Chronic Cigarette Smoke Exposure Generates Pathogenic T Cells Capable of Driving COPD-like Disease in Rag2$^{-/-}$ Mice

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Rationale: **Pathogenic T cells drive, or sustain, a number of inflammatory diseases. Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disease associated with the accumulation of activated T cells. We previously demonstrated that chronic cigarette smoke (CS) exposure causes oligoclonal expansion of lung CD4$^+$ T cells and CD8$^+$ T cells in a mouse model of COPD, thus implicating these cells in disease pathogenesis.**

Objectives: **To determine whether T cells are pathogenic in a CS-induced mouse model of COPD.**

Methods: **We transferred lung CD3$^+$ T cells from filtered air (FA)- and CS-exposed mice into *Rag2*$^{-/-}$ recipients. Endpoints associated with the COPD phenotype were then measured.**

Measurements and Main Results: **Here, we demonstrate that chronic CS exposure generates pathogenic T cells. Transfer of CD3$^+$ T cells from the lungs of CS-exposed mice into *Rag2*$^{-/-}$ recipients led to substantial pulmonary changes pathognomonic of COPD. These changes included monocyte/macrophage and neutrophil accumulation, increased expression of cytokines and chemokines, activation of proteases, apoptosis of alveolar epithelial cells, matrix degradation, and airspace enlargement reminiscent of emphysema.**

Conclusions: **These data formally demonstrate, for the first time, that chronic CS exposure leads to the generation of pathogenic T cells capable of inducing COPD-like disease in *Rag2*$^{-/-}$ mice. This report provides novel insights into COPD pathogenesis.**

Keywords: mouse model; inflammation; lung; emphysema

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The role of T cells in chronic obstructive pulmonary disease (COPD) is ill-defined, but it has been speculated that T cells become pathogenic. Whether chronic cigarette smoke exposure leads to T-cell–mediated, lung-specific pathology is unknown.

What This Study Adds to the Field

This study demonstrates that chronic cigarette smoke exposure leads to the generation of pathogenic T cells capable of driving COPD-like disease in *Rag2*$^{-/-}$ mice.

Chronic obstructive pulmonary disease (COPD) is used to describe two distinct pathological conditions of the lung, chronic bronchitis and emphysema. The pathological features result from substantial inflammation, mucus metaplasia, fibrotic remodeling of the airways, and alveolar destruction that collectively act to impair lung function (1). Long-term exposure to cigarette smoke (CS) is the predominant risk factor for COPD development. However, in addition to COPD, cigarette smoking causes a broad range of lung injury with diffuse histopathologic, radiographic, and physiologic abnormalities (2, 3). As cigarette usage spreads to emerging markets, COPD prevalence and related mortality are predicted to increase substantially worldwide (4). There are few available therapies, and treatments are mainly supportive. The chronic inflammation that characterizes this disease is perhaps the best known correlate of disease severity (1). Earlier studies focused on the roles of macrophages and neutrophils in disease development. Additionally, our previous reports provided novel insights into roles for natural killer (NK) cells (5) and T cells (6, 7) in the development of COPD.

The likelihood that pathogenic T cells influence the progression of COPD is supported by reports that the number of T cells in the lung correlates with the extent of emphysema (8, 9). Patients with COPD exhibit increased numbers of both CD4$^+$ and CD8$^+$ T cells in their lungs and airways (10, 11). The number of pulmonary CD8$^+$ T cells correlates with disease severity (10, 11). Experimentally, mouse models of COPD display significant increases in pulmonary T cells, and *CD8*$^{-/-}$ mice, but not *CD4*$^{-/-}$ mice, are afforded protection from many of the hallmark features of COPD (6, 12). It is likely that T-cell–mediated processes are antigen driven based on reports that oligoclonal CD4$^+$ and CD8$^+$ T cells have been detected in the lungs of patients with COPD (13, 14). Furthermore, we have demonstrated that after chronic CS exposure, T-cell oligoclonal expansions also occur in a mouse model of COPD (7). This finding strongly implicates antigen-specific T cells as mediators of disease pathogenesis. Although the antigen specificity or function of T cells in COPD remains wholly uninvestigated, it is probable that oligoclonal expansions reflect recognition of self-antigens.

In light of the evidence presented above, Cosio and colleagues have proposed that COPD has an autoimmune component (15). This hypothesis is supported by the recent identification of elastin-reactive T cells and antibodies, as well as autoantibodies specific for epithelial cells, in patients with COPD (16, 17). In agreement with the concept that COPD may have an autoimmune component, it has been observed that patients with severe disease who quit smoking exhibit sustained inflammation and lung function decline (18, 19). Similarly, in COPD mouse models,

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lymphocytic and neutrophilic inflammation was sustained for up to 17 weeks after cessation of smoking (20).

The exact role T cells play in the development and progression of COPD is poorly defined. Furthermore, whether T cells are sufficient for recapitulating any of the disease phenotypes is completely unknown. Here, we transferred T cells from mice chronically exposed to CS to *Rag2*$^{-/-}$ mice, and found that the recipients developed many of the phenotypic characteristics of COPD. Thus, in this report, we provide the first direct evidence of that chronic CS exposure generates pathogenic T cells capable of driving COPD-like disease in *Rag2*$^{-/-}$ mice.

METHODS

Mice

BALB/c wild-type (WT) (female, aged 8–12 wk) and BALB/c *Rag2*$^{-/-}$ (female, 8–12 wk) mice used in these studies were obtained from Taconic Farms and bred in University of Cincinnati facilities. In all instances, mice were killed with an intraperitoneal injection of sodium pentobarbital followed by exsanguination. All mice were housed in accordance with institutional guidelines, and all experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Cincinnati Medical Center.

Cigarette Smoke Exposure

Mice were exposed to either filtered air or the smoke generated from 3R4F Kentucky Reference Cigarettes (University of Kentucky). Cigarette smoke exposures were performed using a TE-10z smoking machine connected to an exposure chamber (Teague Enterprises, Woodland, CA). Mice were acclimated to smoke exposure for 1 week before full smoke exposure. Mice were exposed whole body in a chamber maintained at a concentration of 150 ± 15 mg/m^3 total suspended particulates for 4 hours per day, 5 days per week. Concentration of CO at this concentration of total suspended particulates was 400 ± 30 ppm. Exposure details are further described in the RESULTS and DISCUSSION and depicted in Figure 1.

Isolation, Purification, and Transfer of Mouse Lung T Cells

After the initial 6-month exposure (Figure 1), pooled (n = 18–20 per group per experiment) mouse lung leukocytes from either FA- or CS-exposed mice were isolated in a manner previously described (7). Lung leukocytes were plated for 90 minutes in complete RPMI in a humidified cell culture chamber kept at 37°C and 5% CO_2 to enrich for nonadherent cells. Nonadherent lung leukocytes were washed and resuspended with 1× phosphate-buffered saline (PBS)/0.5% bovine serum albumin followed by incubation with functional grade anti-mouse FcγIII/II (CD16/CD32; eBioscience, San Diego, CA). Cells were subsequently stained with hamster anti-mouse APC-CD3 (clone 145–2C11; eBioscience). CD3$^+$ cells were purified by fluorescence-activated cell sorting at the Cincinnati Children's Hospital Research Flow Cytometry Core using a FACSaria II. Purified CD3$^+$ cells were more than 99.7% pure (Figure 1). CD3$^+$ T cells from either FA- or CS-exposed mice were washed twice, resuspended in 1× Hanks' balanced salt solution (HBSS), and 5 × 10^5 cells per mouse were injected intraperitoneally into BALB/c *Rag2*$^{-/-}$ mice (n = 16 per recipient group per experiment).

Bronchoalveolar Lavage

Lungs were lavaged two times with 1 ml of 1× HBSS. The first bronchoalveolar lavage (BAL) return was centrifuged at 300 × *g* for 10 minutes, and the supernatant was removed and stored at −80°C until assayed. The remaining cell pellet was combined with the second BAL return and centrifuged at 300 × *g* for 10 minutes. The cell pellet was resuspended in 1 ml 1× HBSS containing 2% fetal bovine serum. Total cell counts were determined with a hemocytometer. Differential leukocyte counts (>300 cells) were determined on Hemacolor-stained (EM Science, Gibbstown, NJ) cytospin slides (Cytospin3; Shandon Scientific Ltd, Waltham, MA).

Measurement of Cytokines

IL-17 in the BAL samples was measured by ELISA using a mouse IL-17 DuoSet (R&D Systems, Minneapolis, MN). All other analytes in the BAL samples were measured using Milliplex Multiplex kits according to manufacturer's protocol (Millipore, Billerica, MA). In a multiscreen filter plate, samples were incubated with antibody-coated beads overnight at 4°C while shaking. Plates were then washed two times on a vacuum apparatus, and secondary antibody was added and incubated at room temperature for 1 hour while shaking. Streptavidin-RPE was added and incubated for 30 minutes at room temperature with shaking. Plates were then washed two more times, sheath fluid was added, shaken for 5 minutes, and then read using Luminex technology on the Bio-Plex (Bio-Rad, Hercules, CA). Concentrations were calculated from standard curves using recombinant proteins and expressed in pg/ml. The Cytokine and Mediator Measurement Core at the Cincinnati Children's Hospital Medical Center in the laboratory run by Dr. Marsha Wills-Karp conducted the Multiplex assay.

Hyaluronan Assay

Hyaluronan fragments were determined in the BAL samples by ELISA using a hyaluronan DuoSet (R&D Systems).

Matrix Metalloproteinase Activity

Matrix metalloproteinase (MMP) activity in the BAL was determined using a fluorometric SensoLyte 520 Generic MMP Activity Kit (Anaspec, Freemont, CA). This kit uses a 5-FAM and QXL520-labeled FRET peptide substrate. Cleavage of the FRET peptide by MMPs can be monitored by fluorescence. BAL samples were mixed 1:1 with substrate solution and incubated for 60 minutes at room temperature

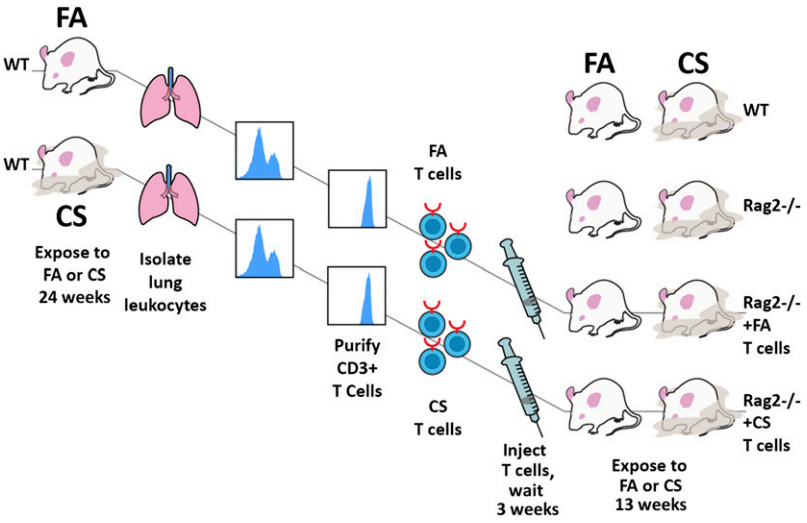

Figure 1. Experimental design. BALB/c wild-type (WT) mice were exposed to either filtered air (FA) or cigarette smoke (CS) for 24 weeks as described in the METHODS section. Leukocytes from the perfused lungs of these mice were pooled, and CD3$^+$ T cells were purified by fluorescence-activated cell sorting (>99.7% pure). Purified T cells from FA- or CS-exposed WT mice were then injected into BALB/c *Rag2*$^{-/-}$ mice. BALB/c *Rag2*$^{-/-}$ FA and CS T cell–recipient mice were rested 3 weeks to allow for reconstitution of the lymphopenic environment. BALB/c WT, BALB/c *Rag2*$^{-/-}$, BALB/c *Rag2*$^{-/-}$ FA T cell–recipient, and BALB/c *Rag2*$^{-/-}$ CS T cell–recipient mice were then exposed to either FA or CS (eight experimental groups, n = 16 per group) for 13 weeks (1 wk of smoke acclimation followed by 12 wk of exposure). At the end of the study, tissues were harvested and analyzed as described in the METHODS section. The CD3$^+$ T-cell purification histograms depicted are actual sort data representative of all sorts. This experimental protocol was performed independently two times.

with the FRET peptide substrate. Stop solution was added, plates were gently shaken, and samples were read using a Flexstation II (Molecular Devices, Sunnyvale, CA).

Preparation and Analysis of Mouse Lungs

For some experiments mouse lungs had their left lungs inflation-fixed in buffered formalin as previously described (7). Tied off unfixed/uninflated right lungs of the same mouse were snap frozen in liquid nitrogen for RNA and protein analysis. For frozen sections, both mouse lungs were inflated with 1:1 OCT:20% sucrose in 1× PBS, briefly frozen in liquid nitrogen, and stored at −80°C until sectioned. Paraffin embedding and all sectioning were performed by AML Laboratories (Rosedale, MD). The mean linear intercept (MLI), a measure of interalveolar distance, was determined as previously described (21). Ten to 15 fields per mouse from five to eight mice per group were measured using horizontal and vertical lines.

Immunohistochemistry

Mac-3 immunohistochemistry analysis was performed with a rat anti-mouse Mac-3 antibody (BD Pharmingen, San Jose, CA). Mac-3$^+$ cells were detected in paraffin-embedded tissue sections (5 μm) of mouse lungs with a Vectastain ABC IgG kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol and developed with 3,3′-diaminobenzidine (DAB) as the substrate. Counterstaining was performed using hematoxylin. CD3$^+$ immunohistochemistry was performed with a hamster anti-mouse AlexaFluor 647-CD3 antibody (Invitrogen, Carlsbad, CA) on frozen sections (5 μm) of mouse lungs. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining on lung frozen sections was performed using the TUNEL kit (Roche, Indianapolis, IN). TUNEL$^+$ cells were counted at 100× magnification and more than 15 fields were counted per mouse. In some instances, before TUNEL labeling, frozen sections were stained with rabbit polyclonal pro–SP-C antibody (Chemicon, Billerica, MA) followed by incubation with goat anti-rabbit F(ab′)2 fragment conjugated with AlexaFluor 594. All slides used for fluorescence were mounted using Flouromount-G mounting medium (Southern Biotech, Birmingham, AL). All fluorescent images were taken at the Center for Biological Microscopy within the Department of Cell and Cancer Biology. Images were taken with a Zeiss Axioplan Imaging 2 infinity-corrected, upright scope with differential interference contrast and epifluorescence, coupled to an Orca-ER cooled, BandW CCD camera. Images were acquired using the Metamorph software. Final preparation of images (adjustments to brightness and contrast; overlays) was performed using Adobe Photoshop.

RNA Isolation and Quantitative Reverse Transcriptase–Polymerase Chain Reaction

Mouse lung RNA was isolated using TRIzol (Invitrogen), followed by DNase digestion (TURBO DNA-free; Applied Biosystems, Foster City, CA) and cDNA synthesis (cDNA archive kit; Applied Biosystems) according to manufacturers' protocols. Mouse monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated upon activation, normal T-cell expressed and secreted (RANTES), inducible protein (IP)-10, MMP-12, and Cathepsin-S TaqMan primer sets were purchased from Applied Biosystems. β-actin was used as an endogenous control. All quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) was conducted using the TaqMan Universal PCR Master Mix (Applied Biosystems). TaqMan qRT-PCR began with an initial step of 50°C for 2 minutes, followed by one cycle of 95°C for 10 minutes, and 40 cycles of 60°C for 1 minute and 95°C for 15 seconds. All PCR was performed using a 7300 Real-Time PCR System (Applied Biosystems).

Statistics

Significant differences among groups were identified by analysis of variance wherever appropriate, and individual comparisons between groups were confirmed by a *post hoc* Bonferroni-Dunn test. Differences between means were considered significant when the P value was less than 0.05.

RESULTS

T Cells from CS-Exposed Mice Drive Lung Inflammation

Based on observations that T-cell clones can drive inflammatory processes in numerous disease models (22–25), we hypothesized that antigen-specific, clonally-expanded T-cell populations generated during chronic CS exposure (7) are pathogenic and contribute to disease progression in a mouse model of COPD. To test this hypothesis, we purified CD3$^+$ T cells from the perfused lungs of WT BALB/c mice chronically exposed to FA or CS for 24 weeks. Although pathogenic T cells are likely present in secondary lymphoid organs and peripheral blood, lung T cells were used to enrich for antigen-specific T cell clones (7). Purified lung T cells derived from either FA-exposed (FA T cells) or CS-exposed (CS T cells) mice were injected into BALB/c *Rag2*$^{-/-}$ mice (Figure 1). It is possible the antigenic stimulus driving T-cell clonal expansion is either a component of CS or the result of smoke-modified self, thus requiring subsequent CS exposure for antigen generation and pathogenic T cell stimulation. Therefore, we subchronically exposed BALB/c *Rag2*$^{-/-}$ T-cell recipients to either FA or CS for 13 weeks (1 wk of acclimation followed by 12 wk of exposure) (Figure 1). Subchronic FA and CS exposures were performed in tandem using BALB/c WT and BALB/c *Rag2*$^{-/-}$ as control animals for exposures and T-cell transfer. We specifically chose this time point because subchronic CS exposure does not result in significant pathology or significant activation and accumulation of T cells in WT mice (26). Therefore, a 13-week subchronic CS exposure would prevent *de novo* activation of T cells in *Rag2*$^{-/-}$ FA T-cell recipients, but allow for activation of pathogenic T cells derived from 24-week CS-exposed donor WT mice. Throughout the experiments, the body weight of all mice was monitored. No differences were observed among groups receiving T cells, and no mice lost weight (data not shown).

We expected *Rag2*$^{-/-}$ mice that received CS antigen-specific T cells would develop a more severe and pronounced COPD phenotype after subsequent CS exposure, as compared with all other groups and exposures. In contrast to our initial expectations, *Rag2*$^{-/-}$ CS T cell recipients had marked inflammation in their lungs, regardless of smoke exposure (Figures 2 and 3). Relative to *Rag2*$^{-/-}$ FA T cell recipients, *Rag2*$^{-/-}$ CS T cell recipients exhibited an approximately 20-fold increase in total cells in the BAL, an effect independent of recipient smoke exposure (Figure 2A). The BAL of *Rag2*$^{-/-}$ CS T cell recipients revealed significant increases in total numbers of monocytes/macrophages, neutrophils, and lymphocytes (Figures 2B−2D). Nominal increases of eosinophils were detected in *Rag2*$^{-/-}$ CS T cell recipients (data not shown), which distinguishes this inflammation from pathways involved in limited CD4$^+$ T-cell diversity or asthma (27). Examination of the BAL revealed a bias toward accumulation of neutrophils in *Rag2*$^{-/-}$ CS T cell recipients, as neutrophils composed approximately 45% of the recovered leukocytes (Figures 2E and 2F). Interestingly, *Rag2*$^{-/-}$ mice did not display an accumulation of neutrophils in their BAL after smoke exposure, and transfer of FA T cells into *Rag2*$^{-/-}$ mice contributed slightly to the accumulation of neutrophils after CS exposure (Figure 2C). These data are in agreement with D'Hulst and colleagues, who have shown that SCID mice have impaired neutrophil recruitment after subchronic CS exposure (28). Monocyte/macrophage accumulation still occurred, although to a somewhat lesser degree, in *Rag2*$^{-/-}$ mice after CS exposure (Figure 2B). Together, these data demonstrate that, independent of subsequent smoke exposure, CS T cells contribute significantly to neutrophil and monocyte/macrophage recruitment in the BAL of *Rag2*$^{-/-}$ recipients.

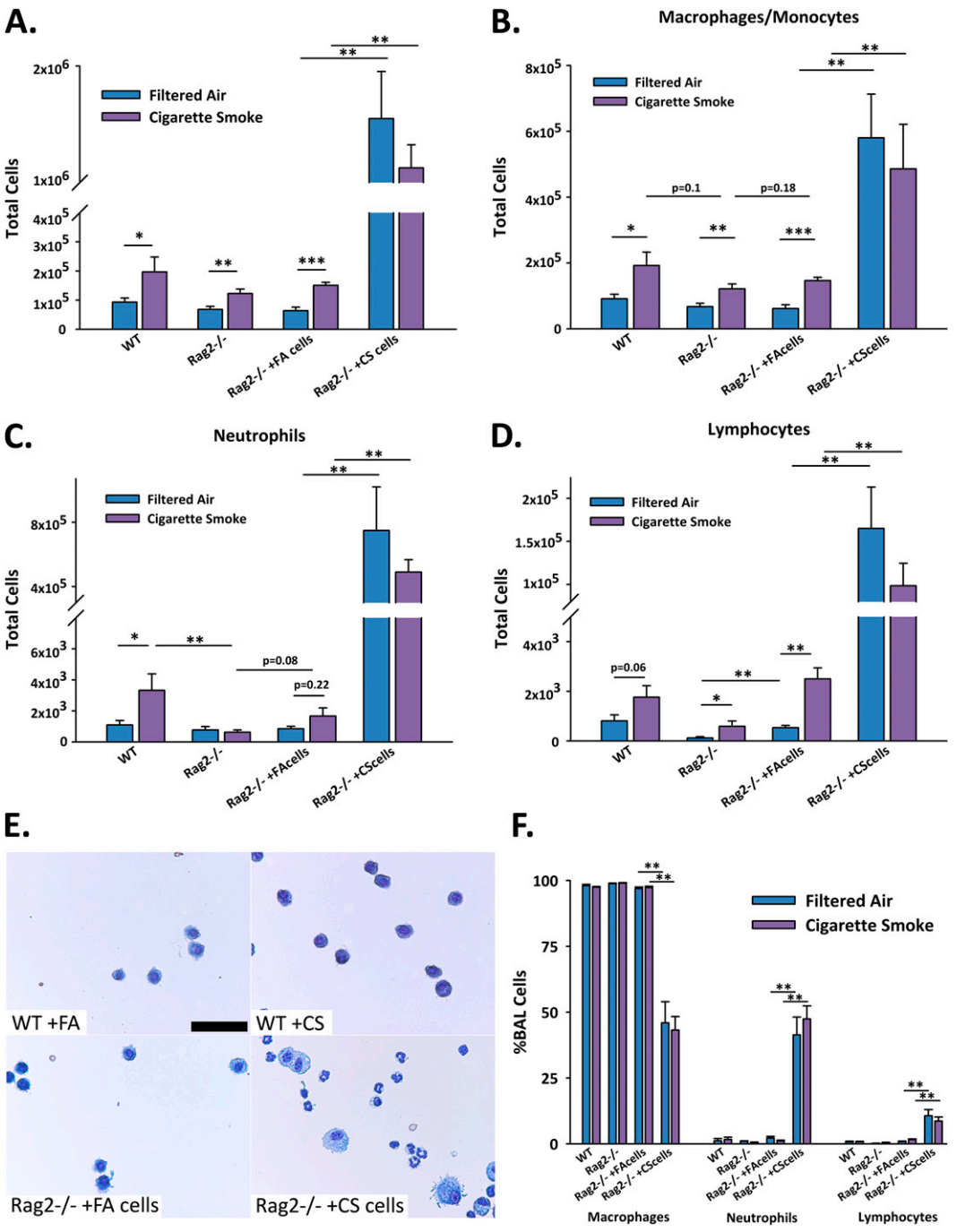

Figure 2. Cigarette smoke (CS) T cells drive lung inflammation in the bronchoalveolar lavage (BAL). (*A*) Mice were lavaged and total BAL cells were counted as described in METHODS. (*B*) Total monocyte/macrophage numbers in the BAL. (*C*) Total neutrophil numbers in the BAL. (*D*) Total lymphocyte numbers in the BAL. (*E*) Representative photomicrographs from BAL cytospins showing neutrophil accumulation and "foamy" macrophages. Original magnification 400×. Bar, 50 μM. (*F*) Percentage of leukocytes found in the BAL. All data representative of two experiments. n = 5–8 mice per group. Data are presented as means ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Macroscopic examination of lungs from CS-exposed mice clearly indicated an increase in lung size and superficial trauma. In contrast, macroscopic examination of other organs (skin, eyes, spleen, liver, and GI tract) of *Rag2*$^{-/-}$ CS T cell recipients revealed no pathologic manifestations, thus indicating the response was lung specific (data not shown). Microscopic examination of lung sections revealed perivascular and peribronchiolar inflammatory aggregates in conjunction with focal pulmonary alveolitis in *Rag2*$^{-/-}$ CS T cell–recipient mice (Figures 3B and 3D). This effect did not occur in *Rag2*$^{-/-}$ FA T cell recipients (Figures 3A and 3C). Again, this phenotype was present in CS T cell recipients regardless of additional smoke exposure. We observed that some inflammatory aggregates from *Rag2*$^{-/-}$ CS T cell recipients were punctuated with monocytes/macrophages. This was confirmed by anti–Mac-3 antibody staining of lung sections (Figure 3E). Interestingly, multinucleated giant cells of inflammation were observed on occasion throughout the lungs of *Rag2*$^{-/-}$ CS T cell recipients (Figure 3F). These cells are believed to be of monocyte/macrophage origin resulting from chronic stimulation with cytokines, notably IFN-γ and tumor necrosis factor (TNF)-α (29). Supporting this hypothesis, multinucleated giant cells in the lungs of *Rag2*$^{-/-}$ CS T cell recipients also stained positively for Mac-3 (data not shown).

Our initial time course was based on the assumption that *Rag2*$^{-/-}$ CS T cell recipients required subsequent CS exposure for T-cell activation. Examination of mice at an earlier time point after CS T cell transfer (5 wk earlier) revealed a similar, albeit milder, inflammatory phenotype in *Rag2*$^{-/-}$ recipients (*see* Figures E1A and E1B in the online supplement).

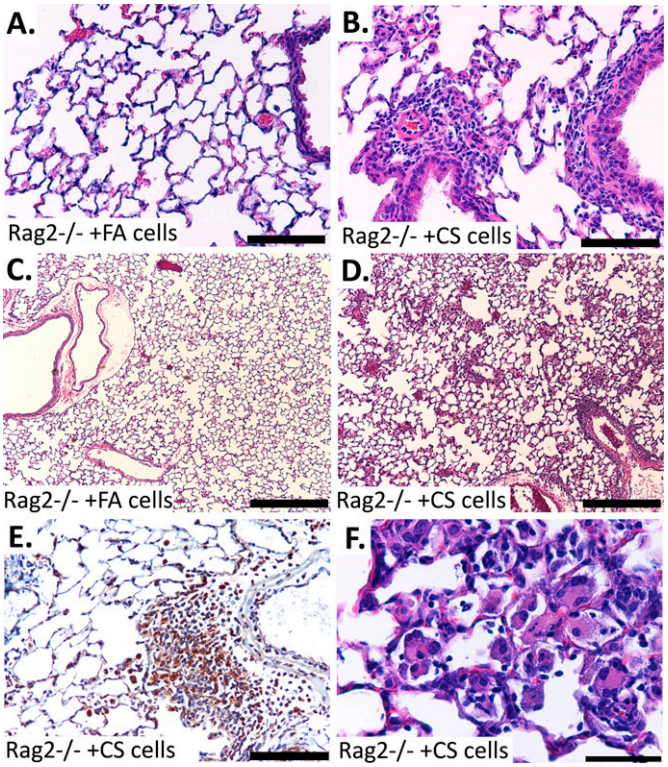

Figure 3. Histological examination of lungs. (*A*) Representative photomicrographs of hematoxylin and eosin (H&E)-stained lung sections from *Rag2*$^{-/-}$ filtered air (FA) T cell recipients. Original magnification 200×. Bar, 100 μM. (*B*) Representative photomicrographs of H&E-stained lung sections of *Rag2*$^{-/-}$ cigarette smoke (CS) T cell recipients showing alveolitis as well as perivascular and peribronchiolar inflammatory aggregates. Original magnification 200×. Bar, 100 μM. (*C*) Representative photomicrographs of H&E-stained lungs from *Rag2*$^{-/-}$ FA T cell recipients. Original magnification 40×. Bar, 500 μM. (*D*) Representative photomicrographs of H&E-stained lungs of *Rag2*$^{-/-}$ CS T cell recipients showing alveolitis. Original magnification 40×. Bar, 500 μM. (*E*) Representative photomicrograph of lungs of BALB/c *Rag2*$^{-/-}$ CS T cell recipient mice showing inflammatory aggregates that contain significant numbers Mac3^{+} cells. Original magnification 100×. Bar, 200 μM. (*F*) Representative lung photomicrograph of CS T cell BALB/c *Rag2*$^{-/-}$ recipient mice highlighting multinucleated giant cells of inflammation. Original magnification 400×. Bar, 50 μM.

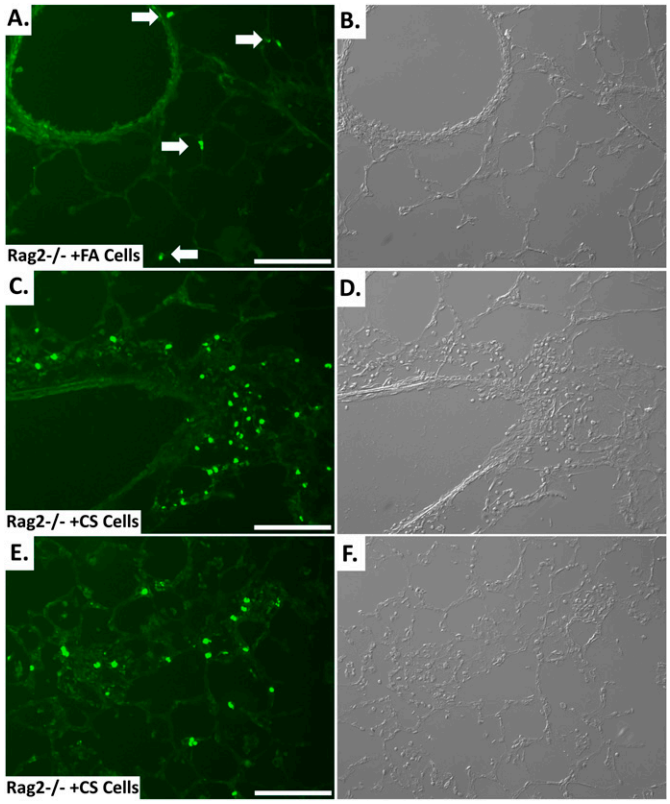

Figure 4. CD3^{+} T cells in the lungs of *Rag2*$^{-/-}$ recipients. (*A*) Fluorescence image showing CD3^{+} T cells in *Rag2*$^{-/-}$ filtered air (FA) T cell recipients. Original magnification 200×. Bar, 100 μM. Positive cells are indicated by *white arrows*. (*B*) Differential interference contrast (DIC) image of *A*. (*C*) Fluorescence image showing CD3^{+} T cells in inflammatory aggregates of *Rag2*$^{-/-}$ cigarette smoke (CS) T cell recipients. Original magnification 200×. Bar, 100 μM. (*D*) DIC image of *C* showing inflammatory aggregate. (*E*) Fluorescence image showing CD3^{+} T cells in parenchymal inflammation of *Rag2*$^{-/-}$ CS T cell recipients. Original magnification 200×. Bar, 100 μM. (*F*) DIC image of *E* showing inflammation. All data representative of two experiments. n = 5–8 mice per group.

Immunohistochemical staining of lungs from *Rag2*$^{-/-}$ FA and CS T cell–recipient mice with anti-CD3 antibody revealed that peribronchiolar, perivascular, and alveolar inflammatory aggregates contained high numbers of CD3^{+} cells, further implicating their role in the inflammatory process (Figure 4). As expected, CD3^{+} cells were also detected in the lungs of *Rag2*$^{-/-}$ FA T cell recipients, but were distributed similarly to those in FA-exposed WT BALB/c lungs (Figure 4).

CS T-Cell Transfer Triggered the Expression of Inflammatory Cytokines and Chemokines

Recognition of the pulmonary epithelium by antigen-specific T cells can result in the elaboration of cytokines and chemokines from antigen-specific T cells and from the epithelium (30, 31). This interaction is an important consequence of antigen recognition, which regulates much of the monocyte/macrophage and neutrophil accumulation and subsequent lung pathology (30). Given the significant accumulation of inflammatory cells in the lungs of *Rag2*$^{-/-}$ CS T cell recipients, we characterized the inflammatory milieu in the lungs by measuring key cytokines and chemokines in the BAL using a bead-based multiplex assay. Levels of IFN-γ, TNF-α, and IP-10 increased in the BAL of *Rag2*$^{-/-}$ CS T cell recipients (Figure 5 and Table E1). This is in agreement with the proposed role of these cytokines in the pathogenesis of COPD (32–34). IL-17 was not detected in the BAL samples. IL-10 levels were reduced in *Rag2*$^{-/-}$ CS T cell recipients relative to *Rag2*$^{-/-}$ FA T cell recipients. Significant increases in the neutrophil chemoattractants keratinocyte chemoattractant, lipopolysaccharide-induced CXC chemokine, and MIP-2 in the lungs of CS T cell recipients were detected. MCP-1, MIP-1α, and MIP-1β levels were also increased in the lungs of CS T cell recipients, providing a mechanistic link to the observed increases in monocytes/macrophages (Figure 5 and Table E1). Increases in the expression of several of these inflammatory molecules also occurred 11 weeks after transfer (Figure E1C). Together these data demonstrate that T cells derived from CS-exposed mice are capable of generating a complex inflammatory cytokine environment that recapitulates the phenotype of COPD.

CS T Cell Transfer Results in Increased Protease Expression and Activation

An important hypothesis in the development of COPD is that a protease–antiprotease imbalance occurs after chronic smoke

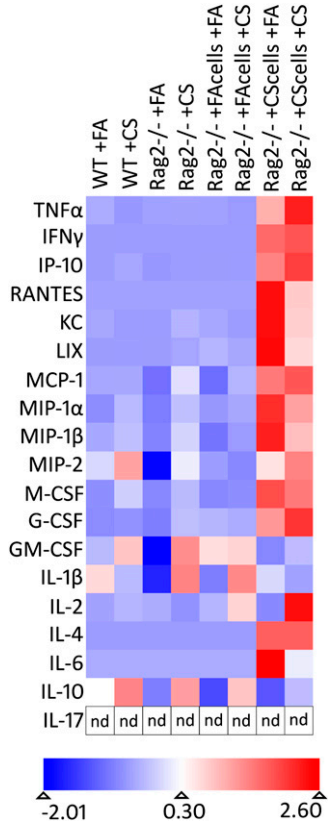

Figure 5. Cigarette smoke (CS) T cells induce expression of inflammatory cytokines. Bronchoalveolar lavage from mice was assayed for cytokines as described in the METHODS section. Raw data were analyzed using the Mayday software (http://www-ps.informatik.uni-tuebingen.de/mayday/wp/). The means of the raw data for each group were *z*-score transformed and are presented as a heatmap. The transformation was performed for each analyte individually. nd = not detectable. Data representative of two experiments. n = 4–6 mice per group. FA = filtered air; G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte-macrophage colony-stimulating factor; IP = inducible protein; KC = keratinocyte chemoattractant; LIX = lipopolysaccharide-induced CXC chemokine; M-CSF = macrophage colony-stimulating factor; MCP = monocyte chemoattractant protein; MIP = macrophage inflammatory protein; RANTES = regulated upon activation, normal T-cell expressed and secreted; TNF = tumor necrosis factor; WT = wild type.

exposure (1). Previous studies have shown that lung-specific expression of IFN-γ alone leads to the expression and activation of proteases in the lung (33). Given that CS T cell transfer resulted in significant accumulation of monocyte/macrophages, as well as increased expression of cytokines and chemokines, we examined the expression of proteases important to COPD pathology. Transcript abundance of two proteases, MMP-12 and CATHEPSIN-S, both important in extracellular matrix degradation and lung tissue destruction in COPD, was measured (35, 36). Relative to *Rag2*$^{-/-}$ FA T cell recipients, *Rag2*$^{-/-}$ CS T cell–recipient mice had an approximately 100-fold increase MMP-12 mRNA expression, and an approximately 10-fold increase in CATHEPSIN-S mRNA expression (Figures 6A and 6B). MMPs in *Rag2*$^{-/-}$ CS T cell recipients were functionally activated, as determined by cleavage-dependent fluorescence of a labeled broad-spectrum MMP target peptide (Figure 6C). *Rag2*$^{-/-}$ CS T cell recipients had an approximately fourfold increase in broad-spectrum MMP protease activity. These data demonstrate that CS T cells can activate pathologically relevant proteases that contribute to extracellular matrix breakdown.

CS T Cells Drive Lung Damage, Alveolar Epithelial Cell Apoptosis, and Emphysematous Changes, But Not Fibrosis or Mucus Hypersecretion

Inflammation, cytokine and chemokine production, and monocyte/macrophage and neutrophil activation are important mechanistic pathways for lung tissue destruction and development of emphysema. Microscopic examination of the lungs revealed alveolar enlargement in the lungs of *Rag2*$^{-/-}$ CS T cell–recipient mice, which was more severe than that found in CS-exposed WT lungs (Figure 7A). Subchronic CS exposure of WT, *Rag2*$^{-/-}$, and *Rag2*$^{-/-}$ FA T cell–recipient mice only caused marginal increases in MLI (WT: 35.7 ± 0.4 μm to 39.0 ± 0.6 μm; *Rag2*$^{-/-}$: 36.0 ± 0.7 μm to 39.9 ± 0.7 μm; *Rag2*$^{-/-}$ FA T cell recipients: 36.7 ± 0.3 μm to 39.9 ± 0.5 μm). In contrast, *Rag2*$^{-/-}$ CS T cell–recipient mice exposed to FA had a large increase in MLI relative to *Rag2*$^{-/-}$ FA T cell recipients exposed to FA (36.7 ± 0.3 μm to 51.5 ± 1.2 μm; 40.3% increase) (Figure 7B). A similar increase was seen in *Rag2*$^{-/-}$ CS T cell–recipient mice exposed to CS relative to *Rag2*$^{-/-}$ FA T cell recipients exposed to CS (39.9 ± 0.5 μm to 55.6 ± 2.7 μm; 39.3% increase) (Figure 7B).

As an additional measure of lung damage, we measured BAL hyaluronan fragments, which are matrix breakdown products produced after chronic smoke exposure or inflammation (37). *Rag2*$^{-/-}$ CS T cell–recipient mice had an approximately 100-fold increase in hyaluronan fragments over *Rag2*$^{-/-}$ FA T cell recipients (Figure 7C). Moreover, we observed a significantly higher number of epithelial cells recovered in the BAL of *Rag2*$^{-/-}$ CS T cell–recipient mice (data not shown), as compared with *Rag2*$^{-/-}$ FA T cell–recipient mice, which is an additional indicator of lung damage (38).

We further investigated whether CS T cell transfer induced apoptosis in the lungs of recipients. CS T cell transfer was sufficient to increase the amount of TUNEL^{+} cells in the lungs of recipients (Figure 8A and 8B). There was also a trend toward increased apoptosis in CS T cell recipients that were subsequently exposed to CS. This is likely due to other important mechanisms of cell death after CS exposure, such as oxidative stress and activation of NK cells through NKG2D (1, 5). Importantly, the TUNEL^{+} cells were frequently identified as type II alveolar epithelial cells, as determined by dual TUNEL and pro–SP-C staining (Figure 8C).

To determine the role of T cells in the development of other COPD phenotypes in the lungs, we examined whether CS T cell transfer influenced small-airway remodeling and collagen deposition. Similar to other reports that inflammation and emphysema are uncoupled with airway remodeling (39), we did not observe airway remodeling in mice receiving CS T cells (Figure E2). However, localized collagen deposition around focal areas of inflammation was observed (data not shown). Furthermore, significant changes in mucus-producing cells after CS T cell transfer were not identified (data not shown). This observation is in agreement with our previous demonstration that mucus production occurs independently of T cells in a mouse model of COPD (6).

DISCUSSION

In summary, these findings are the first demonstration that chronic CS exposure generates pathogenic T cells capable of recapitulating many of the phenotypic changes associated with COPD in *Rag2*$^{-/-}$ mice. Here, we demonstrate that T cells are capable of eliciting pathology independent of subsequent CS

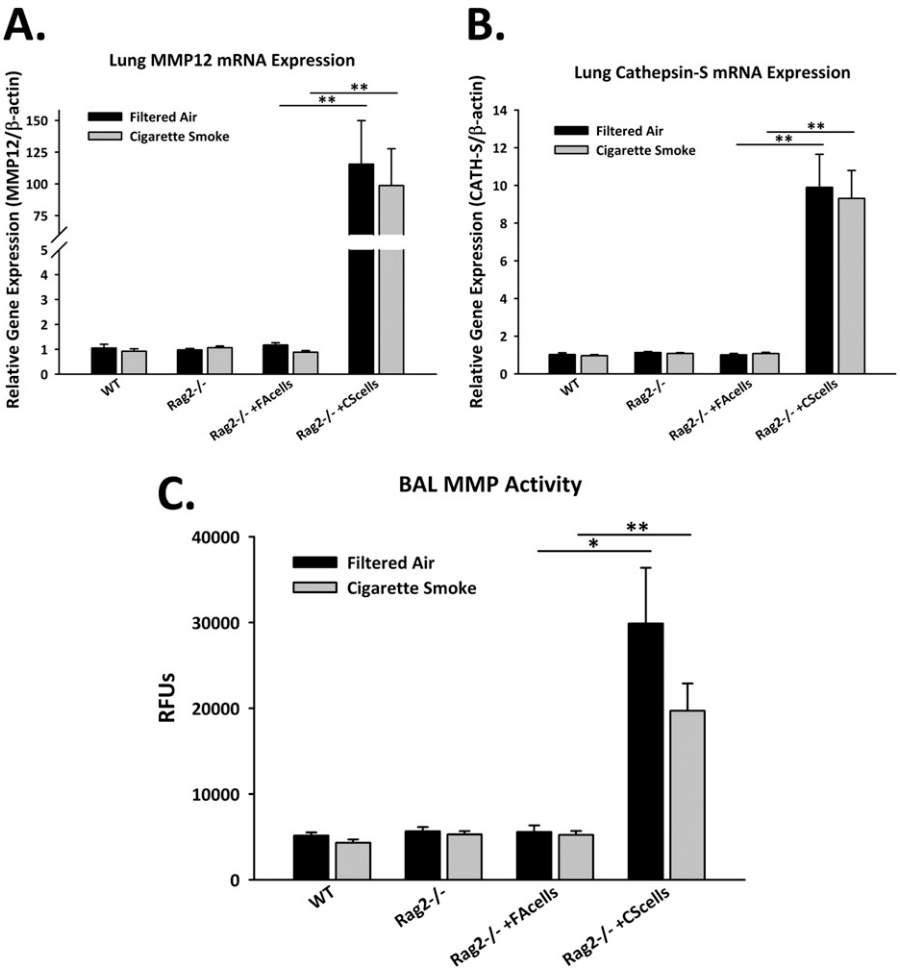

Figure 6. Cigarette smoke (CS) T-cell transfer results in protease activation. (*A*) Matrix metalloproteinase (MMP)-12 mRNA expression in whole lungs. (*B*) CATHEPSIN-S mRNA expression in whole lungs. (*C*) Broad-spectrum MMP activity in bronchoalveolar lavage (BAL). All data representative of two experiments. n = 5–8 mice per group. Data are presented as means ± SEM. * $P < 0.05$; ** $P < 0.01$. RFU = relative fluorescent units.

exposure, a feature indicative of autoreactivity. A rodent model of experimental autoimmune emphysema has been previously developed (40); however, it relied on active immunization with xenogeneic antigens (human endothelial cells) in the presence of potent adjuvants. Thus, in our present model, we provide a potential new system to dissect the mechanisms of CS exposure-induced T-cell autoreactivity in COPD.

In this study, *Rag2*$^{-/-}$ mice were used as T cell recipients. After transfer of T cells into lymphopenic hosts, T cells undergo a program of homeostatic proliferation (41). In irradiated hosts, transferred T cells rapidly proliferate until T-cell numbers reach normal levels, but in *Rag2*$^{-/-}$ mice, proliferation of transferred T cells is slower and numbers reach a low ceiling over several weeks (41). In our own preliminary studies, we recovered 5×10^5 $CD3^+$ T cells from the lungs of *Rag2*$^{-/-}$ mice injected with FA T cells 4 weeks after transfer, about half that typically recovered from WT mice (data not shown). We intentionally exploited homeostatic proliferation to enrich for pathogenic T cells in recipient mice. We believed that subsequent CS exposure would activate the antigen-specific pathogenic T cells and drive a more severe COPD phenotype in *Rag2*$^{-/-}$ CS T cell recipients relative to all other groups and exposures. In contrast to our expectations, *Rag2*$^{-/-}$ CS T cell recipients developed severe lung pathology 16 weeks after transfer even in the absence of secondary CS exposure.

The pathologies exhibited by CS T cell transfer recipient mice are more severe and diverse than those of the CS-exposed WT mice. There are several possible reasons that disease phenotypes are far greater in the *Rag2*$^{-/-}$ recipients of CS T cells than WT mice. The transferred T cells were isolated from lung tissue that presumably is enriched in lung-specific/reactive T-cell clones as we have previously described (7). It is likely that these clonal populations are held in check in WT mice by multiple regulatory mechanisms. These mechanisms may not be similarly active in recipient *Rag2*$^{-/-}$ mice relative to the extensive homeostatic proliferation of the pathogenic T-cell clones in these mice. This observation is not without precedent. In other models of autoimmunity, transfer of pathogenic T cells generates more severe disease in both immunocompromised and WT recipients compared with active disease in a model of multiple sclerosis (24). It is important to note that the severity of disease is clearly a function of time. In our present model, inflammation is milder at 11 weeks versus 16 weeks after T-cell transfer, which more accurately reflects human COPD. Although the mechanisms are not fully understood, this complication represents a limitation in the interpretation of the present study but invokes the concept that autoreactivity is ensconced in long-term smokers and may be revealed in susceptible individuals after a breakdown in regulatory pathways.

Aspects of the lung pathologies induced by transfer of CS T cells are reminiscent of multiple smoking-related pulmonary diseases, including respiratory bronchiolitis, desquamative interstitial pneumonia, and emphysema, but not airway fibrosis or chronic bronchitis (i.e., mucus cell metaplasia) (3). Diffuse alveolitis and multinucleated giant cells are features that can also be found in the biopsies of patients with chronic hypersensitivity pneumonitis, but the lack of granuloma formation,

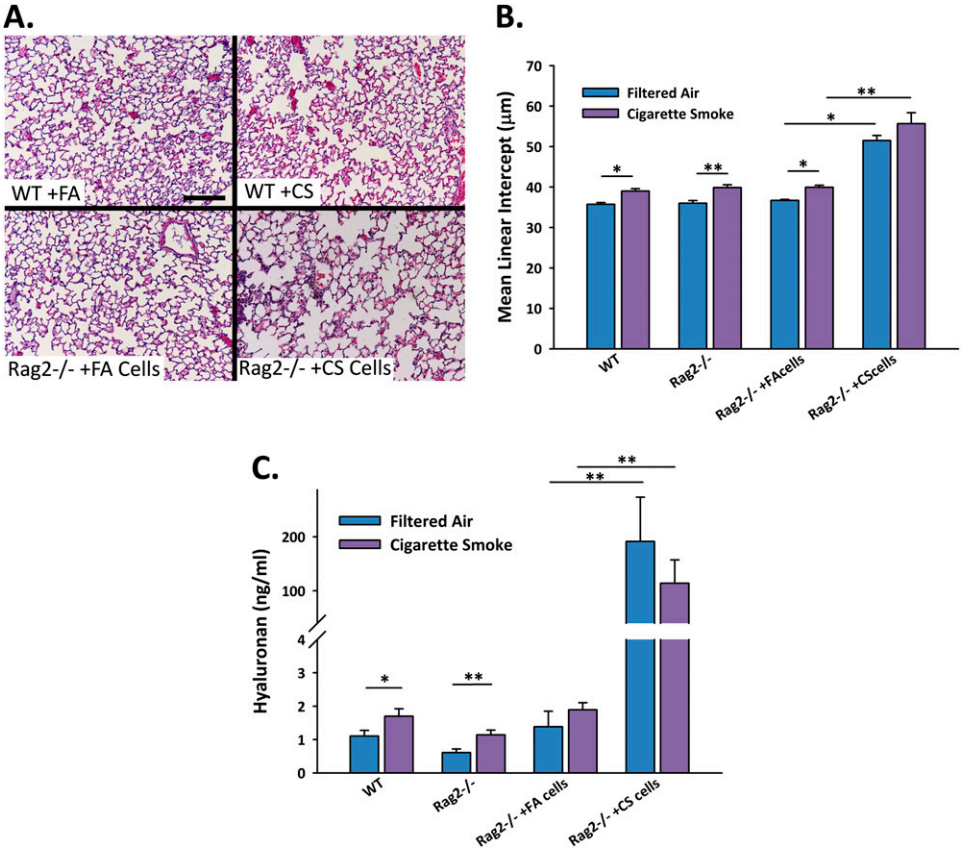

Figure 7. Cigarette smoke (CS) T cell transfer causes lung damage and emphysematous changes. (*A*) Representative photomicrographs of hematoxylin and eosin–stained lungs highlighting emphysematous changes in lungs of CS T cell BALB/c *Rag2*$^{-/-}$–recipient mice. Original magnification 100×. Bar, 200 μM. (*B*) Quantification of emphysema. (*C*) Hyaluronan ELISA of bronchoalveolar lavage. All data representative of two experiments. n = 5–8 mice per group. Data are presented as means ± SEM. * $P < 0.05$; ** $P < 0.01$.

lymphocyte predominance, and robust IL-17 production in our model is less consistent with this pathological pattern (42–44). This permeation of histopathologies in recipient mice leads us to hypothesize that smoking-related pulmonary diseases represent a continuum of disease that manifests in a vast array of phenotypes dependent primarily on other environmental exposures and genetic variability. Whereas human smokers are genetically diverse and encounter unique environmental condi-

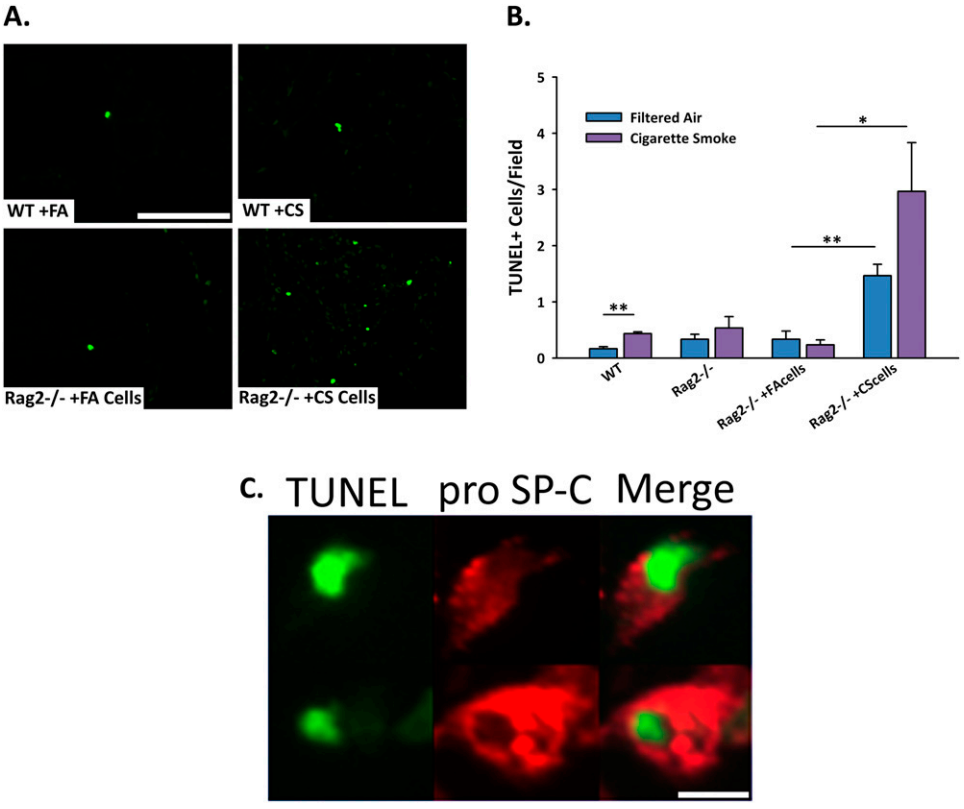

Figure 8. Cigarette smoke (CS) T-cell transfer causes apoptosis. (*A*) Representative fluorescence images highlighting increase in terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)$^{+}$ cells in lungs of CS T cell BALB/c *Rag2*$^{-/-}$–recipient mice. Original magnification 200×. Bar, 100 μM. (*B*) Quantification of TUNEL^{+} cells. (*C*) TUNEL labeling and pro–SP-C double staining shows apoptosis of type II alveolar epithelial cells. Representative fluorescence images presented are of the lungs of CS T cell BALB/c *Rag2*$^{-/-}$ recipient mice. Original magnification 200×. Bar, 5 μM. All data representative of two experiments. n = 5–8 mice per group. Data are presented as means ± SEM. * $P < 0.05$; ** $P < 0.01$.

tions (including infections) over their lifetime, the mice used in these studies were genetically identical and shared identical environmental conditions throughout their lifetime (with the exception of CS exposure). Furthermore, the fact that T cells from CS-exposed mice elicited this multitude of phenotypes may indicate a common T-cell–based cause for smoking-related disease, which discloses uniquely among cigarette smokers.

Presently, mechanistic data supporting a causal role for T cells in the development or progression of COPD are limited. The prevailing theory is that cytotoxic lymphocytes are aberrantly activated in COPD and contribute to tissue destruction through direct and indirect effector mechanisms. Direct mechanisms may involve lymphocyte-mediated apoptosis of type II alveolar epithelial cells (5). Increased expression of perforin, a pore-forming protein that causes apoptosis of cells via cytolytic granules, has been reported in $CD8^+$ T cells in smokers with COPD (45). Additionally, alveolar epithelial cell apoptosis correlates with $CD8^+$ T cells in patients with emphysema (46). Indirect mechanisms, whereby lymphocyte activation contributes to COPD, are based on data that indicate production of Th1 cytokines, such as IFN-γ, is increased in T cells of patients with COPD (34, 47). IFN-γ induction may amplify inflammation and increase macrophage metalloproteinase secretion, which can lead to elastin degradation and alveolar destruction (33, 47). Effector T-cell–derived TNF-α leads to the production of large amounts of MCP-1 by type II alveolar epithelial cells, which is responsible for a robust inflammatory response (30). Therefore, in COPD and our present model, T-cell recognition of antigens present on the lung epithelium may result in robust inflammation and the production of proinflammatory cytokines (30, 48).

The generation of an adaptive immune response in patients and in mouse models of COPD is likely to be pluricausal. Based on time course data of lymphocytic inflammation and lymphoid aggregate formation in mice after smoke exposure (26, 39), combined with our own data that T-cell clonal expansions do not occur after acute exposures (unpublished data), it is probable that generation of antigen-specific T-cell clones requires chronic CS exposure. Stress and physical damage to the lung due to CS exposure mimics the natural course of infections (5) and is likely to be a critical initiating step in an adaptive immune response. We have shown that stress-induced ligands for the cytotoxic lymphocyte-activating receptor, NKG2D, are up-regulated in patients with COPD, as well as in mouse models of COPD (5). Chronic activation of this receptor leads to progressive alveolar apoptosis and emphysema by activating innate cytotoxic functions of the responder cells (5). It has been proposed that γδ T-cell function may be impaired in patients with COPD (49), leading to enhanced epithelial cell death after smoke exposure (50). Importantly, oxidative stress itself may lead to epithelial cell death through exhaustion of the antioxidant capacity of the cell, a mechanism largely independent of the immune system (51).

Cell death and damage in the lung is not without consequence. Stimulation of Toll-like receptors (TLRs) on dendritic cells (DCs) by damage-associated molecular patterns released from smoke-damaged lungs, may lead to DC activation, representing an important link between the innate and adaptive immune systems (52). Indeed, endogenous ligands for TLRs have been detected after smoke exposure (53). Although the role of DCs in COPD is not completely clear, activated DCs have been detected in mouse models of COPD, as well as in patients with COPD (26, 54). Additionally, maturation of DCs has been correlated with disease severity, strongly suggesting a mechanistic role for DCs in COPD (54).

The antigen(s) responsible for the generation of oligoclonally expanded T cells in patients with COPD and mouse models of COPD remain undefined. Here, we have shown that T cells transferred into *Rag2*$^{-/-}$ mice are pathogenic and do not require subsequent CS exposure for activation. This suggests that these antigen-specific T cells recognize self-antigens. This does not preclude the possibility that the initial activation of T cells relies on neoantigen formation. T-cell clones generated against neoantigens may simply cross-react with self-antigens (55). Moreover, during chronic lung damage and inflammation caused by CS, the specificity of the immune response against neoantigens may spread to self-epitopes or to bystander activation of self-specific T cells (56). Strong support for the concept that antigen-specific T cells recognize self-antigens comes from our own observations that oligoclonal expansions persisted up to 6 months after smoking cessation in a mouse model of COPD (7). Furthermore, patients with severe COPD who have ceased smoking continue to have a prolonged and sustained inflammatory response (1, 19). It is likely that a number of self-antigens are recognized by pathogenic T cells in COPD. This concept is supported by evidence of autoantibodies to multiple components in the lung and to multiple cell types (16, 17). Identification of individual autoantibodies or autoantigens in patients will undoubtedly aid the progress toward tailored therapeutic strategies, such as autoantibody deletion, cytotoxic lymphocyte modulation, or antigen masking/removal.

The defining criteria for autoimmune diseases are classically defined by Witebsky's postulates (57). These include (*1*) circumstantial evidence consisting of lymphocyte accumulation in the target organ or association with specific MHC haplotypes, (*2*) indirect evidence consisting of the isolation of autoantibodies or self-reactive T cells from the organ or identification of human antigens and using immunization processes to reproduce the disease in animals, and (*3*) direct proof consisting of reproducing the disease in a normal recipient by the direct transfer of autoantibodies or T cells. The circumstantial evidence and indirect proof of autoimmunity in COPD has been established as described above. These include several reports of T-cell infiltration associated with disease severity and the presence of autoantibodies reactive against elastin and pulmonary epithelial cells. Here, we provide additional supporting evidence that chronic CS exposure may lead to autoreactivity.

Although there is strong evidence for autoreactivity in the present model, there exists the possibility that antigen-independent mechanisms may cause the observed effects. T cells activated in CS-exposed WT donors may persistently produce proinflammatory cytokines in the absence of T cell receptor ligation, possibly through permanent epigenetic modifications. Additionally, activated T cells may induce an inflammatory response from other immune cells (e.g., macrophages, dendritic cells, NK cells), lung epithelial, or endothelial cells that continue to function in the absence of persistent T-cell stimulation (30, 48). A number of unknown additional mechanisms may also be responsible. It is also worth noting that *Rag2*$^{-/-}$ FA T cell recipients did not develop an inflammatory response beyond *Rag2*$^{-/-}$ mice after CS exposure. Therefore, it is entirely likely that antigen-independent functions of T cells are dispensable for the development of COPD phenotypes. T-cell cloning and identification of putative antigens will therefore be invaluable next steps in assessing the antigen dependence of the pathological changes presented here.

Numerous endpoints after CS exposure were similar in WT and *Rag2*$^{-/-}$ mice. This is in agreement with the observation that *scid* mice still develop inflammation and emphysema after chronic CS exposure (28). Superficially, this is in contrast to published reports that *CD8*$^{-/-}$ mice are protected from monocyte/macrophage accumulation and the development of emphysema in models of COPD (6, 12). It is possible that

differences in genetic background (BALB/c vs. C57BL/6) of the mouse strains used may be responsible. Alternatively, *Rag2*$^{-/-}$ mice lack CD4^{+}CD25^{+}Foxp3^{+} Tregs, a population present in *CD8*$^{-/-}$ mice that has been recently demonstrated to control lung inflammation (58).

Together, these data demonstrate a unique system whereby chronic inflammation and damage results in the generation of pathogenic T cells capable of driving disease. Characterization of the events leading to the development of pathogenic T cells will undoubtedly provide key therapies for those at-risk individuals not yet manifesting autoimmune characteristics of COPD. Furthermore, this work will have an important positive impact on COPD research by aiding in the identification of novel, antigen-specific therapeutic targets. Significantly, the finding here that CS-induced COPD has autoimmune characteristics suggests that existing therapeutics currently available for well-characterized autoimmune disorders may prove beneficial for patients suffering from COPD.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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