Exposure to Cigarette Smoke Inhibits the Pulmonary T-Cell Response to Influenza Virus and Mycobacterium tuberculosis

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Smoking is associated with increased susceptibility to tuberculosis and influenza. However, little information is available on the mechanisms underlying this increased susceptibility. Mice were left unexposed or were exposed to cigarette smoke and then infected with Mycobacterium tuberculosis by aerosol or influenza A by intranasal infection. Some mice were given a DNA vaccine encoding an immunogenic M. tuberculosis protein. Gamma interferon (IFN-γ) production by T cells from the lungs and spleens was measured. Cigarette smoke exposure inhibited the lung T-cell production of IFN-γ during stimulation in vitro with anti-CD3, after vaccination with a construct expressing an immunogenic mycobacterial protein, and during infection with M. tuberculosis and influenza A virus in vivo. Reduced IFN-γ production was mediated through the decreased phosphorylation of transcription factors that positively regulate IFN-γ expression. Cigarette smoke exposure increased the bacterial burden in mice infected with M. tuberculosis and increased weight loss and mortality in mice infected with influenza virus. This study provides the first demonstration that cigarette smoke exposure directly inhibits the pulmonary T-cell response to M. tuberculosis and influenza virus in a physiologically relevant animal model, increasing susceptibility to both pathogens.

It is estimated that 5.5 trillion cigarettes are produced globally each year and are smoked by more than 1.1 billion people (http://www.who.int/tobacco/en/atlas8.pdf). An even greater number of people are exposed to second-hand cigarette smoke, when smoke exhaled by the smoker is inhaled by others. Epidemiological studies have shown that tuberculosis and influenza are both more common in smokers than nonsmokers (1, 12, 19, 20, 27, 30). However, it is unclear how cigarette smoke exposure increases susceptibility to these infections. Candidate vaccines against both tuberculosis and influenza are being tested in clinical trials, but it is uncertain if exposure to cigarette smoke reduces the efficacy of these vaccines. Tuberculosis claims 1.9 million lives annually world-wide, and influenza kills an estimated 40,000 persons annually in the United States. Pandemic influenza has killed up to 50 million persons (45), and the recent pandemic of H1N1 swine influenza underscores the potential for this infection to spread rapidly and cause substantial morbidity and mortality. Given the prevalence of exposure to cigarette smoke, it is critical to determine if cigarette smoke increases susceptibility to tuberculosis and influenza or if it affects the efficacy of vaccination against these diseases.

T cells contribute significantly to host defenses against both Mycobacterium tuberculosis and influenza virus. CD4+ cells produce gamma interferon (IFN-γ), which activates macrophages to kill intracellular M. tuberculosis, in part through the induction of nitric oxide (3, 5, 39). CD8+ T cells are critical for the clearance of influenza virus infection (9, 10, 44), and recent evidence suggests that CD4+ cells also contribute to immunity against influenza (4, 7, 42). Limited information is available on the effects of cigarette smoke on T-cell responses to Mycobacterium tuberculosis and influenza infection.

Most studies of the effects of smoking on the immune response have used in vitro assays, such as the addition of tobacco extracts to cell cultures or nicotine exposure through subcutaneous pumps, which impairs T-cell proliferation and T-cell receptor-mediated signaling (14). We evaluated the effect of cigarette smoke exposure on the lung immune response to Mycobacterium tuberculosis and influenza A virus, using a physiologically relevant model in which animals were exposed to environmental cigarette smoke. Using this model, we also investigated how cigarette smoke exposure affected the mucosal response to vaccination against Mycobacterium tuberculosis.

MATERIALS AND METHODS


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Animal specific-pathogen-free 6- to 8-week-old female DR4 transgenic mice expressing the human HLA-DRB1*0401 allele (DR4) or their wild-type

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C57BL/6 counterparts were purchased from Taconic (Albany, NY) or bred at the University of Texas Health Science Center at Tyler. The Institutional Animal Care and Use Committee approved all of the protocols for the animal experiments.

**Exposure to second-hand cigarette smoke.** Mice were randomly divided into an unexposed (control) and cigarette smoke-exposed groups. A smoke exposure chamber (Teague Enterprises, Davie, CA) was used to expose animals to a combination of fresh air and a defined level of cigarette smoke, mimicking natural exposure to environmental cigarette smoke in daily life. We exposed the mice to cigarette smoke from a 1R4F cigarette (University of Kentucky Reference Cigarette) for 120 min, twice (with a 2-h break in between) daily, 5 days a week for 6 weeks. The total smoke particulate concentration during exposure was approximately 80 mg/m³. Control animals were kept in an adjacent room.

Mice that were infected with M. tuberculosis were housed in the biosafety level 3 laboratory, where a smoke exposure chamber is not available. Therefore, mice were not exposed to cigarette smoke after infection. Mice that were infected with influenza A virus continued to be exposed to cigarette smoke after infection.

**Isolation of T cells.** Cell suspensions generated from lungs and spleens were centrifuged, red blood cells were lysed, and the cells were washed twice with complete RPMI medium. T cells were isolated by immunomagnetic separation according to the manufacturer’s recommendations (Miltenyi Biotech). Cell purity was confirmed by flow cytometry.

**T-cell stimulation.** Cells were stimulated with suboptimal concentrations of anti-CD3/anti-CD28. Cells were added to 96-well plates, precoated with 3 µg/ml anti-mouse CD3 and 1 µg/ml anti-mouse CD28, and incubated at 37°C, 95% relative humidity, and 5% CO₂. The supernatants were collected 72 h later and stored at −70°C for the measurement of IFN-γ levels by enzyme-linked immunosorbent assay (ELISA). For the enzyme-linked immunospot (ELISPOT) assay, plates were coated with anti-CD3, anti-CD28, and anti-IFN-γ. Cells were then added and incubated for 24 to 48 h, as noted below.

**ELISPOT assay to determine the frequency of IFN-γ-producing T cells.** The ELISPOT assay was performed as previously described (21). To evaluate stimulated cells, plates were coated with 3 µg/ml anti-CD3, 1 µg/ml anti-CD28, and 10 µg/ml anti-IFN-γ. After overnight incubation and blocking, cells were added to the plates and incubated for 24 to 48 h. After being washed, the detection antibody (Ab) R4-6A2-Biotin (Mabtech) was added, followed by streptavidin–alkaline phosphatase (Mabtech) and a substrate solution (5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium [BCIP-NBT]; BD Bioscience). Plates were rinsed with tap water and air dried, and spots were counted with a stereomicroscope (Olympus SZ2-L2B).

**ELISA to measure IFN-γ concentrations.** The ELISA was performed as previously described (21), using coating and detection monoclonal Abs (Mabs) to mouse IFN-γ (Mabtech). The sensitivity of the assay was 50 pg/ml.

**DNA immunization.** The construction of the pcDNA3.1(+) cukaryotic expression vector to express the M. tuberculosis 10-kDa culture filtrate protein (CFP10) and lysosomal integral membrane protein II and vaccination with this construct were performed as described previously (21). Briefly, endotoxin-free plasmid was diluted in endotoxin-free PBS and incubated to 1 mg/ml, and 100 µg of plasmid DNA was mixed with linear polyethyleneimine (PEI: Bridge Bioscience) solution according to the manufacturer’s instructions, and 200 µl of the mixture containing 100 µg of plasmid DNA was administered once intravenously (i.v.) via the retro-orbital route, as described elsewhere (21).

**Animal infections. (i) Tuberculosis.** Mice were infected with M. tuberculosis H37Rv via aerosol, using an exposure chamber made to order by the University of Wisconsin. To confirm the accuracy of infection, 24 h after infection, 5 mice were sacrificed, their lungs were homogenized and plated on 7H10 agar, and the number of CFU was determined. We reproducibly infected mice with 10 to 25 CFU.

(ii) Influenza. Mouse-adapted influenza A/PR/8/34 (PR8) virus was grown in the allantoic cavity of 10-day-old embryonated hen eggs and stored at −70°C. Uninfected allantoic fluid also was collected and stored at −70°C as a control inoculum. In all experiments, mice were anesthetized and intranasally inoculated with 50 µl of influenza virus or uninfected allantoic fluid. Infection was monitored daily, and clinical signs of disease and weight were recorded. Pilot studies with 2-fold serial dilutions of virus stocks were carried out to determine the 50% lethal infectious dose (LD₅₀) of the PR8 virus in C57BL/6 mice.

**Influenza virus titers.** Virus titers were measured by infecting Madin-Darby canine kidney cells with serially diluted supernatants from lung homogenates. After incubation at 37°C for 24 h, 0.02% tosylsulfonphenyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) was added and incubated for an additional 48 h. Virus titers were determined based on the presence or absence of cytopathic effect, and the median tissue culture infectious dose was calculated.

**Flow cytometry.** Lung cells and splenocytes were stained with allophycocyanin anti-CD3, fluorescein isothiocyanate anti-CD4, and phycocerythrin anti-CD8 (all from BD Pharmingen) and then analyzed with a FACSCalibur flow cytometer and CellQuest pro software (BD Biosciences). At least 10,000 gated events were analyzed in each experiment.

**Evaluation of transcription factors by Western blotting.** Whole-cell protein extracts of T cells from lungs and spleens were prepared as described previously (36–38) and were quantified by bicinchoninic acid assay (Pierce Biotechnology). SDS-PAGE and Western blotting were performed as previously described (36). The blot then was stripped and rebotted with anti-glyceraldehyde-3-phosphate dehydrogenase. All antibodies were obtained from Santa Cruz Biotechnology.

**Statistical analysis.** All of the data were processed in Excel and Prism. Student’s t test or the Mann-Whitney t test was used to test for statistical significance. P < 0.05 was considered statistically significant.

**RESULTS**

Cigarette smoke exposure reduces IFN-γ production in response to stimulation through the T-cell receptor. To determine if cigarette smoke reduces the cell-mediated immune response, we evaluated its effects on the production of IFN-γ, which is a key cytokine in mediating protective immunity against intracellular pathogens. T cells from control or cigarette smoke-exposed C57BL/6 mice were stimulated with suboptimal concentrations of anti-CD3 and anti-CD28. Cigarette smoke exposure markedly reduced IFN-γ production by lung T cells by >80% (P < 0.0001) (Fig. 1A), with similar effects on CD4⁺ and CD8⁺ T cells (Fig. 1B and C). Exposure to cigarette smoke did not significantly reduce IFN-γ production in anti-CD3-stimulated splenocytes (Fig. 1D to F).

We next used ELISPOT assays to measure the frequency of IFN-γ-producing cells upon stimulation with anti-CD3 and anti-CD28. Cigarette smoke exposure significantly reduced the number of IFN-γ-producing lung and CD4⁺ T cells (P = 0.025 for both comparisons) (Fig. 2). However, spleen cells were not affected.

Using flow cytometry, we found that cigarette smoke exposure did not affect the total number of CD3⁺ cells from the lungs and spleens or the percentages of CD4⁺ and CD8⁺ T cells (data not shown), indicating that cigarette smoke did not reduce IFN-γ production by decreasing T-cell numbers.

**Cigarette smoke exposure reduces expression of transcription factors that control IFN-γ production.** We have shown previously that the transcription factors cyclic AMP response element binding protein (CREB), activating transcription factor w (ATF-2), and c-Jun bind to the IFN-γ proximal promoter and upregulate IFN-γ production by T cells (37, 38). The phosphorylation of these transcription factors increases their binding to the transcriptional complex. To evaluate the effect of cigarette smoke exposure on these transcription factors, we isolated T cells from the lungs and spleens of cigarette smoke-exposed and control mice, stimulated them with anti-CD3 and anti-CD28, and performed Western blotting on cellular protein extracts. The expression of phosphorylated CREB, ATF-2, and c-Jun in the lung T cells of cigarette smoke-exposed mice was markedly reduced compared to that of nonexposed control mice (Fig. 3A and C). Similarly, cigarette smoke exposure reduced expression in lung T cells of T-bet, which controls IFN-γ production. The effect of cigarette smoke exposure was specific, as it did not affect NF-κB expression (Fig. 3A). In contrast to the effects on lung T cells, cigarette smoke exposure...
did not reduce the splenic T-cell expression of any transcription factors that control IFN-\(\gamma\)/H9253 production (Fig. 3B).

Cigarette smoke exposure reduces T-cell responses to DNA vaccination against \textit{M. tuberculosis}. The studies described
above indicate that cigarette smoke reduces the capacity of T cells to produce IFN-γ in response to T-cell receptor activation. To determine if cigarette smoke also affects the T-cell response to a specific antigen, we used C57BL/6 mice transgenic for human HLA-DRB1*0401 (DR4). We have demonstrated previously that the M. tuberculosis 10-kDa culture filtrate protein (CFP10) contains several epitopes for human CD4+ T cells that are recognized in the context of DRB1*0401 (41), and that DR4 mice recognize human epitopes of CFP10 (21). Cigarette smoke-exposed and control DR4 mice were immunized with a pcDNA3.1 plasmid expressing CFP10 and the lysosomal integral membrane protein II, which targets antigens for presentation by the MHC class II pathway (35). Mice also were vaccinated with an empty pcDNA3.1 plasmid as controls. Two weeks after immunization, CD4+ T cells from lungs and spleens were isolated and incubated on an ELISPOT plate together with splenocytes as antigen-presenting cells, either unpulsed (No Ag) or pulsed with CFP10. Five mice per group were used. Mean values and standard errors are shown.

Because IFN-γ is central to immunity against M. tuberculosis (16, 47), we next studied if cigarette smoke exposure affected the bacterial burden after infection. Mice were exposed to cigarette smoke for 6 weeks and then infected with M. tuberculosis H37Rv by aerosol (10 to 25 CFU/mouse). Ten weeks after infection, CFU in the lungs of cigarette smoke-exposed mice were significantly higher than those in control mice (P = 0.002) (Fig. 5D). The bacterial burdens in the spleens of both groups were only 3 to 10% of those in the lungs and were comparable in both groups (data not shown).

Cigarette smoke exposure inhibits T-cell IFN-γ production in response to influenza virus infection. The data described above demonstrate that cigarette smoke exposure reduces the T-cell response to M. tuberculosis. To determine if this effect extended to another pulmonary pathogen, cigarette smoke-exposed and unexposed C57BL/6 mice were immunized with a sublethal dose (0.1 LD50) of live influenza A virus (PR8) intranasally. One week later, lung and spleen T cells were isolated by positive immunomagnetic selection and incubated on an ELISPOT plate together with splenocytes from naïve mice on ELISPOT plates precoated with anti-IFN-γ without stimulation in vitro. The number of IFN-γ+ cells from lungs and spleens of cigarette smoke-exposed mice were reduced by more than 90% (Fig. 5A and B) (P < 0.05 for both comparisons). Cigarette smoke also reduced the number of IFN-γ+ CD4+ splenic T cells (Fig. 5C).

We next evaluated the effect of cigarette smoke exposure on viral replication in the lungs of infected mice. Three days after
infection with 0.1 LD$_{50}$ lungs from cigarette smoke-exposed mice had slightly higher viral loads than their control counterparts, but this difference was not statistically significant ($P = 0.14$) (Fig. 6B). By 6 days after infection, viral burdens were essentially identical in control and cigarette smoke-exposed mice. These findings indicate that the reduced number of IFN-γ-producing T cells due to cigarette smoke exposure was not due to a reduction in the viral burden and antigen load.

Cigarette smoke exposure increases weight loss and mortality from influenza virus infection. We next examined the effect of cigarette smoke on weight loss due to IAV infection as a clinical indicator of the efficacy of the immune response. Cigarette smoke-exposed and unexposed C57BL/6 mice were immunized with a sublethal dose (0.1 LD$_{50}$) of IAV PR8 intranasally and weighed daily. Ten days after immunization, the control group stopped losing weight and soon started to gain weight, with some reaching their preinfection weight 2 weeks after infection (Fig. 7A). Cigarette smoke-exposed animals also lost weight during the first 10 days after infection, but most failed to regain weight by 2 weeks postinfection (Fig. 7A). At 2 weeks postinfection, cigarette smoke-exposed mice lost significantly more weight than their control counterparts ($P = 0.006$). Unexpectedly, sublethal infection resulted in the death of 10 to 20% of cigarette smoke-exposed mice but no unexposed mice (data not shown).

The studies described above evaluated the effects of cigarette smoke exposure on sublethal infection with influenza virus. To determine if cigarette smoke exposure affects more severe infection, we infected cigarette smoke-exposed and control mice with a lethal dose of IAV PR8 virus ($\approx$ 1 LD$_{50}$). All cigarette smoke-exposed mice succumbed to infection, whereas approximately one-third of control mice survived ($P = 0.008$) (Fig. 7B). Mice that survived until day 15 all recovered completely (data not shown).

DISCUSSION

In this report, we provide the first evidence that exposure to cigarette smoke directly inhibits the lung T-cell production of IFN-γ during stimulation in vitro with anti-CD3/CD28, after vaccination with a construct expressing an immunogenic mycobacterial protein, and during infection with M. tuberculosis...
and influenza A virus in vivo. Reduced IFN-γ production was mediated through the decreased phosphorylation of CREB, ATF-2, and c-Jun, which positively regulate IFN-γ transcription. The effects of cigarette smoke exposure on the T-cell production of IFN-γ were associated with reduced immunity, as manifested by increased bacterial burden in the case of tuberculosis and increased weight loss and mortality in the case of influenza virus infection. These findings provide the first demonstration that cigarette smoke exposure reduces resistance to tuberculosis and influenza in an animal model, and they suggest that increased susceptibility is mediated in part through direct inhibitory effects on T cells.

The effects of smoking on the immune response are multifaceted and complex. On the one hand, smoking favors the development of pulmonary inflammation and chronic obstructive pulmonary disease, and cigarette smoke exposure in animals increases the expression of the proinflammatory cytokines IL-18 (18) and IL-1 (11) and activates natural killer cells (26). Furthermore, smoking in humans is associated with the increased expression of STAT4 and IFN-γ by lymphocytes in bronchial biopsy specimens and bronchoalveolar lavage fluid (8). In contrast to its proinflammatory effects, cigarette smoke can inhibit dendritic cell maturation and the production of IL-12 in animal models (22, 33), suggesting the potential to inhibit Th1 responses that depend on IL-12.

The effects of cigarette smoke on T-cell responses have varied in different studies. Some authors found no effect of mainstream cigarette smoke on splenic T-cell production of IFN-γ (46), whereas others found the marked inhibition of splenocyte proliferation and the ability to mount a Ca²⁺ flux in response to T-cell receptor ligation (17). The few studies of the effects of cigarette smoke on pulmonary T-cell responses have focused on Th2 responses that contribute to allergic inflammation and have yielded contradictory results. Mainstream...
cigarette smoke has been reported to reduce (24, 34) and enhance (25, 40) T-cell-mediated allergic airway inflammation. These differences may reflect differing levels of smoke exposure, as one study showed that higher levels inhibited T-cell cytokine production, whereas lower levels did not (43).

Previous studies have evaluated the effects of mainstream cigarette smoke on T-cell function, but our current work provides the first evaluation of the effects of environmental cigarette smoke on T cells. Cigarette smoke exposure inhibited both the lung and splenic CD4+ and CD8+ T-cell production of IFN-γ in response to M. tuberculosis and influenza (Fig. 4, 5, and 6A), suggesting that both local and systemic T-cell function are reduced during infection. However, exposure to cigarette smoke inhibited the capacity of the lung but not splenic T cells to produce IFN-γ in response to stimulation through the T-cell receptor independently of antigen-presenting cells (Fig. 1). Anti-CD3 and anti-CD28 provide a stronger T-cell stimulus than bacterial and viral antigens and may overcome the inhibitory effects of cigarette smoke on splenic but not lung cell responses, since the concentrations of immunosuppressive components of cigarette smoke are highest in the lung and are likely to have the greatest effect on local T cells. Although cigarette smoke exposure affected antigen-specific responses, this was not due to global effects on T-cell recruitment, as exposure did not reduce the trafficking of CD4+ or CD8+ T cells to the lungs or mediastinal lymph nodes, either before or after influenza infection (H. Shams, unpublished data). Furthermore, cigarette smoke exposure did not significantly affect the recruitment of naïve or effector memory T cells, or T cells expressing the integrins LFA-1 and VLA-1, to the lungs after influenza infection (H. Shams, unpublished data).

Smoking and tuberculosis are strongly epidemiologically linked. One recent study of 17,700 Taiwanese persons showed a 2-fold increase in the risk of tuberculosis among current smokers, with a significant dose-response relationship with the number of cigarettes smoked per day and the number of pack-years of smoking (23). Cigarette smoke exposure also was independently associated with a 70% increase in the likelihood of the development of culture-proven tuberculosis among 15,500 nonsmoking women (2). Despite these epidemiologic data, no published information is available on the mechanisms through which smoking increases susceptibility to tuberculosis. IFN-γ plays a central role in immunity against tuberculosis, as mice with a deleted IFN-γ gene rapidly succumb to tuberculosis (6, 13), and T cells from tuberculosis patients with ineffective immunity produce low concentrations of IFN-γ compared to that of healthy tuberculin reactors with protective immunity (16, 47). In this report, we immunized mice with a plasmid DNA vaccine that has been shown previously to deliver an immunogenic mycobacterial protein to the lung and to elicit strong T-cell responses (21). Cigarette smoke exposure inhibited the lung and splenic T-cell production of IFN-γ in response to vaccination (Fig. 4). Furthermore, when mice were infected with M. tuberculosis, cigarette smoke exposure reduced IFN-γ production (Fig. 5A to C) and the bacillary burden was greater in cigarette smoke-exposed animals (Fig. 5D), suggesting that the inhibition of the T-cell response significantly impacted the capacity of the immune system to control bacterial infection.

Smoking predisposes healthy adults to the development of influenza, which is more severe in smokers (20, 28). Of three studies of smoking and influenza in mice in vivo, one used miniosmotic pumps to administer nicotine and another administered extremely high smoke exposure for only 4 days prior to influenza infection (15, 31), situations that are unlikely to mimic conditions in humans. The third study found that mainstream cigarette smoke reduced the local airway inflammatory response after infection with a low dose of influenza virus but enhanced inflammation and increased mortality after high-dose infection (32). Our study is the first to evaluate the effects of cigarette smoke on the T cell response to influenza in animals. Similarly to our findings for tuberculosis, cigarette smoke reduced the local and systemic T-cell production of IFN-γ in mice infected with influenza A virus (Fig. 6A), and this effect was associated with increased weight loss and mortality (Fig. 7A and B).

IFN-γ is critical for human defenses against M. tuberculosis and other bacterial, fungal, and viral intracellular pathogens,
and the proximal promoter of IFN-γ is necessary and sufficient for its transcription in activated T cells (29). In the present report, we demonstrated that cigarette smoke exposure reduced the phosphorylation of CREB, ATF-2, and c-Jun (Fig. 3), which are known to bind to and positively regulate the proximal promoter of IFN-γ in primary human T cells in response to mycobacterial antigen (38). It will be important to delineate the upstream mechanisms through which cigarette smoke exposure reduces the expression of these transcription factors.

In summary, we provide the first evidence that cigarette smoke exposure directly inhibits the pulmonary and systemic T-cell production of IFN-γ during infection with M. tuberculosis and influenza A virus in vivo, at least in part through the decreased phosphorylation of CREB, ATF-2, and c-Jun. The inhibition of IFN-γ production was associated with an increased bacterial burden in the case of tuberculosis and increased weight loss and mortality in the case of influenza virus infection. Given the enormous numbers of people exposed to cigarette smoke and the tremendous morbidity and mortality attributable to tuberculosis and influenza world-wide, future studies will be critical to fully understanding the molecular mechanisms of these effects of cigarette smoke exposure, so that immunomodulatory strategies can be developed to correct these defects.

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