T cell depletion protects against alveolar destruction due to chronic cigarette smoke exposure in mice

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The role of T cells in chronic obstructive pulmonary disease (COPD) is not well understood. We have previously demonstrated that chronic cigarette smoke exposure can lead to the accumulation of CD4+ and CD8+ T cells in the alveolar airspaces in a mouse model of COPD, implicating these cells in disease pathogenesis. However, whether specific inhibition of T cell responses represents a therapeutic strategy has not been fully investigated. In this study inhibition of T cell responses through specific depleting antibodies, or the T cell immunosuppressant drug cyclosporin A, prevented airspace enlargement and neutrophil infiltration in a mouse model of chronic cigarette smoke exposure. Furthermore, individual inhibition of either CD4+ T helper or CD8+ T cytotoxic cells prevented airspace enlargement to a similar degree, implicating both T cell subsets as critical mediators of the adaptive immune response induced by cigarette smoke exposure. Importantly, T cell depletion resulted in significantly decreased levels of the Th17-associated cytokine IL-17A, and of caspase 3 and caspase 7 gene expression and activity, induced by cigarette smoke exposure. Finally, inhibition of T cell responses in a therapeutic manner also inhibited cigarette smoke-induced airspace enlargement, IL-17A expression, and neutrophil influx in mice. Together these data demonstrate for the first time that therapeutic inhibition of T cell responses may be efficacious in the treatment of COPD. Given that broad immunosuppression may be undesirable in COPD patients, this study provides proof-of-concept for more targeted approaches to inhibiting the role of T cells in emphysema development.

T-helper 17; chronic obstructive pulmonary disease; cigarette smoke

CHRONIC OBSTRUCTIVE PULMONARY disease (COPD) is the term used to describe a progressive, nonreversible inflammatory condition of the lungs that predominantly occurs as a direct result of chronic exposure to tobacco smoke (33). COPD is characterized by airflow limitation associated with chronic bronchitis that may be accompanied by destruction of the alveoli (5). Although it is well established that chronic pulmonary inflammation is central to the progressive nature of COPD, the precise cellular mechanisms that contribute toward this state are complex, and the understanding of its pathogenesis continues to evolve (1, 26, 41). Whereas neutrophils and macrophages have historically been the focus as effectors of COPD, it is now recognized that infiltrating lymphocyte subsets, including T cells (6, 39), B cells (15, 49), and NK cells (30) (48), may play an important role in disease progression. Indeed, it has recently been postulated that the progressive nature of COPD, which continues even after smoking cessation, may result from an autoimmune response and loss of self-tolerance resulting from chronic tobacco smoke exposure (6, 16, 23, 43).

Along these lines, Motz et al. have demonstrated that CD3+ T cells generated through chronic cigarette smoke exposure in one mouse can transfer a COPD-like disease into an immunodeficient Rag2−/− recipient mouse (29). Although these results clearly demonstrate the ability of T cells to mediate cigarette smoke-induced alveolar destruction, the relative roles of CD4+ T helper vs. CD8+ T cytotoxic subsets and whether these cells could be targeted therapeutically were not addressed. Whereas a number of groups have previously demonstrated a positive correlation between the numbers of infiltrating CD8+ cytotoxic T cells in the lungs and the degree of airflow limitation and disease progression in COPD patients (32, 37), there is increasing evidence that CD4+ T helper cells may also play an important role in orchestrating the chronic inflammatory response (11, 12, 14). It is accepted that CD4+ T cell help plays an important role in driving CD8+ T cell responses, and oligoclonal CD4+ and CD8+ T cells have been described in the lungs of COPD patients (19, 45). Furthermore, CD4+ T cells, which have specificity for the autoantigen elastin, have recently been identified in the peripheral blood of COPD patients (23). In addition, we have previously reported that chronic cigarette smoke exposure led to the infiltration of both Th1- and Th17-type CD4+ T cells in the pulmonary airspaces in a mouse model of cigarette smoke-induced emphysema (12). Despite this accumulating evidence of an important role for T cell subsets in mediating disease progression in COPD, whether these cells can be targeted therapeutically has not been addressed.

In this study, we have examined the effects of specifically targeting the T cell response in a mouse model of cigarette smoke-induced emphysema. Using T cell-depleting antibodies or cyclosporin A, we demonstrate that inhibition of T cell subsets offers significant protection against alveolar destruction and pulmonary neutrophil infiltration arising from chronic cigarette smoke exposure. Furthermore, selective depletion of CD4+ T helper cells offers levels of protection similar to those achieved through the selective depletion of CD8+ T cytotoxic cells. Importantly, T cell depletion also results in significantly decreased pulmonary expression of IL-17A and IL-17A/F heterodimers, as well as of caspase 3 and caspase 7 gene expression and activity, implicating Th17-type responses and apoptosis as important mediators of disease. Finally, protection

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from emphysema is also observed when T cell responses are targeted in a therapeutic manner. These data identify a role for both CD4+ T helper and CD8+ T cytotoxic cells in mediating cigarette smoke-induced emphysema and define the T cell response as a potential therapeutic target for the treatment of COPD.

MATERIALS AND METHODS

Antibodies and reagents. PE rat anti-mouse CD4 (clone H129.19), fluorescein isothiocyanate (FITC) rat anti-mouse CD8 (clone 53–6.7), PerCP hamster anti-mouse CD3e (clone 145–2C11) and appropriate isotype control antibodies PE rat IgG2a, FITC rat IgG2b, and PerCP hamster IgG1, respectively, were purchased from BD Bioscience (San Jose, CA). T cell-depleting antibodies specific for Thyl.2 (clone 30H12), CD4 (clone GK1.5), and CD8 (clone 2.43), and a rat IgG2b isotype control (clone LTF-2), were purchased from BioXCell (West Lebanon, NH). Cyclosporin A (CsA) was purchased from Enzo Life Sciences (Plymouth Meeting, PA).

Mouse cigarette smoke exposure and drug administration. Cigarette smoke exposure was performed as previously described (12). Briefly, beginning at 4 mo of age, female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) received nose-only exposure to 4% cigarette smoke from 3R4F cigarettes (College of Agriculture, Reference Cigarette Program, University of Kentucky), for 2 h/day, 5 days/wk, for up to 24 wk. Smoke was generated by a Baumgartner-Jaeger CSM 2070i Smoking Machine (CH Technologies, Westwood, NJ) containing a circular head that holds 30 cigarettes and performs one revolution per min. A 4% concentration of smoke was produced via a 2×, 35-ml puff of smoke taken from each cigarette once per minute; the resulting 1 liter of smoke was then mixed with 24 liters of air and delivered to the exposure tower. The concentration of total particulate matter, measured by gravimetric filter analysis, was 500 mg/m3. During exposure to smoke or air only (controls), mice were maintained in restraining tubes containing stainless steel nose cone inserts. Twenty hours following the final smoke exposure, bronchoalveolar lavage (BAL) fluid or lung tissue (n = 8/treatment group) was collected, and serum (n = 8) was harvested for cotinine measurement by enzyme-linked immunosorbent assay (ELISA) (Bio-Quant, San Diego, CA). Cotinine levels exhibited by smoke-exposed mice (52.4 ± 11.2 ng/ml) were significantly elevated compared with those exhibited by air-exposed mice (1.47 ± 0.71 ng/ml) (P < 0.001) and were similar to levels reported in human smokers (7). Twice weekly mice received intraperitoneal administration of T cell-depleting antibodies (0.1 ml of Fatal Plus (Vortech Pharmaceuticals, Dearborn, MI), and the trachea was cannulated with a 3-in. section of PE-90 tubing (BD, Franklin Lakes, NJ), to which was attached a blunted 21-gauge needle connected to a three-way stopcock (Baxter Healthcare, Deerfield, IL). Four 1-ml aliquots of ice-cold PBS were injected and removed sequentially through the tubing separately, and the BAL fluid was centrifuged at 140 g for 2 min. Cell pellets isolated from the four aliquots were combined, and total cells were counted using a hemocytometer. Differential cell analysis was performed on cytospins using Wright-Geimsa stain.

Lung tissue preparation and quantification of airspace enlargement. Eight animals from each treatment group were taken for morphometric analysis. Following administration of 0.1 ml of Fatal Plus intraperitoneally, mice were exsanguinated by severing the carotid artery, the trachea was cannulated, and the lungs were inflated to a constant pressure of 25 cm water with 10% formalin for 2 h. The inflated lungs were processed whole, embedded in paraffin, and following a random start were step sectioned at 750-μm intervals until block exhaustion (8–10 steps/lung). At each step, two serial sections (of 3 μm thickness) were collected and used for quantitation. Tissue sections were stained using Harris’ hematoxylin and eosin, and cover slips were applied using the Symphony staining system (Ventana Medical Systems, Tucson, AZ). Slide superimages were generated using an Aperio scanner (Aperio Technologies, Vista, CA). With the use of the Visiopharm system (Visiopharm, Hoersholm, Denmark), random meander-based sampling at ×10 magnification was performed on each lung superimage. Measurements of mean linear intercept (MLI) were performed on a Power Macintosh G3 using Stereology Toolbox 1.3.3 (kindly provided by Kent Pinkerton, University of California Davis) with a 21-line grid. The number of alveolar septa intersecting with a counting line were quantified, with a minimum of 600 intercepts being counted per lung. MLI was calculated according to the method of Kawakami et al. (17).

Measurement of total lung volume. Lungs (n = 6) were inflated to a constant pressure of 25 cm water with 10% formalin for 2 h, and the lung volume was measured by fluid displacement.

Spleen and BAL cell preparation for flow cytometric analysis. Spleens were harvested, cleaned of surrounding tissue, teased apart with forceps, and filtered through a 70-μm mesh filter with the assistance of a 5-ml syringe plunger. The collected cells were then centrifuged at ~600 g for 5 min, and red blood cells were lysed by resuspension of the pellet in 1× M-Lyse Buffer (R&D Systems, Minneapolis, MN) at 2 ml/spleen for 10 min, followed by the addition of 1× Wash Buffer (R&D Systems) at 8 ml/spleen. The cells were centrifuged as above and resuspended in 2 ml of cold antibody diluent (DPBS, 1% FBS, 0.1% azide; all remaining steps at 4°C or on ice). BAL/alveolar samples were centrifuged at ~600 g for 5 min, and the cells were resuspended in cold antibody diluent.

Assessment of cell surface markers by flow cytometry. Spleen or BAL cells (1 × 106) were transferred to a 96-well round-bottom plate, and the samples were centrifuged as above. Each sample was resuspended in 50 μl of cold diluent containing 1 μg of anti-mouse CD16/CD32 monoclonal antibody (clone 2.4G2; BD Biosciences) to block FcR binding. Following incubation for 10 min at 4°C, 50 μl of the appropriate antibody combination were added. The samples received a combination containing each 1 μg each of PE H129.19, FITC 53–6.7, and PerCP 145–2C11 or 1 μg each of the appropriate isotype controls. The samples were incubated for 30 min at 4°C on a plate shaker. Diluent (100 μl) was then added to each sample, and the plate was centrifuged at 1000 g for 5 min. The supernatants were aspirated, and two more washes were performed in a similar fashion using 200 μl of diluent. Each sample was then resuspended in 100 μl of diluent and transferred to 900 μl of diluent containing 0.5 μM ToPro-3 (Invitrogen Molecular Probes, Carlsbad, CA) for the purpose of eliminating nonviable cells from the analysis. Events were collected on a fluorescence-associated cell sorter (FACS) Cantos flow cytometer (BD Biosciences) and analyzed using FACS Diva software.

Culture of lung cells for cytokine assessment. Following perfusion with DPBS, mouse lungs were removed and cleaned of other tissue by dissection. The lungs were rinsed with DPBS and minced into small pieces. For each sample (n = 1), the lung pieces from two mice were pooled into a single gentleMACS C tube (Miltenyi Biotech, Auburn, CA) containing 4.9 ml of prewarmed (37°C) Digestion Medium [HEPES buffer (Lonza, Walkersville, MD) containing 2 mg/ml col-

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Assays were performed according to the manufacturers’ instructions. Total Nitric Oxide and Nitrite/Nitrate assay kit (R&D Systems). The samples were then filtered through separate 70-μm strainers, and the cells were pelleted at ~600 g. Erythrocytes were lysed using a Mouse RBC Lysis kit (R&D Systems); the cells of each sample were resuspended in 2 ml of 1× Lysis Buffer for 10 min followed by addition of 8 ml of 1× wash buffer. The samples were pelleted as before, and each was resuspended in 10 ml of medium [RPMI-1640 (Mediatech, Manassas, VA) supplemented with 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate, 50 μg/ml 2-mercaptoethanol, 10% heat-inactivated FBS, and antibiotic mixture (Invitrogen)]. The cells were counted, pelleted, and resuspended in medium at 1×10^6 cells/ml before being placed in 24-well plates for 4 h. The supernatants were then collected, clarified at 10% heat-inactivated FBS, and antibiotic mixture (Invitrogen). The plate was incubated for an additional 5 min. The samples were then filtered through separate 70-μm strainers, and the cells were pelleted at ~600 g. Erythrocytes were lysed using a Mouse RBC Lysis kit (R&D Systems); the cells of each sample were resuspended in 2 ml of 1× Lysis Buffer for 10 min followed by addition of 8 ml of 1× wash buffer. The samples were pelleted as before, and each was resuspended in 10 ml of medium [RPMI-1640 (Mediatech, Manassas, VA) supplemented with 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate, 50 μg/ml 2-mercaptoethanol, 10% heat-inactivated FBS, and antibiotic mixture (Invitrogen)].

The cells were counted, pelleted, and resuspended in medium at 1×10^6 cells/ml before being placed in 24-well plates for 4 h. The supernatants were then collected, clarified at ~15,000 g, and stored at ~80°C until cytokine analysis was performed. Analysis was performed using ELISA kits for IL-17A, IL-17A/F, monocyte chemotactic protein (MCP)-1, interferon (IFN)-γ, KC, macrophage inflammatory protein (MIP)-1α, and MIP-2 (R&D Systems) as directed by the manufacturer.

mRNA analysis. Mouse lung tissue was collected and placed immediately in 5 ml of RNAlater Stabilization Reagent (Applied Biosystems/Ambion, Austin, TX), cut into small pieces <5 mm in thickness, incubated overnight at 2–8°C, and then stored at ~80°C. Total RNA was isolated from lung using Trizol reagent (Life Technologies, Grand Island, NY) and homogenized using a gentleMACS Dissociator (Miltenyi Biotec). RNA quantity and integrity were assessed by UV Spectroscopy and an Agilent Gel RNA 6000 Nano Kit (Agilent Technologies, Foster City, CA), respectively. Intact RNA was then normalized and reverse transcribed into cDNA using a QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA).

Quantitative RT-PCR was performed in triplicate using an Applied Biosystems 7900HT Fast Real-Time PCR system (Foster City, CA). Transcripts were amplified using TaqMan Gene Expression Assays (Applied Biosystems) that contained primers and a fluorogenic FAM-labeled probe specific for murine matrix metalloproteinase-12 (MMP-12) (Assay ID Mm00432045_m1), glutathione peroxidase 1 (GPx1) (Mm00500821_m1), B cell lactic dehydrogenase (Bak) (Mm00432045_m1), glutathione peroxidase 1 (GPx1) (Mm00439154_m1), superoxide dismutase 1 (SOD1) (Mm01344233_g1), superoxide dismutase 2 (SOD2) (Mm00433237_m1), and FasL (Mm00438864_m1). Eukaryotic 18S RNA (part no. 4310893E) was used for normalization. The 20-μl PCR reactions were composed of 1× TaqMan Fast Universal Master Mix (Applied Biosystems), 1× primers/probes, and 2 μl cDNA from reverse transcription reaction and were made up to a volume with diethyl pyrocarbonate-treated H2O. Relative quantification of data was performed using the comparative cycle threshold (Ct) method (25).

Assay of lipid peroxidation products and nitric oxide. The lipid peroxidation products malondialdehyde (MDA) and 4-hydroxynonenals (4HDA) were measured in lung homogenate samples using the Bioxytech LPO-586 kit (OxisResearch, Burlingame, CA). Nitric oxide levels were determined in lung homogenate samples using the Total Nitric Oxide and Nitrite/Nitrate assay kit (R&D Systems). Assays were performed according to the manufacturers’ instructions.

Caspace 3/7 assay. To measure the activities of caspase 3 and caspase 7, 10 μl of lung homogenate samples, diluted 1:25 in Apo-One Caspase 3/7 substrate buffer (Promega), and 10 μl of 50 μM z-DEVD-R110 substrate (Invitrogen) were assayed in triplicate in a 384-well plate. The plate was incubated in the dark at room temperature for 60 min. Fluorescence was measured at excitation and emission wavelengths of 499 and 535 nm, respectively.

Statistical analysis. Data are presented as means ± SE. Statistical significance was determined using a one-way ANOVA with a Dunnett’s posttest. Values of P < 0.05 were considered significant.

RESULTS

Establishment of a model to examine the role of T cell responses as mediators of COPD in mice chronically exposed to mainstream cigarette smoke. We have previously demonstrated that mice exposed to mainstream cigarette smoke for 2 h/day, 5 days/wk, for a period of 24 wk, exhibited significant levels of alveolar destruction as measured by morphometric analysis (12). As in human smokers, both gaseous constituents and particulate matter (500 mg/m³) found in mainstream cigarette smoke in this model are likely to play a role in contributing toward tissue destruction and inflammation observed. The T cell subsets identified in the BAL fluid of these mice were characterized as CD8⁺ T cytotoxic cells, CD4⁺ T helper cells of both the Th1 and Th17 subsets, and double-negative T cell subsets (12). To determine what role, if any, these T cell subsets play in the pathogenesis of COPD, we sought to specifically target T cell responses, initially through prophylactic administration of T cell-depleting antibodies. Antibodies were first administered beginning 2 wk before the commencement of smoke exposure. We used the GK1.5 (anti-CD4) antibody to deplete CD4⁺ T cells, the 2.43 (anti-CD8) antibody to deplete CD8⁺ T cells, and both of the above in combination with an anti-Thy1.2 (clone 30H12) antibody to deplete all T cell subsets (Fig. 1A). To further examine the role of adaptive immunity, and T cell responses in particular, in the pathogenesis of COPD, we also examined whether the immunosuppressant drug CsA had a similar effect on disease progression to that observed with T cell-depleting antibodies. Although CsA can mediate pleiotropic effects on a variety of cell types, it has been approved since the early 1980s as an immunosuppressant and is commonly used to inhibit transplant rejection through its profound inhibition of allogeneic T cell responses (28). Furthermore, we extended our analysis by administering T cell-depleting antibodies or CsA in a therapeutic fashion at the midpoint in the study, after 12 wk of cigarette smoke exposure, and continuing through 24 wk (Fig. 1B). To confirm efficient depletion of T cell subsets under both prophylactic and therapeutic strategies, the percentages of both CD4⁺ and CD8⁺ T cell subsets in both the spleens and BAL of treated mice were analyzed after 6 and 24 wk. Figure 2, A–D, shows the sequential gating strategy used for flow cytometric analysis of the T cell subsets. Dead cells and debris were excluded from analysis by gating (P1) on live whole cells (Fig. 2A). Within the live P1 gate, the lymphocyte-containing region (P2) in the light scatter plot was gated on to exclude larger, more granular, and more autofluorescent cell types from subsequent analysis (Fig. 2B). CD3⁺ cells from the lymphocyte region were then gated on (P3) (Fig. 2C), and CD3⁺ cells were assessed for CD4⁺ and CD8⁺ cells (P5 and P6, respectively) (Fig. 2D). In mice exposed to smoke and treated prophylactically for 6 wk with the pan T cell–, CD4–, or CD8-depleting antibodies, efficient T cell depletion was observed, with splenic
CD4+ cells undergoing reductions of 100% following depletion with either pan T cell or anti-CD4 antibodies and splenic CD8+ cells undergoing reductions of 100 and 96% following depletion with pan T cell or anti-CD8 antibodies, respectively, compared with smoke-exposed/isotype control-treated mice (Fig. 2E). BAL CD4+ cells underwent reductions of 100 and 96% following pan T cell or CD4+ T cell depletion, respectively, whereas BAL CD8+ cells underwent reductions of 100 and 93% following pan T cell or CD8+ T cell depletion, respectively (Fig. 2F). Additionally, this study confirmed that the anti-CD4 and anti-CD8 antibodies did not nonspecifically deplete CD8+ and CD4+ cells, respectively (Fig. 2, E and F), although, interestingly, treatment with the anti-CD4 antibody resulted in a sixfold increase in BAL CD8+ cells compared with smoke-exposed/isotype control-treated mice. We hypothesize that this is a secondary effect of the anti-CD4 antibody, with depletion of CD4+ cells resulting in a redistribution of CD8+ cells. Importantly, efficient depletion of T cell subsets was maintained throughout the study duration, as demonstrated by similar analysis at the study endpoint after 24 wk (data not shown).

Prophylactic depletion of T cell subsets results in significant protection from cigarette smoke-induced airspace enlargement and neutrophil infiltration. To determine whether T cells play a significant role in driving disease pathogenesis in this model, T cell subsets were depleted through repeated administration (two times weekly) of all three T cell-depleting antibodies or either anti-CD4 or anti-CD8 antibodies in isolation. Depletion of total T cell subsets beginning 2 wk before commencement of chronic cigarette smoke exposure was found to result in significant protection from alveolar destruction (76%) as assessed by MLI (Fig. 3A) as well as changes in lung volume (data not shown). This protection occurred in association with a significant inhibition of neutrophil influx in the bronchoalveolar airspaces of smoke-exposed mice, whereas numbers of mononuclear cells (largely macrophages) were not affected (Fig. 3, B and C). Similarly, depletion of either CD4+ or CD8+ subsets in isolation also offered significant protection from disease albeit at reduced levels from those observed upon total T cell depletion (39 and 59%, respectively) (Fig. 3D). Again this protection occurred in association with reduced numbers of infiltrating neutrophils, whereas mononuclear cell numbers were not altered (Fig. 3, E and F). Similar to our observations upon total T cell depletion, administration of CsA was found to offer protection from disease (80%) as assessed by MLI (Fig. 3G), and changes in lung volume (data not shown), as well as protection from neutrophil influx (Fig. 3, H and I).

Together these data demonstrate that T cell subsets play an important role in disease pathogenesis in response to chronic cigarette smoke exposure. Moreover, both CD4+ T helper and CD8+ T cytotoxic subsets contribute to alveolar tissue destruction, potentially by facilitating the recruitment of neutrophils in the bronchoalveolar airspaces.

Expression of T cell effector cytokines in the lungs of mice after chronic cigarette smoke exposure. We have previously demonstrated that both Th17- and Th1-type T cells are present in the bronchoalveolar airspace of mice after chronic smoke exposure. To determine which of these effector T cell subsets may play a role in driving disease pathogenesis, we examined the levels of expression of their signature cytokines (IL-17A and IFN-γ, respectively) by lung cells of smoke-exposed mice. At the midpoint in the study (12 wk), single cell suspensions from the lungs of control air- and smoke-exposed mice that had been treated with isotype control or total T cell-depleting antibodies or vehicle beginning at 12 wk.
Fig. 2. Efficient depletion of peripheral and lung infiltrating T cells with specific antibodies. A–D show the sequential gating strategy used for flow cytometric analysis of T cell subsets. A: live whole cell gate (P1). B: lymphocyte-containing region (P2) within the P1 gate. C: CD3+ cells (P3) within the P2 gate. D: assessment of CD3+ cells for CD4+ and CD8+ cells (P5 and P6, respectively). The percentage of CD3+ cells is with respect to the grandparent gate, P1. The CD4 and CD8 percentages shown are with respect to CD3+ cells. CD4+ and CD8+ T cells as a percentage of total live cells in the spleens (E) and bronchoalveolar lavage (F) of mice treated with isotype control, total T cell, or specific CD4+ or CD8+ subset-depleting antibodies. Data are presented as means ± SE with 8 mice/group. Nos. above columns represent %reduction compared with relevant smoke-exposed/isotype control-treated mice.
antibody regimens were isolated and placed in culture. After 48 h, expression levels of T cell cytokines were examined in the supernatants by ELISA. The rationale for use of this approach, rather than intracellular cytokine staining as detected by flow cytometric analysis, was that in the T cell-depleted mice the cells of interest would be largely absent (e.g., there would be few or no cells on which to gate). Chronic smoke exposure led to a significant increase in the levels of both IL-17A and IL-17A/F heterodimers expressed in the lungs of mice. Critically, these increases were reversed when total T cell subsets were depleted (Fig. 4, A and B, respectively). Levels of IFN-γ expressed were low and did not appear to be significantly altered upon chronic smoke exposure (Fig. 4C).

These data are in agreement with our earlier observations identifying Th17-type cell infiltrates in the lungs of chronically smoke-exposed mice (12). Importantly, they also implicate IL-17A and IL-17A/F as mediators of disease, possibly via recruitment of neutrophils. To further investigate the role of T cells in driving disease pathogenesis, we also examined the expression levels of a number of chemokines that have previously been implicated in this model (2, 4). Although chronic smoke exposure led to significant increases in the expression of MCP-1, MIP-1α, and MIP-2, these levels were not altered in the absence of T cell subsets (Fig. 5, A, B, and C, respectively). Because both MCP-1 and MIP-1α are known to be important mediators of macrophage recruitment to inflammatory sites, these data are consistent with our observations (Fig. 3) that T cell depletion does not affect macrophage recruitment in this model. We also examined expression of KC by lung infiltrating cells, and, although its expression appears to be increased upon smoke exposure, these levels did not reach statistical significance (Fig. 5D).

Pulmonary expression of molecules relevant to proteolytic, oxidative stress, and apoptotic pathways after chronic cigarette smoke exposure. In addition to the inflammatory response, the primary physiological mechanisms believed to underlie the pathogenesis of COPD are proteolysis, oxidative stress, and apoptosis. To determine if these mechanisms contribute to the protection from alveolar destruction observed following T cell depletion, the gene/protein expression and/or activity of mol-
ecules relevant to these mechanisms were examined in the lungs of air- or smoke-exposed mice treated with isotype control or total T cell-depleting antibody regimens for 12 wk. Gene expression of MMP-12, a protease that has been reported to play a role in both animal models of COPD and in human disease (9, 13), was significantly increased as a result of smoke exposure, and somewhat unexpectedly, was increased further following total T cell depletion (Fig. 6 A). As previously reported (35), the antioxidant genes HO-1 and NQO1 were increased in expression following smoke exposure; however, they were not further modulated as a result of T cell depletion (Fig. 6 B and C). Levels of lipid peroxidation products (MDA and 4HDA) and nitric oxide, indicative of oxidative/nitrosative stress, were not significantly modulated by smoke exposure or T cell depletion. Similarly, gene expression of the antioxidant enzymes GPx1, GPx2, GR, SOD1, and SOD2 did not change as a result of smoke exposure or T cell depletion (data not shown).

Gene expression of the proapoptotic enzymes caspase 3 and caspase 7 increased significantly as a result of smoke exposure,
and these increases were reversed upon T cell depletion (Fig. 7, A and B). These changes in caspase 3 and caspase 7 mRNA levels were consistent with the changes observed in an assay measuring the combined enzymatic activities of caspase 3 and caspase 7 (Fig. 7C). Similar to the modulation of the antioxidant genes HO-1 and NQO1, expression of the antiapoptotic gene Bcl-2 was significantly increased following smoke exposure but not changed as a result of T cell depletion (Fig. 7D). Expression levels of the proapoptotic genes Bim, Bak, Fas, and FasL were not modulated as a result of smoke exposure or T cell depletion (data not shown).

These data suggest that regulation of the apoptotic pathway, but not of oxidative/nitrosative stress, contributes to the mechanism by which total T cell depletion protects from cigarette smoke-induced alveolar destruction.

**Therapeutic depletion of T cell subsets or treatment with CsA protects mice from cigarette smoke-induced alveolar airspace enlargement.** Because we have demonstrated that prophylactic depletion of T cell subsets or treatment with CsA can attenuate the development of disease, we sought to investigate whether therapeutic inhibition of T cell responses could affect disease progression in a similar manner. To achieve this, we administered T cell-depleting antibodies or CsA beginning at the midpoint of the study, after 12 wk of cigarette smoke exposure. This time point was chosen given the significant levels of inflammation and tissue destruction already present.

**Fig. 6.** Pulmonary expression of molecules relevant to proteolytic and oxidative stress pathways in the lungs after chronic cigarette smoke exposure. Levels of expression of matrix metalloproteinase (MMP)-12 (A), heme oxygenase-1 (HO-1) (B), and NAD(P)H-quinone oxidoreductase 1 (NQO1) (C) mRNA in the lungs of mice that had undergone 12 wk of cigarette smoke exposure and prophylactic treatment with either total T cell-depleting or isotype control antibodies. Data are presented as means ± SE with 8 mice/group. Statistical significance was determined using a one-way ANOVA with a Dunnett’s posttest. *P < 0.05.

**Fig. 7.** Pulmonary expression of molecules relevant to apoptosis in the lungs after chronic cigarette smoke exposure. Levels of expression of caspase 3 mRNA (A), caspase 7 mRNA (B), caspase 3/7 activity (C), and B cell lymphoma 2 (Bcl-2) (D) mRNA in the lungs of mice that had undergone 12 wk of cigarette smoke exposure and prophylactic treatment with either total T cell-depleting or isotype control antibodies. Data are presented as means ± SE with 8 mice/group. Statistical significance was determined using a one-way ANOVA with a Dunnett’s posttest. *P < 0.05, **P < 0.01, and ***P < 0.001.
As shown in Fig. 8, E–J, therapeutic depletion of T cell subsets, or administration of CsA, resulted in significant attenuation of alveolar tissue destruction and neutrophil infiltration, comparable to levels observed upon treatment throughout the entire period of cigarette smoke exposure (Fig. 3). Specific depletion of either CD4$^+$ or CD8$^+$ T cell subsets beginning at 12 wk offered similar levels of protection from alveolar destruction (Fig. 8G). Similar to our prophylactic studies, depletion of each T cell subset led to a reduction in neutrophil infiltration, which achieved statistical significance.

Fig. 8. Therapeutic depletion of T cell subsets inhibits alveolar tissue destruction and neutrophil infiltration of the airways of cigarette smoke-exposed mice. Representative histology (A) and morphometric analysis (B) of alveolar airspace (mean linear intercept) from 12-wk air- or cigarette smoke-exposed mice. Nos. of neutrophils (C) and mononuclear cells (D) in the bronchoalveolar lavage of 12-wk air- or smoke-exposed mice. Morphometric analysis of alveolar airspace (mean linear intercept) from 24-wk air- or cigarette smoke-exposed mice following therapeutic treatment with total T cell-depleting antibodies (E), CD4$^+$ or CD8$^+$ T cell-depleting antibodies (G), or CsA (I), beginning after 12 wk. Nos. of neutrophils in the bronchoalveolar lavage of 24-wk air- or smoke-exposed mice following therapeutic treatment with total T cell-depleting antibodies (F), CD4$^+$ or CD8$^+$ T cell-depleting antibodies (H), or CsA (J). Data are presented as means ± SE with 8 mice/group. Statistical significance was determined using a one-way ANOVA with a Dunnett’s posttest. **P < 0.01 and ***P < 0.001.
upon CD4$^+$ but not CD8$^+$ T cell depletion (Fig. 8H). Furthermore, total T cell depletion in a therapeutic manner reversed the increased IL-17A and IL-17A/F heterodimer expression induced upon chronic cigarette smoke exposure (Fig. 9, A and B). Similar to our prophylactic studies described above, no significant alterations in the expression levels of IFNγ, KC, MCP-1, MIP-1α, and MIP-2 were observed upon therapeutic treatment (data not shown). Importantly, these results indicate that therapeutic targeting of T cell subsets may represent a viable strategy in preventing progressive alveolar destruction among emphysema patients.

**DISCUSSION**

The role of the adaptive immune response, and T cells in particular, in the pathogenesis of COPD has until recent times been somewhat controversial. Historically, the focus has been on cells of the innate immune response in mediating disease, since these cells are found in greater abundance at the site of inflammation and in the pulmonary airspaces (15). However, recent advances in our understanding of the cellular mechanisms that contribute toward the chronic inflammation observed in COPD have revealed a previously unappreciated role for T cell subsets as possible mediators of disease pathogenesis (18).

Recently, we have identified two distinct CD4$^+$ T helper cell subsets expressing IL-17 (Th17) or IFN-γ (Th1) in the pulmonary airspace of mice exhibiting significant levels of alveolar destruction as a result of chronic cigarette smoke exposure (12). Although these T cell subsets represented a relatively small proportion of the overall inflammatory cell infiltrate, which is dominated by neutrophils and macrophages, we hypothesized that these cells play an important role in the pathogenesis of disease. In this study we have taken a number of different approaches to inhibit T cell responses in a murine model of cigarette smoke-induced emphysema. First, we have demonstrated that prophylactic depletion of all T cell subsets using a cocktail of depleting antibodies results in protection against airspace enlargement. This effect occurs in association with a decrease in neutrophil infiltration, and in pulmonary IL-17A and IL-17A/F expression, induced upon chronic smoke exposure. Because IL-17A plays an important role in recruiting neutrophils to the site of cigarette smoke-induced inflammation (42), and a significant body of literature demonstrates a causative role for neutrophils in emphysema (31, 34, 44), this observation provides a potential mechanistic link to our previous studies identifying Th17 cells in the airways of emphysematous mice (20). Similar to the IL-17A homodimer, the IL-17A/F heterodimer is biologically active, inducing neutrophil recruitment and pulmonary expression of the CXC chemokines KC (CXCL1) and ENA-78 (CXCL5) in vivo (24) and the expression of KC and IL-6 from mouse fibroblasts in vitro (3). Additional studies are required to determine if the IL-17A/F heterodimer plays a role distinct from that of the IL-17A homodimer in the etiology of COPD. Consistent with these data implicating IL-17A and IL-17A/F in the mechanism by which T cells mediate alveolar destruction, a number of recent reports have highlighted the importance of Th17 cells in the pathogenesis of COPD in both humans and mouse models of disease (4, 40). Using IL-17RA-deficient mice, Chen et al. demonstrated that the IL-17 receptor-α chain was required for cigarette smoke-induced macrophage recruitment and alveolar airspace enlargement in a model similar to that used in this study. However, no analysis of infiltrating neutrophils was reported (4). In contrast, we did not observe any changes in the levels of macrophage infiltration upon T cell depletion. Furthermore, we did not detect any changes in cigarette smoke-induced levels of the chemokines MCP-1 (CCL2) and MIP-1α (CCL3), which are thought to play a central role in recruiting macrophages to the site of inflammation. These discrepancies may reflect differences between T cell-derived and innate sources of IL-17, as well as redundancy of function between IL-17-related cytokines and their respective receptors. Our data clearly demonstrate the role that T cell subsets play in facilitating neutrophil recruitment to the lungs upon chronic cigarette smoke exposure. Further studies will be necessary to determine whether T cells, in addition to their role in the recruitment of other leukocyte subsets, directly induce tissue destruction in response to chronic cigarette smoke exposure.

Underlying the development of COPD are complex interactions between the inflammatory, proteolytic, oxidative stress, and apoptotic pathways (10, 46). To determine which, if any, of these pathways play a role in the protection from alveolar destruction seen following T cell depletion, molecules/activities relevant to the proteolytic, oxidative stress, and apoptotic pathways were examined. Components of each of these pathways exhibited a cigarette smoke-induced upregulation, including MMP-12, HO-1, NQO1, caspase 3, caspase 7, and Bcl-2. Interestingly, only caspase 3 and caspase 7 were significantly decreased in expression/activity upon T cell depletion, indicating a downregulation of the apoptotic pathway in the...
absence of T cells. Although this study does not formally demonstrate a role of T cell-driven caspase activity in disease pathogenesis, these results are consistent with the reduced levels of alveolar destruction and neutrophil infiltration observed in T cell-depleted animals, given that apoptosis has been widely reported to underlie emphysema development (36, 38, 50). In contrast to the other molecules examined, whose expression remained unchanged or decreased upon T cell depletion, MMP-12 gene expression increased. Although several groups have reported a positive association between MMP-12 expression/activity and cigarette smoke-induced neutrophil recruitment (21, 22), using lipopolysaccharide as a stimulus, Dean et al. demonstrated an early proinflammatory role of MMP-12, inducing recruitment of neutrophils, and a late anti-inflammatory role, causing termination of neutrophil influx (8). This dichotomy is consistent with our observation of elevated MMP-12 expression under conditions of both smoke-induced neutrophil increase and T cell depletion-induced neutrophil reduction.

In addition to the protection from disease afforded by prophylactic T cell depletion, we demonstrate for the first time that therapeutic depletion of T cell subsets results in significant protection against disease. These data indicate that T cells play an important role in disease progression, since significant levels of tissue destruction were already evident at the time chosen to commence therapeutic treatment. We extended this analysis to examine the relative contribution of CD4+ T helper vs. CD8+ T cytotoxic cells toward disease pathogenesis and demonstrated that specific depletion of either subset resulted in similar levels of protection from airspace enlargement. While on the one hand these data may suggest participation of the cell subsets in parallel but independent processes leading to a common endpoint, they may also reflect a requirement of CD8+ T cells for appropriate CD4+ T cell “help” to mount an efficient response. Our observation that depletion of CD4+ T cells can inhibit emphysema is in contrast to a previous report by Maeno et al. demonstrating that CD4 knock-out mice did not show any protection in a similar chronic model of cigarette smoke-induced emphysema (27). However, because these mice have been characterized as having severe abnormalities in T cell subset development, and indeed retain a significant repertoire of major histocompatibility class II-restricted T cells (47), we believe our depletion strategy to be a more accurate approach. Finally, we also examined the ability of the well-characterized T cell immunosuppressant drug CsA (28) to inhibit disease progression and found that treatment could inhibit emphysema development, as well as neutrophil infiltration, to the same extent as total T cell depletion. This demonstrates that a therapeutic agent approved specifically to target T cell responses in human disease also inhibits disease progression in this model.

There are several potential limitations to the approach described herein that should be noted. Chronic administration of rat T cell-depleting antibodies in the mouse can lead to the production of neutralizing antibodies to the xenogeneic proteins. Based on the high level of T cell depletion also confirmed at the termination of the study, it appears that the rat antibodies did not lose effectiveness, although an effect of antibody complex formation on the disease phenotype cannot be ruled out. In addition, cells other than T cells express CD4, CD8, and Thy1.2, raising the potential for the antibodies to deplete or alter the function of these cells, and thus affect the disease phenotype. Finally, although IL-17A and IFN-γ are widely recognized as the signature cytokines of Th17 and Th1 cells, respectively, these cytokines are produced by additional cell types, including CD8+ T cells, γδ T cells, NK cells, and NKT cells (IL-17A) and CD8+ T, NK cells, macrophages, and dendritic cells (IFN-γ). Thus one or more these cell types may contribute to the cytokine expression reported in this study and warrant further investigation.

In summary, the data presented in this study provide, to our knowledge, the first demonstration that depletion of either CD4+ T helper or CD8+ T cytotoxic T cells can offer significant protection from emphysema in a mouse model of chronic cigarette smoke exposure. Importantly, strategies to inhibit T cell responses were equally effective in preventing disease progression whether administered before the commencement of the study or at the midpoint in the study in a therapeutic fashion. Together these data indicate that the T cell response may represent a previously unappreciated therapeutic target in COPD patients. Although it should be noted that broad immunosuppression elicited by such strategies may be undesirable in COPD patients, given their susceptibility to infection and exacerbation, this study provides proof of concept for more targeted approaches to inhibit the central role that T cell subsets play in promoting emphysematous tissue destruction.

DISCLOSURES

All authors are either current or former employees of GlaxoSmithKline.

AUTHOR CONTRIBUTIONS


REFERENCES


