous PPARγ agonist in the plasma of smokers with emphysema. The potent antiinflammatory function of ciglitazone in vivo and the reduction in *PPARG* expression in lung mDCs of smokers prompted us to examine whether endogenous lipid–based PPARγ agonists (28) might also be altered in smokers with emphysema. In particular, commercially available human AB

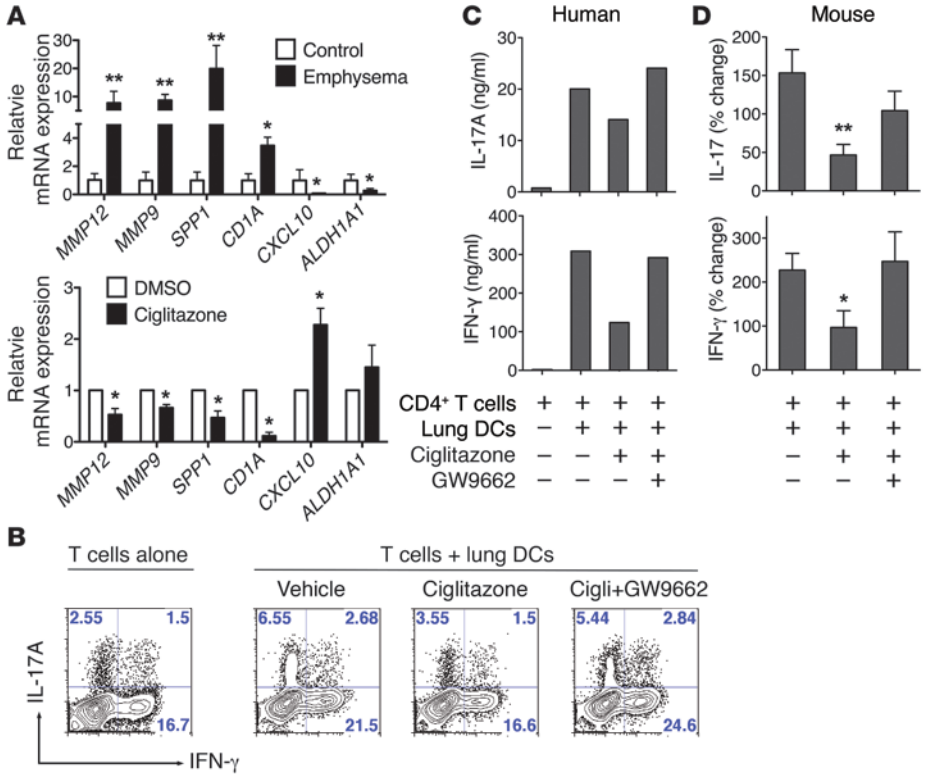

Figure 4

PPARγ agonist treatment in vitro inhibits pathogenic lung DCs. Human lung DCs from control ($n = 8$) and emphysematous lungs ($n = 8$) were isolated, and (**A**) selected emphysema-related genes were measured by qPCR, normalized to 18S expression (left panel). mRNA expression of the same selected genes after a 24-hour treatment with ciglitazone (10 μM) or vehicle control (0.1% DMSO) using human emphysematous lung mDCs ($n = 6$–8) (right panel). Data represent the mean ± SEM. $^{**}P < 0.001$, $^{*}P < 0.01$, by Mann-Whitney *U* test. (**B**) Representative flow cytometry of ICC staining of CD4 T cells from PBMCs (5×10^5) alone or cocultured with mDCs (5×10^4) isolated from emphysematous lungs that were pretreated for 24 hours with vehicle (0.1% DMSO), ciglitazone (10 μM), or neutralized with the PPARγ antagonist GW9662 (10 μM). CD4 T cells were stimulated with 1 μg/ml anti-CD3 for 3 days, and cells were removed for ICC staining with IL-17A and IFN-γ (**B**). Numbers in each quadrant indicate the percentage of positive cells for the indicated cytokines. Supernatants were collected for cytokine measurement (**C**). Data are representative of three independent studies. (**D**) Lung APCs ($CD11c^+CD11b^{hi}$) were isolated from mice that were exposed to 4 months of smoke and treated as in **B**. Lung APCs were then cultured for 3 days with congenic naive mouse spleen CD4 T cells plus 1 μg/ml anti-CD3. Culture supernatant was measured for IL-17A and IFN-γ production using a Milliplex kit. Data represent the percentage of cytokine change compared with T cells alone and are pooled from three independent studies performed in duplicate. $^{**}P = 0.002$, $^{*}P = 0.03$, by 1-way ANOVA and Bonferroni's multiple comparison test.

serum has been shown to inhibit CD1a expression in MDDCs, a process that is mediated through PPARγ activation (23). Therefore, we next examined the relative functional activity of PPARγ agonists in the plasma of smokers with and without emphysema. Using a well-established in vitro model system, we incubated MDDCs with human plasma, and inhibition of CD1a expression was used as an indication of endogenous PPARγ agonist activity. As expected, ciglitazone inhibited CD1a expression, and its effect was rescued in the presence of the PPARγ antagonist GW9662 (Figure 6A). Likewise, plasma from smokers without emphysema (controls) inhibited CD1a expression, and was similarly reversed by GW9662 treatment (Figure 6A). In contrast, compared with controls, plasma from smokers with emphysema showed an attenuated ability to decrease CD1a expression (Figure 6, B and C). The difference in relative inhibition of CD1a expression was independent of gender, because significant differences between the two groups persisted when we examined male subjects (Supplemental Figure 9). Collectively, these findings suggest that reduced PPARγ agonists in the plasma of smokers with emphysema in part explain the reduced PPARγ activity that potentially contributes to enhanced chronic lung inflammatory changes and emphysema.

Finally, similar to its reduced relative expression, we found a significant negative correlation between plasma-mediated CD1a inhibition and emphysema severity, as determined by the percentage of low attenuation area (%LAA) (ref. 29 and Figure 6D), suggesting a relationship between endogenous PPARγ agonist activity and the pathogenesis of smoking-related lung disease.

Discussion

Macrophages and mDCs comprise some of the most abundant inflammatory cells in the lungs of smokers and in response to IL-17A can express proteolytic enzymes (e.g., MMP12 and MMP9) that are strongly associated with emphysema (3, 11, 30). They are also potent inducers of acquired immune responses that can propagate autoimmunity, even in the absence of ongoing smoke exposure (18, 31). The upstream molecular events that promote activation of lung APCs and expression of proinflammatory OPN in smokers remain unknown, but knowledge of these molecular pathways is critical to understanding the pathogenesis of smoking-related disease and to developing effective preventative and therapeutic strategies. We show here that smokers with emphysema have both reduced expression PPARγ in lung mDCs and lower endogenous PPARγ agonist activity. Furthermore, treatment of WT mice with the PPARγ agonist ciglitazone reversed BAL fluid inflammatory cells, decreased Th17 in the lung parenchyma, and decreased emphysema despite continual exposure to smoke. These findings both clarify the pathogenesis of smoking-related emphysema and suggest novel means to treat, and potentially reverse, lung disease.

A major goal of these studies was to determine the mechanism by which PPARγ mediates its antiinflammatory activity in the lungs. Lung APCs tightly control the cellular expression of OPN that is required for proinflammatory changes in response to smoke. Furthermore, we show that APC-specific deletion of PPARγ results in spontaneous Th17 lung parenchyma inflammation and emphysema in mice, a process that required expression of *Spp1*. In vitro, we

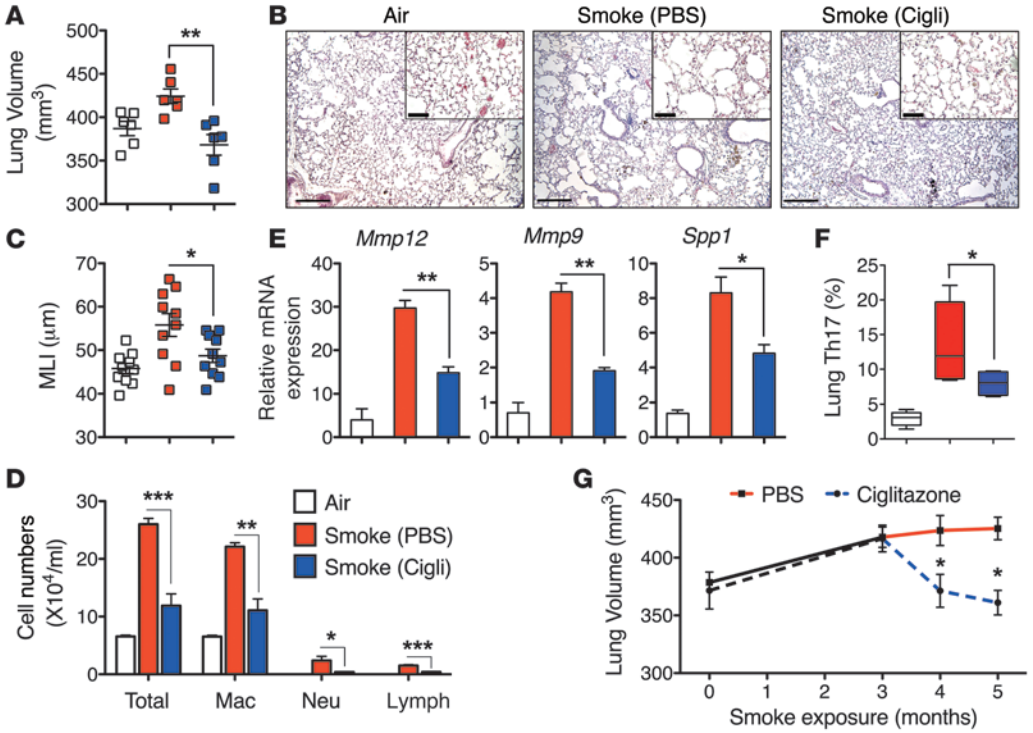

Figure 5

PPARγ agonist i.n. treatment attenuates cigarette smoke–induced emphysema. C57/BL6 mice were exposed to 3 months of smoke and treated with i.n. ciglitazone (Cigli), 3.3 μg/50 μl twice weekly or with PBS. (**A**) Micro-CT quantification of lung volume and lung density; data are from two pooled experiments. $^{**}P < 0.01$, by 1-way ANOVA and Bonferroni's multiple comparison test. (**B**) H&E staining of lung showing airway enlargement. Data are representative of three independent studies ($n = 3$ in each group), with similar results. Scale bars: 400 μm (insets: 100 μm). (**C**) MLI was determined in the same group of mice using unbiased morphometry. $^{**}P < 0.01$, $^{*}P < 0.05$, by 1-way ANOVA and Bonferroni's multiple comparison test. (**D**) Total number of cells in BAL fluid from the indicated treatment groups ($n = 3$ in each group). Data are representative of three independent studies ($n = 3$ in each group), with similar results. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, by 1-way ANOVA and Bonferroni's multiple comparison test. (**E**) *Mmp12*, *Mmp9*, and *Spp1* mRNA expression (normalized to 18S expression) in total BAL fluid cells ($n = 3$ in each group). $^{**}P < 0.01$, by 1-way ANOVA and Bonferroni's multiple comparison test. (**F**) Relative abundance (%) of Th17 cells in the lung parenchyma from the same group of mice detected using intracellular IL-17. (**G**) Serial repeated measurements of lung volume in a separate group of WT mice ($n = 3$ in each group) treated as described in **A**, but undergoing four micro-CT measurements over 5 months. $^{*}P < 0.05$, by 1-way ANOVA and Bonferroni's multiple comparison test. $^{*}P < 0.05$, by Mann-Whitney *U* test.

show that PPARγ agonists inhibit activated human mDCs and lung APCs from mice exposed to smoke, rendering both cells incapable of inducing Th1 and Th17 cell differentiation. Thus, the beneficial, antiinflammatory function of PPARγ appears to be mediated primarily through the regulation of OPN expression in APCs.

Our report documents, to the best of our knowledge, the first use of an antiinflammatory agent (e.g., ciglitazone) that can reverse smoke-induced emphysema despite active smoke exposure.

Deficiency in inducible NO synthase (NOS2) has been shown to result in attenuation of emphysema, and administration of an NOS2 inhibitor after 8 months of smoke exposure reversed lung damage within 3 months (32). However, reversal of emphysema in these mice was only seen after 3 months of smoking cessation, suggesting that rather than specifically inducing disease reversal, inhibition of the NO pathway promotes a faster recovery from smoke-induced injury (32). Similarly, inhibition of myeloperoxidase, a neutrophil and macrophage product, stopped the progression of emphysema and small airway remodeling in guinea pigs exposed to smoke long term (33). Therefore, these findings suggest potential interactions between PPARγ and different innate immune mediators that deserve further study.

Although we have shown that early-stage experimental emphysema can be reversed, it is not clear that end-stage smoking-related emphysema is also reversible. Beyond its activity in APCs as shown here, PPARγ expressed in epithelia has been shown to play a critical role in lung development (34) and maturation (35). Human lungs possess c-Kit–positive stem cells that have self-renewing properties and that are capable of regenerating normal lung architecture over a relatively short (2-week) period in mice (36). An intriguing possibility, therefore, is that PPARγ agonists enhance the recruitment or function of lung-specific stem cells, with the potential to regenerate damaged lung tissue at any clinical stage. Combined with the ability of PPARγ to directly inhibit Th17 cell differentiation (37), PPARγ likely controls diverse inflammatory and developmental pathways that are relevant to lung health in the context of smoke exposure.

Should emphysema prove to be poorly reversible in later stages, efforts to diagnose the disease at earlier stages will be of paramount importance. Currently, no screening strategies exist that can identify smokers with the potential to develop end-stage disease, although we have recently shown that the determination of elastin-specific IL-6 and IFN-γ responses from peripheral blood

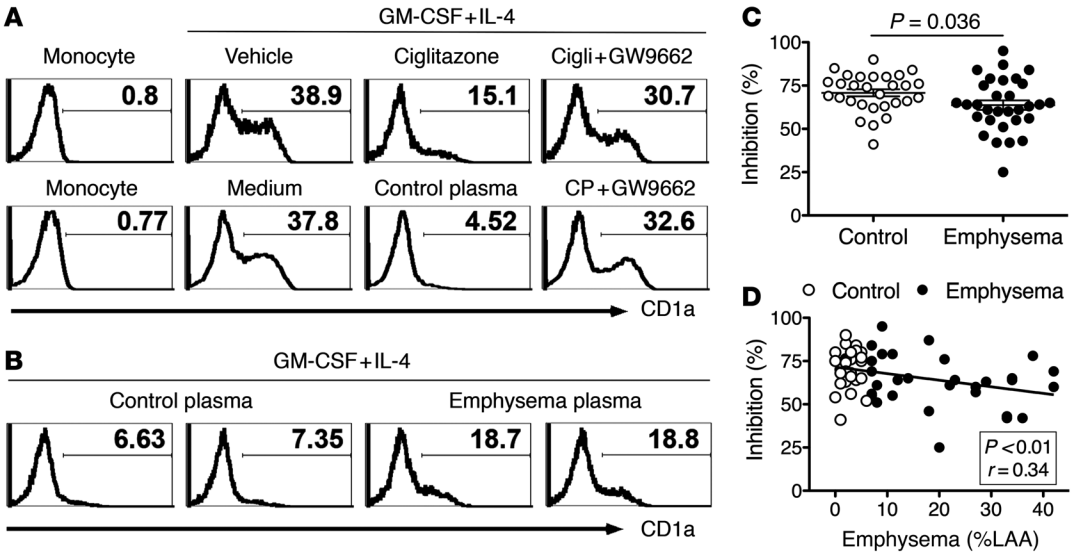

Figure 6

Reduced activation of PPARγ in plasma from emphysema patients. Human monocytes were isolated from PBMCs and cultured for 3 days with human GM-CSF and human IL-4 in complete medium containing 10% FBS to induce DC differentiation. (**A**) Histogram showing the expression of CD1a on MDDCs, as detected by flow cytometry. Cells were treated with vehicle (0.1% DMSO), 10 μM ciglitazone, or 10% control (smokers with no emphysema) plasma, with or without 10 μM GW9662. Numbers in the right upper corner represent the percentage of relative expression of CD1a. CP, control plasma. (**B**) Cell culture was supplemented with 10% human plasma from control or from smokers with emphysema. Data are representative of at least three different experiments with more than eight samples in each group. (**C**) Cumulative data of CD1a expression inhibition induced by human plasma obtained from ever-smokers participating in the LES-COPD study conducted at Baylor College of Medicine, in which emphysema was quantified using the %LAA in a high-resolution chest CT, as previously described in detail (17, 29) (control plasma, $n = 29$; emphysema plasma, $n = 31$). Percentage of CD1a inhibition was calculated using the formula: Inhibition (%) = (V – P) / V, where V represents the percentage of CD1a in the vehicle-treated group, and P represents the percentage of CD1a in the plasma-treated group. *P* determined using the Mann-Whitney *U* test. (**D**) Correlation between CD1a inhibition induced by human plasma and disease severity (percentage of emphysema) in smokers (control plasma, $n = 29$; emphysema plasma, $n = 31$). Statistics were derived by linear regression.

T cells has this potential (17). Because endogenous plasma PPARγ agonist activity correlated with disease activity, our current findings suggest an alternative diagnostic strategy based on detecting serum PPARγ agonist activity. Further studies are warranted to fully understand the predictive value of this activity in emphysema and other smoking-related disorders.

Our genome-wide analysis of activated lung mDCs in smokers with emphysema revealed a reversal of the gene signature pattern in human MDDCs treated with a PPARγ agonist. These critical counter-regulatory changes in the signature of human lung DCs suggested pathways that promote activation of acquired immunity unleashed under conditions in which relative deficiency of PPARγ exists. In support of this concept, PPARγ deficiency in macrophages has been shown to increase lung BAL fluid cellularity and enhance Th1 responses (26). Whether older mice with macrophage-specific deletion of PPARγ can develop emphysema has not been reported, but we have previously shown that in response to smoke, activated mDCs are enriched in the lung and play a much stronger proinflammatory role when compared with that of macrophages in inducing pathologic Th1 and Th17 cells in humans and mice (11, 18). Nonetheless, PPARγ deficiency in macrophages may further contribute to the proinflammatory state that contributes to emphysema.

Ciglitazone belongs to the thiazolidinedione family of molecules that have recently been approved for the oral management of diabetes, although off-target effects of these compounds are of significant concern (38, 39). High-dose (700 μg/week) oral PPARγ agonist treatment has also been used with success in a preclinical model of allergic inflammation (40, 41). In contrast, by adopting an i.n. delivery protocol, we demonstrated highly efficient inhibition of BAL fluid cellularity and lung inflammation using 100-fold less drug (7 μg/week i.n.). These observations suggest that targeted delivery of PPARγ agonists to the airway maximizes efficacy, while at the same time minimizing side effects.

Smokers are at increased risk of developing autoimmune diseases other than emphysema, suggesting that a common pathogenic mechanism that inhibits self-tolerance underlies diverse autoimmune syndromes (42–44). Smokers with lung disease are also at much higher risk of developing vascular inflammation and atherosclerosis (43). Intriguingly, a genetic variant of *PPARG* has been associated with metabolic and cardiovascular endpoints, including atherosclerosis (45), while PPARγ agonists have been shown to reduce vascular inflammation in humans (46, 47). Although it is unknown whether genetic variants of PPARγ influence the expression of emphysema, a recent case-control study showed that a slow-activity variant of PPARγ was associated with increased COPD susceptibility in a homogeneous population (48). Similarly, reduced circulating PPARγ in smokers has been linked to the development of other autoimmune diseases such as MS (49, 50), psoriasis (51), and RA (52). Type 2 diabetes mellitus is also increasingly being recognized as an inflammatory disorder of macrophages (53). Thus, PPARγ has been linked in diverse ways to

many inflammatory syndromes encompassing some of the most lethal and chronic of human disorders. Whether due to PPARγ polymorphisms or deficiency in circulating agonists, our findings suggest that PPARγ agonists represent a broadly applicable and effective antiinflammatory strategy for these conditions.

In summary, we have identified PPARγ as an important negative regulator of smoke-induced emphysema. Moreover, we have shown that circulating natural ligands of PPARγ are deficient in smokers with emphysema, implying the existence of a systemic, proinflammatory environment that promotes emphysema and potentially many other chronic, smoking-related afflictions. Among many potential proinflammatory genes regulated by PPARγ, *SPP1* appears to be particularly critical to the generation of pathological Th17 responses that lead to MMP overexpression and the emphysematous state. These findings provide a robust foundation for further exploring diagnostic and therapeutic avenues in smoking-related diseases and the genetic basis of these afflictions.

Methods

Human study subjects. A total of 70 nonatopic current or former smokers were serially entered into the study (Supplemental Table 1); all smokers had a significant (>15 pack-year) history of smoking and had quit smoking for an average of 15 ± 24 years (mean ± SD) and 4 ± 9 years (mean ± SD) in the control and emphysema groups, respectively (Supplemental Table 1). Chronic obstructive pulmonary disease (COPD) was diagnosed according to the criteria recommended by the NIH/WHO workshop summary (54), and emphysema was diagnosed by chest CT scan. Smoking 1 pack of cigarettes per day each year is defined as 1 "pack-year." Subjects were recruited from the chest or surgical clinics at Methodist and Michael E. DeBakey Houston VAMC hospitals. The patients had no history of allergy or asthma, had not received oral or systemic corticosteroids, and were free of acute symptoms suggestive of upper or lower respiratory tract infection during the previous 6 weeks.

Human lung tissue and PBMCs. Human lung tissue was obtained from individuals undergoing lung resection for medical treatment of lung cancer. Emphysema was defined based on the presence of enlarged alveolar space detected by chest CT in former and current (ever) smokers by an unbiased radiologist. mDCs were isolated from lung tissue of controls defined as former smokers without emphysema and emphysema subjects as previously described (11). Alternatively, PBMCs and plasma samples were collected from ever-smokers participating in the Longitudinal Exacerbation Study of COPD (LES-COPD) conducted at Baylor College of Medicine (17, 29). Emphysema was quantified in this cohort using the %LAA observed in high-resolution chest CT scans as previously detailed (17, 29).

Mice. WT, *Spp1*$^{-/-}$, and *Pparg*$^{flox/flox}$ mice (both on a C57BL/6J background) were purchased from The Jackson Laboratory. *CD11c-Cre* transgenic mice (on a C57BL/6 background) were provided by Jonathon Levitt (Baylor College of Medicine). All mice were bred at the transgenic animal facility at Baylor College of Medicine.

Cigarette smoke exposure. Mice were exposed to cigarette smoke as we previously reported (18). Six- to 8-week-old mice were exposed to active smoke from commercial cigarettes (Marlboro 100's). Exposure to 4 cigarettes (approximately 4 to 5 minutes/cigarette) per day, 5 days a week was carried out by intermittently forcing air (4 liters/minute) through the burning cigarette. Intermittent cycles were designed to mimic puffing cycles of actual human smokers and to prevent CO_2-induced asphyxiation. Puffing cycles consisted of 5 seconds of active cigarette smoke followed by 25 seconds of forced air by a timer-controlled 2-way valve (Humphrey). Mice were given 10 minutes of rest between each cycle of cigarette smoke exposure. In total, mice were given 4 cigarettes each day (1 hour), 5 days a week for the indicated number of months (3 and 5 months).

Quantification of an experimental model of emphysema: micro-CT and lung morphometry. The quantification of mouse emphysema was done as we previously described (18). Briefly, the severity of emphysema in mouse lungs was determined based on original CT methods developed for humans (55), with modifications for micro-CT imaging in mice (56). Mice were anesthetized with etomidate (30 mg/kg) and placed in an animal CT scanner (Gamma Medica), and completed images of the chest were obtained by the Animal Phenotyping Core at Baylor College of Medicine. Amira 3.1.1 software was used to process the images and quantification of emphysema in 3D.

The MLI measurements of mouse lung tissue were calculated as previously described (57). Briefly, the lungs were inflated with 1% PFA using 20 cm of water pressure, processed, and embedded in paraffin. Sections (5-μm) were cut and stained with H&E. Ten randomly selected fields from the left lobe of the lung (excluding large airways and vessels) were examined using an unbiased observer (57). ImageJ software (NIH) was used to place parallel lines (40 μm apart) on serial lung sections, and MLI was calculated by multiplying the length and the number of lines per field, divided by the number of intercepts.

Analysis of an experimental model of emphysema. The collection of BAL fluid and lung tissue was done as previously described (58). Briefly, mice were anesthetized with etomidate, and BAL fluid was collected by instilling and withdrawing 0.8 ml of sterile PBS twice through the tracheal cannula. Total and differential cell counts in the BAL fluid were determined using a standard hemocytometer and HEMA3 staining (Biochemical Sciences Inc.) of 200 ml of BAL fluid was performed for cytospin slide preparation. In some experiments, mouse lungs were dissected to prepare single-cell suspensions; alternatively, lungs were fixed with instillation of 4% PFA solution via a tracheal cannula at 25 cm H_2O pressure followed by paraffin embedding and were then sectioned for histopathological studies. H&E staining was performed as previously described (58).

Human immune cell isolation from lung and PBMCs. Human lung single-cell suspensions were prepared as described (30). Briefly, fresh lung tissue was cut into 0.1-cm pieces in Petri dishes and treated with 2 mg/ml of collagenase D (Roche Pharmaceuticals) in HBSS for 30 minutes at 37°C. Single cells were extracted by pressing digested lung tissue through a 40-μm Falcon cell strainer (BD), followed by rbc lysis (ACK lysis buffer) (Sigma-Aldrich). Lung cells were then sorted on a FACSAria (BD Biosciences) using antibodies (CD19, CD20, CD56, CD3) to eliminate lineage-positive cells, and CD11c$^+$/CD1a$^+$ (mDCs) cells were sorted with antibodies against their respective markers (all antibodies were purchased from BD Biosciences). This procedure yielded cell populations that were over 95% pure. Alternatively, single-cell suspensions were labeled with paramagnetic bead–conjugated anti-CD1a (autoMACS; Miltenyi Biotec) to select mDCs. PBMCs were isolated by Histopaque (Sigma-Aldrich) gradient centrifugation. After centrifugation, upper layers were collected as plasma. Human CD4 T cells were selected from peripheral blood using autoMACS.

Mouse immune cell isolation from lung and spleen. Mouse lung or spleen single-cell suspensions were prepared by mincing whole organs through a 40-μm Falcon cell strainer, followed by rbc lysis (ACK lysis buffer) for 3 minutes. To isolate lung APCs, rbc-free whole lung cells were labeled with paramagnetic bead–conjugated anti-CD11c (Miltenyi Biotec) and isolated using autoMACS. To isolate DCs and macrophages, rbc-free whole lung cells were sorted on a FACSAria (BD Biosciences) using antibodies (B220, CD3) to eliminate positive cells, and CD11c$^+$/CD11b^{hi} (mDCs) and CD11c$^+$/CD11b^{lo} (lung macrophages) were sorted with antibodies against their respective markers (all antibodies were purchased from BD Biosciences). Mouse CD4 T cells were selected from peripheral splenocytes using autoMACS.

mRNA isolation and quantitative PCR. Cell pellets were treated with TRIzol (Invitrogen), and mRNA was extracted with chloroform (Sigma-Aldrich), precipitated in isopropanol (Sigma-Aldrich), and washed in 70% alcohol (Sigma-Aldrich). The mRNA concentration was measured using NanoDrop 2000 (Thermo Scientific). Quantitative PCR (qPCR) was performed by one-step, real-time RT-PCR to determine relative gene expression using the ABI PerkinElmer Prism 7500 Sequence Detection System (Applied Biosystems). The following probes, all purchased from Applied Biosystems, were used: MMP9 (Mm00600164_g1); MMP12 (Mm00500554_m1); SPP1 (Mm00436767_m1); PPARG (Mm00440940_m1, Mm00440945_m1); FABP4 (Mm00445878_m1); PPARG (Hs01115512_m1, Hs01115513_m1); MMP12 (Hs00899662_m1); MMP9 (Hs00957562_m1); SPP1 (Hs00960942_m1); CD1a (Hs00381754_g1); CXCL10 (Hs01124251_g1); and ALDH1A1 (Hs00946916_m1).

PPARγ agonist treatment. The PPARγ agonist ciglitazone (230950-5MG) and antagonist GW9662 (370700-5MG) were purchased from Calbiochem. For in vitro cell culture conditions, the PPARγ agonist and antagonist were dissolved in 0.1% DMSO and were then added to the cell culture media at a final concentration of 10 μM. For in vivo experiments, the frequency and dose of PPARγ agonist treatment were modified based on a previously published method (41). Briefly, ciglitazone was resolved in ethanol as a stock solution of 500 μg/ml. Mice were then treated i.n. with ciglitazone (diluted with PBS at a final concentration of 66.67 μg/ml) solution at a final concentration of approximately 3.5 μg/mouse twice per week.

In vitro T cell coculture and cytokine measurements. CD4 T cells isolated from PBMCs were cultured for 3 days in vitro with autologous lung APCs (10:1 ratio) in the presence of soluble anti-human CD3 (1 μg/ml; BD) or anti-mouse CD3 (1 μg/ml; BD) in humans. Similarly, mouse CD4 T cells isolated from spleen were cultured for 3 days in vitro with congenic lung APCs (10:1 ratio) in the presence of soluble anti-mouse CD3 (1 μg/ml; BD). A Milliplex kit (Millipore) was used to measure concentrations of a selected group of cytokines (IL-17, IFN-γ), according to the manufacturer's instructions. Alternatively, cells were stimulated for 3 hours with 10 ng/ml PMA (Sigma-Aldrich) and 200 ng/ml ionomycin supplemented with 10 ng/ml monensin (Sigma-Aldrich) and were then stained for surface markers Pacific Blue anti-human CD3 (BD) or Pacific Blue anti-mouse CD3 (BD). The cells were then fixed with 1% PFA, permeabilized with 0.5% saponin, and stained with Alexa Fluor 700 anti-human IFN-γ (BD) and PE anti-human IL-17A (eBioscience) or with APC anti-mouse IFN-γ (BD) and PE anti-mouse IL-17A (eBioscience) for flow cytometric analysis of ICC production.

Flow cytometry and antibodies. Flow cytometry was performed with a BD LSR II (BD Biosciences), and data were analyzed with FlowJo software (Tree Star Inc.). The following mouse-specific antibodies were used and were purchased from BD Pharmingen: Pacific Blue-CD3 (500A2); APC-Cy7-CD8 (53-6.7); PE-IL-17A (TC11-18H10); APC-IFN-γ (XMG1.2); PE-Cy5-CD4 (PM4-5); and APC-Cy7-GR1 (RB6-8C5). FITC-γδTCR (eBioGL3), eFluro450-B220 (RA3-6B2), PE-CD11b (M1/70), and APC-CD11c (N418) were used and were purchased from eBioscience. The following human-specific antibodies were used: APC-CD19 (SJ25C1); APC-CD3 (SK7); Pacific Blue-CD3 (UCHT1); APC-IFN-γ (B27); APC-CD1a (HI149); FITC-CD1a (HI149); PE-CD11c (B-ly6); and FITC-CD14 (M5E2) (all purchased from BD Pharmingen); and PE-IL-17A (eBio64DEC17) (purchased from eBioscience).

Statistics. For the comparison of cytokine production and gene expression from air- and smoke-exposed mice, we used a Student's *t* test or 1-way ANOVA and Bonferroni's multiple comparison test.

For the comparison of human lung DC gene expression, a nonparametric Mann-Whitney *U* test was used. For the comparison of CT quantification of air- and smoke-exposed mice, 1-way ANOVA and Bonferroni's multiple comparison tests were used. The correlation between gene expression and FEV1%-based emphysema quantification was determined by linear regression. All statistical analyses were performed with GraphPad Prism (GraphPad Software). A Student's *t* test was performed using 2-tailed parameters, and a *P* value of less than 0.05 was considered significant. All data shown are the mean ± SEM.

Study approval. Studies were approved by the IRB of Baylor College of Medicine, and written informed consent was obtained from all patients. All experimental protocols used in this study were approved by the IACUC of Baylor College of Medicine and followed National Research Council guidelines for the care and use of laboratory animals.

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