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Functional Characterization of T Cell Populations in a Mouse Model of Chronic Obstructive Pulmonary Disease

Bryan L. Eppert,* Brian W. Wortham,* Jennifer L. Flury,* and Michael T. Borchers*†

Cigarette smoke (CS) exposure is the primary risk factor for the development of chronic obstructive pulmonary disease (COPD). COPD is characterized by chronic airflow limitation, and inflammation of the airways and lung parenchyma. The inflammatory cells consist primarily of macrophages, neutrophils, and lymphocytes. Although myeloid cells are well studied, the role of lymphocyte populations in pathogenesis of COPD remains unclear. Using a mouse model of CS-induced emphysema, our laboratory has previously demonstrated that CS exposure causes changes in the TCR repertoire suggestive of an Ag-specific response and triggers a pathogenic T cell response sufficient to cause alveolar destruction and inflammation. We extend these findings to demonstrate that T cells from CS-exposed mice of the BALB/cJ or C57B6 strain are sufficient to transfer pulmonary pathology to CS-naive, immunosufficient mice. CS exposure causes a proinflammatory phenotype among pulmonary T cells consistent with those from COPD patients. We provide evidence that donor T cells from CS-exposed mice depend on Ag recognition to transfer alveolar destruction using MHC class I–deficient recipient mice. Neither CD4+ nor CD8+ T cells from donor mice exposed to CS alone are sufficient to cause inflammation or pathology in recipient mice. We found no evidence of impaired suppression of T cell proliferation among regulatory T cells from CS-exposed mice. These results suggest that CS exposure initiates an Ag-specific response that leads to pulmonary destruction and inflammation that involves both CD8+ and CD4+ T cells. These results are direct evidence for an autoimmune response initiated by CS exposure. The Journal of Immunology, 2013, 190: 1331–1340.

From 1970 to 2002, the age-adjusted mortality attributable to chronic obstructive pulmonary disease (COPD) has doubled and is now the third leading cause of disease in the United States (1, 2). Worldwide, COPD affects an estimated 63 million people and was the cause of 3 million deaths in 2004 (3). Approximately 10% of the global population over the age of 40 years exhibits airway obstruction consistent with Global Initiative for Obstructive Lung Disease stage II or greater (4). The predominant risk factor for COPD is exposure to cigarette smoke (CS), although occupational exposures confer a significant risk for COPD development, and indoor burning of biomass fuels is an important risk factor in some developing countries (5).

COPD is a progressive disease of airway obstruction characterized by emphysema, airway remodeling, chronic bronchitis, and frequent respiratory infections. Inflammatory processes are important drivers of COPD, and previous research has identified a role for virtually all major leukocyte populations in the inflammatory response to CS (6). The majority of studies have focused on macrophage- and neutrophil-derived proteases being causative factors in CS-induced tissue destruction (7, 8). Furthermore, alveolar macrophages from COPD patients exhibit a consistent gene expression pattern that is marked by activation of proteases and proinflammatory cytokines (9).

Recent research has lead to an increased awareness for the role of lymphocyte populations in the development and progression of COPD. Our laboratory has shown that CS exposure induces NKG2D ligands that activate NK cells, thereby contributing to tissue destruction (10). Persistent activation of NK cells through NKG2D during CS exposure leads to hyperreactive viral responses in a mouse model (11). A growing body of research has identified changes among the lymphocytes that make up the adaptive immune system, B cells and T cells, in COPD patients compared with never-smokers and smokers without COPD. Circulating T cells in COPD patients produce more IFN-γ, and disease severity is correlated with T cell activation (12). CD4+ T cells in the lungs of COPD patients are skewed toward a Th1 phenotype, and T cell–derived cytokines were linked to the expression of proteases by macrophages (13). The number of CD8+ T cells in the lungs of COPD patients correlate with disease severity (14, 15), and they exhibit enhanced expression of cytokines and cytotoxic proteins (16, 17). Furthermore, mice deficient in CD8+ T cells are protected from CS-induced inflammation and emphysema (18). Mice exposed to CS and patients with COPD both exhibit an increase in the number of B cell follicles in the lung (19). These studies point to the involvement of every major lymphocyte population in the inflammatory response to chronic CS exposure.

In 2003, Agustí et al. (20) published an editorial presenting an argument for an autoimmune component to COPD based on the apparent involvement of T and B cells in COPD and other clinical features. Since this publication, the investigation of potential autoimmune mechanisms in COPD has rapidly increased. Oligoclonal expansions in CD4+ T cells, indicative of an Ag-specific T cell response, have been identified in the lungs of COPD patients (21).
patients (21). CD4+ T cells from the peripheral blood of COPD patients proliferated and produced proinflammatory cytokines when stimulated with elastin fragments (22). A number of recent studies have identified autoantibodies in COPD patients that bind epithelial (23), endothelial (24), smooth muscle (25), or extracellular matrix Ags (22). Autoantibodies against carbonyl-modified proteins generated in response to CS exposure have also been identified in COPD patients (26). Our laboratory has avoided many potential confounders associated with the clinical observations of immune function in COPD patients due to concomitant infections and cancer by using a mouse model of CS exposure. We found evidence for an Ag-specific response among pulmonary T cells of mice chronically exposed to CS in the form of oligoclonal expansions within the CD4+ and CD8+ TCR repertoire that persisted following smoke cessation (27). We have also demonstrated that T cells from the lungs of CS-exposed mice are sufficient to cause pulmonary pathology in CS-naive Rag2−/− recipients, providing direct evidence for an autoimmune response against lung tissue initiated by CS exposure according to Witebsky’s revised postulates (28, 29).

In this study, we provide a comprehensive characterization of the functional consequences of chronic CS exposure on T cells in a mouse model of COPD. We report the effects of chronic CS exposure on the phenotype and function of CD4+, CD8+, and regulatory T cells (Tregs) and demonstrate that the ability of T cells to drive pulmonary pathology is dependent upon Ag recognition by CD8+ T cells. Specifically, we find that CD4+ and CD8+ T cells from the lungs of CS-exposed mice exhibit enhanced proinflammatory cytokine production. Additionally, we perform the most thorough investigation of the effects of CS on Treg function. We demonstrate that T cell dysregulation in CS-exposed mice is not accompanied by defects in the Treg population or resistance among CD4+ and CD8+ T cells to Treg control. Finally, we show that the ability of T cells from CS-exposed donors to transfer pathology to recipients requires both CD4+ and CD8+ T cells and is dependent upon Ag presentation on MHC class I in recipient mice. Together, these findings suggest that CS exposure promotes functional changes among T cell populations that are sufficient to cause lung pathology and inflammation that is Ag dependent.

Materials and Methods

Mice

Female BALB/cJ and C57B6 wild-type (WT) aged 8–12 wk used in this study were obtained from The Jackson Laboratory (Bar Harbor, ME) and Taconic Farms, respectively. C57B6 MHC class II–deficient (Citra−/−) and C57B6 MHC class I–deficient (B2m−/−) recipient mice were obtained from The Jackson Laboratory and bred in University of Cincinnati facilities. Mice were housed according to institutional guidelines, and the Institutional Animal Care and Use Committee at the University of Cincinnati Medical Center reviewed and approved all experimental protocols. Mice were euthanized by i.p. injection of sodium pentobarbital followed by exsanguination.

CS exposure

Mice were exposed to either filtered air (FA) or CS generated from burning 3R4F Kentucky Reference Cigarettes (University of Kentucky) using a TE-10x smoking machine as described previously (28) (Teague Enterprises, Woodland, CA). Mice were exposed whole body for 4 h/d, 5 d/wk, for 24 wk. The exposure chambers were maintained at a concentration of 150 ± 15 mg/m3 total suspended particulates, and CO at this concentration was 400 ± 30 ppm.

Bronchoalveolar lavage

Lungs were lavaged twice with 1 ml HBSS. The first bronchoalveolar lavage (BAL) was centrifuged at 300 × g for 10 min, and the supernatant was recovered and stored at −80°C. The cell pellet was then resuspended with the second lavage and centrifuged at 300 × g for 10 min. The supernatant was discarded, and the cell pellet was resuspended in HBSS/2% FBS. Total cells were counted with a hemacytometer. For differential leukocyte counts, cells were adhered to slides using a Cytospin3 (Shandon Scientific, Waltham, MA) and stained with Hemacolor (EMD Millipore).

Isolation of mouse lung leukocytes

After euthanization, lungs were perfused by injecting 5 ml PBS/0.6 mM EDTA through a cannula inserted into the right tracheal. Lungs were dissected away from the surrounding tissue and chopped before adding media containing digestion enzymes (RFPI 1640, 20 mM HEPES, 10% FCS, 175 U/ml collagenase, 75 U/ml DNase I, 0.2 U/ml elastin tear fluid, 35 U/ml hyaluronidase, 100 U/ml penicillin, and 100 µg/ml streptomycin). Lung pieces were incubated in digestion media at 37°C for 45 min. The resulting suspension was passed through a 19-gauge needle three times to break up clumps and passed through a 40-µm filter to remove debris. Lung leukocytes were then enriched by centrifuging in a discontinuous Percoll gradient and recovering at the interface between the 40% Percoll and 70% Percoll layers.

Preparation of mouse lungs and histology

Mouse lungs were inflation-fixed with buffered formalin instilled through the trachea at a pressure of 25–30 cm H2O. The trachea was then tied off, and the heart lung block was excised and placed in 25 ml buffered formalin. Lungs were embedded in paraffin, sectioned, and slides stained with H&E at the Cincinnati Children’s Hospital Medical Center Pathology Core. Mean linear intercept (MLI) was measured as previously described (30). Focal areas of inflammation were classified as slight (<25 cells), mild (25–100 cells), moderate (750–20,000 µm2), or severe (>20,000 µm2) and according to the morphological features associated with the inflammation. Inflammation scores for individual mice were obtained by summing the instances of inflammation weighted for severity as follows: slight, 1; mild, 2; moderate, 4; and severe, 8.

Measurement of T cell–derived cytokines

Lung leukocytes were isolated from BALB/cJ mice exposed to either FA or CS for 6 mo as described above, and either CD4+ or CD8+ T cells were isolated by negative selection using a Dynabeads magnetic separation kit (Invitrogen). Isolated CD4+ and CD8+ T cells were stimulated with PMA (50 ng/ml; Sigma Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) in the presence of brefeldin A (3 µg/ml; eBioscience). After 5 h, cells were recovered and stained for the surface Ags PE-CD4 (clone GK1.5) or PE-CD8a (clone 53-6.7), fixed with 3% paraformaldehyde for 15 min, and stained for intracellular Ags FITC-IL-4 (clone BVD6-24G2), PerCP-Cy5.5-IFN-γ (clone XMG1.2), allophycocyanin-TNF-α (clone MP6-XT22), and FITC–TNF-α (clone XMG1.2). All washing and staining steps for detection of intracellular Ags were performed with 1% Permeabilization Buffer (eBioscience). Data was acquired on a BD FACSCalibur (BD Biosciences) and analyzed using FlowJo 7.6.2 (Tree Star).

Isolation and adoptive transfer of mouse pulmonary T cells

Lung leukocytes were isolated from BALB/cJ or C57B6 mice exposed to either FA or CS for 6 mo as described above and then T cells were enriched by negative selection using a Dynabeads magnetic separation kit (Invitrogen). Total T cells, CD4+ T cells, or CD8+ T cells were then isolated to >99% purity by FACS on the basis of CD3e expression (allophycocyanin; clone 145-2C11), CD4 expression (FITC; clone GK1.5), or CD8a expression (PE; clone 53-6.7), respectively. Isolated T cells were then expanded ex vivo using Dynabeads T-Activator beads (Invitrogen) coated with Abs against CD3, CD28, and CD137 for 7–10 d. Expanded T cells were washed twice and resuspended in HBSS, and 2 × 106 cells was injected i.p. into WT BALB/cJ, WT C57B6, and C57B6 Cita−/−, or C57B6 B2m−/− mice. Recipient mice were sacrificed 12 wk after transfer, and lungs were inflated and fixed for histology or lavaged. None of the recipient mice died during the 12 wk before they were sacrificed.

Treg suppression assay

BALB/cJ mice exposed to either FA or CS were euthanized, and spleens were harvested and pooled from six mice per group. Spleens were pressed through 100-µm filters, and RBCs were lysed using RBC Lysis Solution (Qiagen). Ninety percent of the splenocytes were used for negative selection of CD4+ T cells using a Dynabeads magnetic separation kit (Invitrogen). Enriched CD4+ T cells were stained with FITC–CD4 (clone GK1.5) and allophycocyanin–CD25 (clone PC61.5). Tregs, CD4+CD25−,
were isolated from the negatively selected CD4+ T cells by FACS. Total T cells were enriched by positive selection from the remaining 10% of splenocytes using a Dynabeads FlowComp Mouse Pan T kit (Invitrogen). Enriched total T cells were stained with FITC-CD4 (clone GK1.5), PE-CD8a (clone 53-6.7), and allophycocyanin-CD25 (clone PC61.5). T responders, CD8+ or CD4+CD25−, were isolated from the enriched T cells by FACS. Tregs were stained with 5 μM CFSE (Invitrogen). T responders and Tregs were cultured at ratios ranging from 16:1 to 0.5:1. T responders were stimulated with Dynabeads M-450 Epoxy (Invitrogen) coated with antimouse CD3e Ab (clone 145-2C11). Four days later, proliferation among T responders was measured by CFSE dilution using a BD FACSCalibur (BD Biosciences). FlowJo 7.6.2 (Tree Star) was used to analyze the results and calculate the division index, which represents the mean number of divisions undergone by the T responders. This experiment was independently performed three different times.

FACS

FACS was performed at Cincinnati Children’s Hospital Medical Center Research Flow Cytometry Core using a BD FACSAria II (BD Biosciences). Cells were stained with allophycocyanin-CD3e (clone 145-2C11), PE-CD8a (clone 53-6.7), FITC-CD4 (clone GK1.5), or allophycocyanin-CD25 (clone PC61.5).

Statistics

Significant differences between groups with respect to MLI, inflammation score, and total cells recovered from the BAL were determined by Student’s t test (SigmaPlot 10.0). Inflammation scores were transformed by taking the square root of the raw scores, and the total numbers of cells were transformed using Log10 of the raw counts. In every instance, the Student’s t test was used, normality was assessed using Kolmogorov-Smirnov test, and homogeneity of variances was tested using SigmaPlot 10.0 to ensure that the assumptions of the Student’s t test were not violated. A p value of <0.05 was considered significant.

For comparisons of the proportion of CD4+ T cells or CD8+ T cells that expressed cytokines by flow cytometry, the odds ratios were calculated to assess the impact of CS exposure on the likelihood of cytokine expression (31). The odds ratio is defined as \[ \frac{P_{CS}(1 - P_{FA})}{P_{FA}(1 - P_{CS})} \]
in which \( P_{CS} \) is the proportion of cells expressing a cytokine from CS-exposed animals, and \( P_{FA} \) is the proportion of cells expressing a cytokine from FA-exposed animals. Odds ratios equivalent to 1 indicate the treatment (CS) has no effect on the proportion. Cytokine expression was measured in T cells pooled from the lungs of six mice and repeated in independent experiments. The results from the two independent experiments were combined to create a pooled odds ratio. Pooled odds ratios and 95% confidence intervals were calculated using the meta package in R version 2.15.1.

Results

Lung-derived T cells from CS-exposed donor mice transfer pathology and inflammation to CS-naive, immunocompetent recipients

We previously reported that T cells isolated from the lungs of CS-exposed mice were sufficient to cause airspace enlargement and drive inflammation in immunodeficient recipient mice (28). To further confirm and extend these findings to the use of immunocompetent recipient mice, we transferred total T cells isolated from the lungs of mice exposed to either FA or CS for 6 mo into immunosufficient, CS-naive BALB/cJ mice. Histopathological examination revealed significant airspace enlargement in mice that received T cells from CS-exposed donors compared with recipients of T cells from FA donors (Fig. 1A–C). Gross macroscopic analysis did not indicate inflammation or pathologies in the gastrointestinal tract, skin, or eyes. The total number of cells recovered in the BAL was elevated in mice that received T cells from CS-exposed donors compared with those that received T cells from FA-exposed donors (Fig. 1D). Cell-differential analysis of the BAL revealed a majority of macrophages and monocytes in both groups (Fig. 1E, 1F). These data confirm and extend our previous findings that CS exposure leads to the generation of pathogenic T cells capable of driving pathology and architectural

![FIGURE 1](http://www.jimmunol.org/DownloadedFrom/1333.1058.500.png)  

**Figure 1.** T cells from CS-exposed donor mice cause emphysema and airway inflammation in immunocompetent recipient mice. Donor BALB/cJ mice were exposed to either FA or CS for 6 mo. T cells were isolated by FACS and expanded ex vivo before transferring 2 × 10^6 cells/recipient. Recipient mice were sacrificed 12 wk posttransfer. (A) Recipients of T cells from CS donors exhibited increased MLIs, indicating alveolar destruction and increased alveolar diameter (n = 4 mice/group; p = 0.011). Representative micrographs of H&E-stained lung sections from mice that received T cells from either FA- (B) or CS-exposed (C) donor mice showing extent of airspace destruction. Scale bars, 100 μm. (D) T cells from donors exposed to CS caused an increase in the number of inflammatory cells recovered from the BAL (n = 6 mice/group; p = 0.041). (E and F) Representative fields showing predominantly macrophages and monocytes recovered from the BAL. Scale bars, 25 μm. *p < 0.05.
changes in the lung independent of subsequent smoke exposure in the recipient mouse.

**Chronic CS exposure increases the proinflammatory capacity of CD8+ T cells**

We examined the cytokine expression profile of CD8+ T cells isolated from the lungs of mice chronically exposed to either FA or CS to better understand the mechanisms through which T cells from CS-exposed mice are able to promote inflammation and alveolar destruction. The proportion of CD8+ T cells that stained double positive for both TNF-α and IFN-γ was higher among cells isolated from the lungs of CS-exposed mice compared with FA-exposed mice (Fig. 2A, 2B). Furthermore, the geometric mean fluorescent intensity of TNF-α staining was higher for CD8+ T cells isolated from the lungs of CS-exposed mice than for those isolated from the lungs of FA-exposed mice, although the statistical significance of this observation cannot be determined based on the study design (Fig. 2C). Similarly, the proportion of CD8+ T cells expressing only TNF-α was higher among cells isolated from the lungs of CS-exposed mice compared with those collected from the lungs of FA-exposed mice (Fig. 2A, 2B). Parallel experiments performed without the addition of brefeldin A also showed a modest increase in IFN-γ production by ELISA among CD8+ T cells isolated from the lungs of CS-exposed mice compared with cells from FA-exposed mice (Fig. 2D). Taken together, these results suggest that chronic CS exposure promotes a proinflammatory phenotype among CD8+ T cells in the lung by enhancing expression of IFN-γ and TNF-α.

**Chronic CS exposure increases the proportion of Th1 and Th17 CD4+ T cells in the lung**

We next sought to understand the effects of chronic CS exposure on the accumulation of Th1-, Th2-, and Th17-polarized CD4+ T cells in lung. CD4+ T cells were isolated from the lungs of mice exposed to either FA or CS and stimulated ex vivo to reveal their cytokine expression profile. Intracellular flow cytometry was used to identify Th1 (CD4+, IFN-γ+, IL-4+, IL-17A-), Th2 (CD4+, IL-4+, IFN-γ-, IL-17A-), and Th17 (CD4+, IL-17A+; IFN-γ-, IL-4-) subsets based on cytokine expression. Chronic CS exposure significantly increased the proportion of pulmonary Th1 and Th17 CD4+ T cells, without any discernible effect on the proportion of Th2 cells (Fig. 3A, 3B). Parallel experiments were performed in the absence of brefeldin A, and ELISA was performed on the cell-culture supernatant to measure the production of IFN-γ and IL-17A. In accordance with the flow cytometry data, CD4+ T cells from CS-exposed mice produced more IFN-γ and IL-17A in the culture supernatant compared with cells isolated from FA-exposed mice (Fig. 3C). These results suggest that chronic CS exposure skews CD4+ T cells

**FIGURE 2.** CS exposure promotes the expression of proinflammatory cytokines IFN-γ and TNF-α among CD8a+ T cells in the lung. BALB/cJ mice were exposed for 6 mo to FA or CS, and CD8+ T cells were isolated from digested lungs by negative selection and stimulated with PMA and ionomycin in vitro with or without the presence of brefeldin A so that cytokine expression could be measured by intracellular flow cytometry or ELISA. CD8+ T cells were pooled from n = 6 mice/group and repeated in an independent experiment. (A) Representative density plots indicate that chronic CS exposure results in an increase in the proportion of CD8a+ T cells that express TNF-α alone or both TNF-α and IFN-γ compared with the CD8a+ T cells from FA-exposed mice. (B) Odds ratios comparing the proportion of CD8+ T cells that are TNF-α+, TNF-α+IFN-γ+, or TNF-α+IFN-γ- between FA- and CS-exposed mice indicate that CS increases the likelihood that CD8+ T cells express TNF-α and IFN-γ. Error bars represent the 95% confidence intervals for the pooled odds ratios from two independent experiments. (C) The geometric mean fluorescent intensity (gMFI) of TNF-α staining is higher among CD8a+TNF-α+ cells from CS-exposed mice compared with FA-exposed mice, indicating higher production of TNF-α. (D) CD8+ T cells from CS-exposed mice stimulated in vitro in the absence of brefeldin A produced more IFN-γ as measured by ELISA. *p < 0.05.
toward Th1 and Th17 phenotypes without affecting the Th2 population.

CS exposure does not reduce the number or suppressive ability of Tregs

We hypothesized that the generation of pathogenic T cells in response to chronic CS exposure may be accompanied by defects in the Treg population given the role of Tregs in peripheral tolerance and immunoregulation. Therefore, we investigated the effects of chronic CS exposure on the number and function of Tregs. We performed flow cytometry to identify the number of Tregs (CD4+, CD25<sup>high</sup>, Foxp3<sup>+</sup>) in the lungs and lung-draining lymph nodes of FA- and CS-exposed mice. We found no difference in the proportion or absolute number of Tregs in the lungs and lung-draining lymph nodes between FA- and CS-exposed mice (Fig. 4A, 4B).

We isolated Tregs (CD4<sup>+</sup>CD25<sup>high</sup>) and T responders (CD8<sup>+</sup> or CD4<sup>+</sup>CD25<sup>+</sup>) from the spleens of mice exposed to CS or FA and cocultured them to define the effects of CS exposure on the ability of Tregs to suppress T responders. The four possible combinations of Tregs and T responders from FA and CS-exposed mice (FA Tregs/FA T responders, FA Tregs/CS T responders, CS Tregs/FA T responders, and CS Tregs/CS T responders) were cocultured to assess whether differences in suppression of proliferation were due to resistance to suppression by the T responders or defective suppression among Tregs.

Tregs from FA- and CS-exposed mice were not different in their ability to suppress the proliferation of CD4<sup>+</sup> T responders from both FA- and CS-exposed mice across a wide range of Treg/T responder ratios (Fig. 4C, 4D). Furthermore, there did not appear to be any resistance to Treg suppression among the T responders based on the combinations of FA Tregs/CS T responders or CS Tregs/FA T responders (Fig. 4C).

Interestingly, we found that CD4<sup>+</sup>CD25<sup>+</sup>T responders and CD8<sup>+</sup>T responders react differently to coculture with Tregs. Tregs suppress CD4<sup>+</sup>CD25<sup>+</sup>T responders by reducing the rate of cellular division; however, CD8<sup>+</sup>T responders are suppressed by inducing cell death without affecting the rate of proliferation among the surviving CD8<sup>+</sup>T responders (data not shown). This suggests that Tregs counter the proliferation of CD4<sup>+</sup>T cells in vitro by inducing cell death, likely through apoptotic mechanisms. In our experiments, the Tregs isolated from mice exposed to CS did not differ in their ability to induce cell death among CD4<sup>+</sup>T responders compared with FA Tregs, and T responders from CS-exposed mice were as susceptible to induced cell death as T responders from FA-exposed mice (Fig. 4D). These findings demonstrate that CS exposure does not impair the ability of Tregs to suppress T responder activation in vitro nor does it cause resistance among T responders to Treg control.

T cells from CS-exposed C57B6 mice transfer alveolar pathology and inflammation to CS-naive recipients

Pathogenic T cells generated during chronic CS exposure may be responding to a specific Ag, or they may be promoting inflammation in a nonspecific manner. We can test if Ag

![FIGURE 3. CD4<sup>+</sup> T cells in the lung are skewed toward a Th1 and Th17 phenotype in response to chronic CS exposure. BALB/cJ mice were exposed for 6 mo to FA or CS, and CD4<sup>+</sup> T cells were isolated from lung digestion by negative selection and stimulated with PMA and ionomycin in vitro with or without the presence of brefeldin A so that cytokine expression could be measured by intracellular flow cytometry or ELISA. CD4<sup>+</sup> T cells were pooled from n = 6 mice/group and repeated in an independent experiment. (A) Representative density plots showing the cytokine expression profile among CD4<sup>+</sup> T cells and the gates used to identify the CD4<sup>+</sup> subsets. (B) Odds ratios comparing the proportion of CD4<sup>+</sup> T cells expressing each cytokine demonstrate that CS exposure increases the likelihood that CD4<sup>+</sup> T cells express IFN-γ and IL-17 but not IL-4. Pooled odds ratios were calculated from two independent experiments, and error bars represent the 95% confidence intervals. (C) CD4<sup>+</sup> T cells isolated from the lungs of FA- and CS-exposed mice were stimulated ex vivo with PMA and ionomycin, and 48 h later, the production of IFN-γ and IL-17A was measured by ELISA in the supernatant. *p < 0.05. FSC, Forward scatter.](http://www.jimmunol.org/content/1335/6/1335)
recognition in the recipient is necessary for total T cells to the transfer pathology by using mice that are deficient in either MHC class I or class II. Furthermore, we can identify the relative contribution of CD8+ T cells and CD4+ T cells to the inflammation and airspace destruction based on their restriction to MHC class I and class II presentation, respectively. Using $B2m^{-/-}$ and $Ciita^{-/-}$ mice on the C57B6 background necessitated the validation of our transfer model using donors and recipient mice of the C57B6 strain. We performed the transfer experiment using C57B6 donors exposed to FA or CS and CS-naive, C57B6 recipient mice. Histological examination of the recipients showed that T cells from CS-exposed donors induce alveolar destruction (Fig. 5A–C). We also observed an increase in focal areas of inflammation in the lungs of mice that received T cells from CS-exposed donors (Fig. 5D), dominated by perivascular inflammation in recipients of T cells from CS-exposed donors (Fig. 5E, 5F). These focal areas of inflammation are a point of divergence between BALB/cJ and C57B6 background. T cells from CS-exposed donors also cause increased recruitment of inflammatory cells, mostly myeloid cells, to the airways, as indicated by an increase in the total number of cells recovered from BAL (Fig. 5G). These results demonstrate that the T cell transfer model can be appropriately applied to the C57B6 mouse strain, opening up the possibility of using transgenic mice on the C57B6 background to explore putative mechanisms. Furthermore, these results indicate that the ability of T cells from CS-exposed donors to transfer pathology to CS-naive recipients is not a phenomenon peculiar to the BALB/cJ strain.

Neither CD4+ nor CD8+ T cells from CS-exposed mice are sufficient to transfer alveolar destruction to CS-naive recipients

We conducted a series of T cell–transfer experiments on the C57B6 background to: 1) identify whether CD4+ or CD8+ T cells from CS-exposed donors are sufficient to cause pulmonary pathology and inflammation; and 2) identify whether Ag presentation in the recipient is necessary for total T cells from CS-exposed donors to cause lung pathology. CD4+ and CD8+ T cells were isolated from FA- or CS-exposed donors and transferred sepa-
MHC class I expression in recipient mice is required for T cells to transfer emphysema, and neither CD4+ nor CD8+ T cells from CS-exposed donor mice are sufficient to cause emphysema when transferred separately. (A–F) Donor C57B6 mice were exposed to FA or CS for 6 mo, and CD3+ T cells were isolated from the lungs. C57B6 recipient mice received 2 × 10^6 T cells/mouse and were sacrificed 12 wk later, and lungs were inflated and fixed for histology. (A) C57B6 mice that received T cells from CS-exposed donor mice exhibited increased MLI suggestive of emphysema (p = 0.01; n = 6–8 mice/group). Representative micrographs of H&E-stained lung sections from mice that received T cells from FA donors (B) and mice that received T cells from CS donors (C) showing the extent of alveolar damage in the mice that received T cells from CS-exposed donors. Scale bars, 100 μm. (D–F) Focal areas of inflammation were scored by number of occurrences and severity in H&E-stained lung sections from C57B6 mice that received total T cells from FA- or CS-exposed donor mice. (D) Mice that received T cells from CS-exposed donors had a higher inflammation score (p = 0.038; n = 8 to 9 mice/group). (E) Focal areas of inflammation were classified according to nearby pulmonary features and the histology score for each category was summed for all nine mice that received T cells from CS-exposed donors. (F) Representative micrograph of H&E-stained lung section showing moderate perivascular inflammation recipient of T cells from CS-exposed donors. Scale bar, 50 μm. (G and H) CD4+ and CD8+ T cells were isolated separately from C57B6 donor mice exposed to FA or CS for 6 mo and injected into recipient mice. Recipient mice were sacrificed 12 wk posttransfer, and lungs were inflated and fixed for histology or lavaged and frozen. (G) CD8+ T cells from CS-exposed donor mice are sufficient to cause inflammation in the airways as evidenced by an increase in the total cells recovered from BAL (p = 0.024; n = 4 mice/group). (H) Neither CD4+ T cells nor CD8+ T cells are sufficient to drive airspace enlargement in recipient mice. No significant difference in MLI is evident, suggesting that both CD4+ and CD8+ T cells from CS-exposed mice are required to cause airspace enlargement or emphysema (n = 3–7 mice/group). (I–K) Donor mice were exposed to FA or CS and total T cells (CD3+) were isolated from their lungs and expanded ex vivo. Total T cells from either FA- or CS-exposed donors were transferred into B2m−/− recipient mice at 2 × 10^6 cells/mouse. Recipient mice were sacrificed 12 wk posttransfer, and lungs were prepared for histology or lavaged and frozen. (I) MHC class I expression in recipient mice is required for T cells from CS-exposed mice to cause increased airspace enlargement compared with recipients of T cells from FA-exposed donors. There is no difference between the MLI of B2m−/− recipients that received total T cells from FA-exposed donors and those that received T cells from CS-exposed mice (n = 3–7 mice/group). (J) MHC class I expression in recipient mice is required for T cells from CS-exposed mice to transfer airway inflammation. There is no difference in total cells recovered from BAL of B2m−/− mice that received T cells from FA-exposed donors and those that received T cells from CS-exposed donors (n = 3 mice/group). (K) MHC class I expression in recipient mice is necessary for T cells from CS-exposed donors to drive focal areas of inflammation. There is no difference in the histological inflammation score of B2m−/− mice that received T cells from FA-exposed donors and those that received T cells from CS-exposed donors (n = 5–9 mice/group). *p < 0.05.
rately into CS-naive recipient mice. CD8\textsuperscript{+} T cells from CS-exposed donors, but not CD4\textsuperscript{+} T cells, were sufficient to cause pulmonary inflammation assessed by total cells recovered from the BAL (Fig. 5G). However, upon histological examination, neither CD4\textsuperscript{+} T cells nor CD8\textsuperscript{+} T cells from CS-exposed donors were capable of causing alveolar destruction (Fig. 5H) or increased leukocytic accumulation in the lungs of recipient mice (data not shown).

**Recipient mice deficient in MHC class I are protected from airspace enlargement and inflammation caused by T cells transferred from CS-exposed mice**

We used CS-naive recipient mice deficient in either MHC class I expression (B2m\textsuperscript{−/−}) or mice that lack MHC class II expression (Ciita\textsuperscript{−/−}) to test whether total T cells from CS-exposed mice are responding to a specific Ag (or Ags) in the recipient mice. Unexpectedly, MHC class II–deficient mice exhibited severe and profound pulmonary pathology including alveolar destruction, alveolar fibrosis, and inflammation regardless of whether the donor T cells were from FA- or CS-exposed mice (data not shown). Ciita\textsuperscript{−/−} is a transcriptional coactivator regulating MHC class II expression; therefore, we hypothesize that the phenomenon we observed when T cells were transferred into Ciita\textsuperscript{−/−} recipients was due to a paucity of CD4\textsuperscript{+} T cells and Tregs or other unanticipated consequences of Ciita\textsuperscript{−/−} deficiency. As such, we were unable to draw any conclusions based on the effect of MHC class II deficiency on the ability of total T cells from CS-exposed donor mice to transfer pathology. When MHC class I–deficient mice received T cells from FA- or CS-exposed mice, there was no significant increase in alveolar diameter among recipients of T cells from CS-exposed mice compared with recipients of T cells from FA-exposed mice. This suggests that MHC class I expression in recipients is required for T cells from CS-exposed mice to cause alveolar destruction (Fig. 5I). Similarly, MHC class I deficiency in recipient mice prevents T cells from CS-exposed donors from eliciting an increase in the total cells recovered during BAL and in the formation of leukocytic accumulation by histology compared with T cells from FA-exposed donors (Fig. 5J, 5K).

Taken together, these results suggest that although CD8\textsuperscript{+} T cells from CS-exposed donors alone are sufficient to recruit immune cells to the airways, they cannot drive alveolar destruction and emphysema. MHC class I–deficient hosts are also protected from alveolar airspace enlargement and airway inflammation caused by total T cells from CS-exposed mice. These experiments define the requirement for both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells from CS-exposed donor mice and Ag presentation to CD8\textsuperscript{+} T cells on MHC class I molecules for transfer of airspace enlargement and inflammation.

**Discussion**

Recent research using data generated from both patients and mouse models has lead to a greater appreciation of the role of T cells in the progression of COPD. Much of the research based on samples obtained from COPD patients is necessarily associative. Using a mouse model of disease, our laboratory was able to build on our previous finding that T cells from the lungs of mice exposed to CS are sufficient to transfer pulmonary pathology to CS-naive recipients (28). In the current study, we demonstrate that recipient mice must present Ag to CD8\textsuperscript{+} T cells and that CD4\textsuperscript{+} T cells and CD8\textsuperscript{+} T cells from CS-exposed donor mice are both necessary to transfer pathology to CS-naive recipients. This is the most direct evidence to date that chronic CS exposure triggers the development of a T cell–mediated autoimmune process that is Ag specific. To our knowledge, this is the first report of a murine autoimmune model initiated in response to an environmental insult in two different strains without genetic manipulation or immunization with self-Ag. In addition, we confirm previous reports from patients with COPD and mice exposed to CS suggesting that the cytokine expression profiles of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells are skewed toward a more proinflammatory phenotype after chronic CS exposure (12, 13, 16, 17, 32–34).

Interestingly, we find no evidence for any accompanying defect among Tregs in terms of their numbers or ability to suppress proliferation after chronic CS exposure. These data suggest that autoreactive T cells do not arise during chronic CS exposure as a simple consequence of compromised Treg function. Our results are in contrast to the report by Deroo et al. (35), which showed an increase in the number of Tregs both in the lungs and in the lung-draining lymph nodes of C57B6 mice after 6 mo exposure to CS. Differences between BALB/cJ and C57B6 mice or differences in the exposure protocol may account for these discordant results. Additionally, Barceló et al. (36) compared the proportion of CD4 T cells that were Tregs in the BAL of never-smokers, COPD patients, and smokers without COPD and found that Tregs were elevated in smokers without COPD, but not in patients with COPD compared with never-smokers. More research needs to be done to better understand the effects of CS on Tregs and how Tregs modulate CS-induced inflammation. However, this manuscript presents the most thorough investigation of the effects of CS on both the number and function of Tregs to date.

CD8\textsuperscript{+} T cells are increased in the Airways of COPD patients, and cytokine and perforin expression among CD8\textsuperscript{+} T cells in the lungs of COPD patients correlates with disease severity (14–17). Furthermore, CD8-deficient mice on a C57B6 background are resistant to airspace enlargement after 6 mo of CS exposure (18). We report that if total T cells from CS-exposed donor mice are transferred into recipients lacking MHC 1 expression, effectively blinding CD8\textsuperscript{+} T cells to Ag, then the ability to transfer airspace enlargement is blocked. This underscores the importance of CD8\textsuperscript{+} T cells in CS-induced inflammation and suggests that their activity is directed by specific recognition of a lung Ag. However, it is still unclear how CD8\textsuperscript{+} T cells promote pulmonary pathology after CS exposure, though it is likely through a combination of both direct and indirect mechanisms. CD8\textsuperscript{+} T cells may directly recognize and eliminate pulmonary epithelial cells through cytotoxic pathways while also producing IFN-γ and TNF-α to damage host tissue indirectly through the activation of neutrophils and macrophage. A large body of research describes the role of macrophages and neutrophils in lung destruction following chronic CS exposure (7–9). These results provide an attractive framework for synthesizing the research on adaptive and innate immune function in the progression of COPD by superimposing a role for CD8\textsuperscript{+} T cells as directors of macrophage and neutrophil activity that persists even after smoking cessation.

Alveolar airspace enlargement and inflammation were not different between MHC class I–deficient mice that received T cells from FA- and CS-exposed donors. However, both groups of MHC class I–deficient recipients had similar, marginal increases in alveolar airspace and inflammation compared with age-matched B2m\textsuperscript{−/−} mice that did not receive T cells. Graft-versus-host disease (GVHD) between the B2m\textsuperscript{−/−} recipients and the WT donor T cells may be one potential explanation for this artifact of the T cell transfers. We did not observe any gross pathology systematically in MHC class I–deficient mice that received either T cells from FA- or CS-exposed mice. Other studies have used B2m\textsuperscript{−/−} mice as recipients in adoptive transfer experiments to determine the requirement for Ag presentation in mouse models of autoimmune disease including NOD mice and experimental autoimmune encephalomyelitis without any reported GVHD (37, 38).
38). The amount of inflammation and airspace enlargement is not severe or accompanied by systemic effects in the B2m−/− mice that received T cells; therefore, the artifact is unlikely to be GVHD or is at least very mild GVHD. Ultimately, we conclude that T cells from CS-exposed mice were unable to cause an increase in MLI in MHC class I-deficient mice compared with recipients of T cells from FA-exposed donors, suggesting that Ag presentation to CD8 T cells in the recipients is required for alveolar destruction caused by CS-exposed T cells.

Although CD8-deficient mice on the C57B6 background were protected from airspace enlargement and inflammation after 6 mo of CS exposure (18), two other reports demonstrated that the adaptive immune system is not required for the development of emphysema or recruitment of macrophage or neutrophils in response to 6 mo of CS exposure using either scid or Rag2−/− mice, which lack both T cells and B cells, on the BALB/cJ strain (28, 39). The conflict between these studies may simply be the result of genetic differences between BALB/cJ and C57B6 strains. However, the studies cast doubt on whether the adaptive immune system, and T cells specifically, are necessary for the development of COPD pathologies. Although the current study does not address this specific question, it is clear from these results, and from our previous work, that T cells from CS-exposed mice are sufficient to cause pulmonary pathology in CS-naive recipient mice.

Awareness of the role of CD4+ T cells, particularly Th17 cells, in COPD and CS-induced inflammation has increased due to recent publications implicating CD4+ T cells and IL-17 in COPD (32–34). As reported previously, we see an increase in the proportion of CD4+ T cells that exhibit a Th17 or Th1 phenotype after CS exposure (32, 34, 40). However, our results indicate that CD4+ T cells may play a supporting role to CD8+ T cells in our model because CD8+ T cells from CS-exposed donors are not sufficient to cause pathology when transferred alone, although total T cells from CS-exposed donors are blocked from driving pathology when recipients lack MHC I expression. Alternatively, the inability of CD8+ T cells from CS-exposed donors to drive pathology when transferred alone may reflect regulatory elements in the host that control the donor CD8+ T cells but are inadequate when CD4+ T cells from the donor are included. CD4+ T cells are necessary for effective and long-lasting CD8+ T cell memory (41–43). It is plausible to suggest that transferring CD8+ T cells from CS-exposed donors without the corresponding CD4+ T cells might deprive the CD8+ T cells of the CD4+ T cells necessary to maintain their pathogenic potential. The perpetuation of CD8+ T cell responses against a chronic viral infection, perhaps the infection model most similar to a CD8+ driven autoimmune disease, requires CD4+ T cell help (44). Our results may then suggest that although CD8+ T cells are the primary cell type promoting lung pathology, CD4+ T cells are nevertheless required for perpetuation of the pathogenic response.

Prior research demonstrating that CS exposure leads to the generation of pathogenic T cells suggested that CS exposure causes a breakdown in peripheral tolerance. Tregs are important in maintaining peripheral tolerance; therefore, we were surprised to find that Tregs were unaffected in number or ability to suppress T cell proliferation. Using all possible combinations of responding T cells and Tregs from both FA- and CS-exposed mice indicated that the responding T cells from FA- and CS-exposed mice were equally susceptible to Treg control. There are two significant implications of these findings: first, there are alternative pathways by which CS exposure causes a breakdown in tolerance; and second, the regulatory pathways activated in T responders by Tregs are intact and present a target for therapeutic intervention. However, it must be noted that our in vitro assay to assess Treg suppression only detects the effects of CS exposure Tregs to directly suppress T cell proliferation. A number of studies have shown that Tregs reduce the expression of costimulatory molecules on the surface of dendritic cells, thereby preventing activation of naive T cells (45, 46). Qureshi et al. (47) have identified a mechanism whereby CTLA-4 on the surface of Tregs binds to CD80 and CD86 on the surface of dendritic cells and leads to endocytosis of the costimulatory molecules. Using flow cytometry, we did not find any differences in the expression of CTLA-4 on the surface of Tregs in the lungs or lung-draining lymph nodes of mice exposed to CS (data not shown). Although we did not find any effects of CS exposure on Treg numbers in the lungs or lung-draining lymph nodes or on the ability of Tregs to suppress T cell proliferation, it is possible that CS exposure impairs the ability of Tregs indirectly to suppress activation of naive T cells by modulating Ag presentation and costimulation by dendritic cells. One potential mechanism whereby CS leads to a breakdown of tolerance is through altering the relationship between dendritic cells and Tregs. Further research is necessary to identify the effects of CS exposure on the complex relationship among Tregs, dendritic cells, and naive T cells.

This report presents evidence that CS exposure drives a pathogenic T cell response that is Ag specific and driven primarily by CD8+ T cells. It details changes in the expression of cytokines by CD4+ and CD8+ T cells in the lungs of mice exposed to chronic CS that are also reflected in T cells recovered from the lungs or sputum of COPD patients. The development of pathogenic T cells in response to CS exposure is not accompanied by any appreciable defect in Tregs, indicating other means through which CS exposure causes a breakdown in peripheral tolerance. These findings are presently the most compelling evidence for the ability of chronic CS exposure to cause an Ag-specific T cell response against self-tissue. Future studies should focus on translating this research toward therapy or screening tools. The T cell–transfer model can also be used to test potential therapies that may slow the progression of COPD in patients whose disease includes an autoimmune component. Identification of the Ag recognized by pathogenic T cells that arise from chronic CS exposure would open the door to study the kinetics of T cell activation and earlier identification of patients with autoreactive T cells, potentially before the onset of clinically relevant symptoms.

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Disclosures

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