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Sustained Effect of Inhaled Diesel Exhaust Particles on T-Lymphocyte–Mediated Immune Responses Against *Listeria monocytogenes*

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Studies have shown that exposure to diesel exhaust particles (DEP) suppresses pulmonary host defense against bacterial infection. The present study was carried out to characterize whether DEP exposure exerts a sustained effect in which inhaled DEP increase the susceptibility of the lung to bacterial infection occurring at a later time. Brown Norway rats were exposed to filtered air or DEP by inhalation at a dose of 21.2 ± 2.3 mg/m^3, 4 h/day for 5 days, and intratracheally instilled with saline or 100,000 *Listeria monocytogenes* (*Listeria*) 7 days after the final DEP exposure. Bacterial growth and cellular responses to DEP and *Listeria* exposures were examined at 3 and 7 days post-infection. The results showed that inhaled DEP prolonged the growth of bacteria, administered 7 days post DEP exposure, in the lung as compared to the air-exposed controls. Pulmonary responses to *Listeria* infection were characterized by increased production of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-12, and IL-10 by alveolar macrophages (AM) and increased presence of T lymphocytes and their CD4$^+$ and CD8$^+$ subsets in lung draining lymph nodes that secreted elevated levels of IL-2, IL-6, IL-10, and interferon (IFN)-γ. Diesel exhaust particles were found to inhibit *Listeria*-induced production of IL-1β and TNF-α, which are responsible for the innate immunity, and IL-12, which initiates the development of T helper (Th)1 responses, but enhance *Listeria*-induced AM production of IL-10, which prolongs *Listeria* survival in these phagocytes. The dual action of DEP on AM production of IL-12 and IL-10 correlated with an inhibition of the development of bacteria-specific T lymphocytes by DEP. Cytokine production by lymphocytes from DEP- and *Listeria*-exposed rats showed a marked decrease in the production of IL-2, IL-10, and IFN-γ compared to *Listeria* infection alone, suggesting either that DEP inhibit the production of cytokines by lymphocytes or that these lymphocytes contained T-cell subsets that are different from those of *Listeria* infection alone and less effective in mediating Th1 immune responses. This study demonstrates that inhaled DEP, after a 7-day resting period, increase the susceptibility of the lung to bacterial infection occurring at a later time by inhibiting macrophage immune function and suppressing the development of T-cell–mediated immune responses. The results support the epidemiological observations that exposure to DEP may be responsible for the pulmonary health effects on humans.

Key Words: **diesel exhaust particles; host defense; *Listeria monocytogenes*; alveolar macrophages; T lymphocytes.**

INTRODUCTION

Exposure to increased ambient particulate matter (PM) is associated with an increase in morbidity and mortality from cardiopulmonary conditions, especially in industrialized areas (Dockery *et al.*, 1993). The Committee on the Environmental and Occupational Health Assembly of the American Thoracic Society (1996a, 1996b) has reported a correlation of the daily ambient PM concentrations with increased incidence of respiratory symptoms, hospitalization, and premature death among the general population. Studies have suggested that fine fractions of PM, with diameters less than 2.5 μm ($PM_{2.5}$), are predominant in emissions from the combustion of fossil fuels and are more closely associated with mortality and adverse health effects than coarse fractions of PM (PM_{10}) (Delfino *et al.*, 1997; Peters *et al.*, 1997). Diesel exhaust particles (DEP), which are generated through use of heavy-duty diesel engines, are the major constituent of the atmospheric $PM_{2.5}$ in urban and industrialized areas. Therefore, the effect of DEP exposure on pulmonary immune responses is an important environmental and occupational health concern.

Exposure of rats to DEP resulted in particle distribution in the alveolar region as well as in the lung-draining lymph nodes (LDLN) through particle translocation in the local lymphoid system (Chan *et al.* 1981; Yu and Yoon 1991). We have shown previously that DEP strongly suppress host defense mechanisms, including both innate and cell-mediated immune responses against bacterial infections that occurred immediately after lung exposure (Castranova *et al.*, 2001; Yang *et al.*, 1999, 2001; Yin *et al.*, 2002, 2003, 2004a, 2004b). Diesel

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exhaust particles increase the susceptibility of the lung to infection by inhibiting the phagocytic activity and bacterial killing of alveolar macrophages (AM) and by altering AM responses to bacteria that are crucial for the development of T-cell–mediated immune responses. In particular, DEP exposure was found to suppress interleukin (IL)-12, but enhance IL-10 production by AM in response to bacterial infection, thus prolonging the survival of pathogens in the lung and inhibiting the development of bacteria-specific T lymphocyte, including the $CD4^+$ T helper (Th)1 and the $CD8^+$ T cells (Yin *et al.*, 2003, 2004a, 2004b). These studies, however, have been designed to characterize the acute effects of DEP on the susceptibility of the lung to pulmonary infections. Acute pulmonary exposure to DEP as well as other particles, particularly at high concentrations, induces evident inflammatory responses in the lung. These inflammatory responses, although generally unspecific, may interfere with the determination and assessment of DEP effects on pulmonary immunity against an ongoing infection. Under normal environmental conditions, however, DEP exposure occurs chronically at low dose levels that are unlikely to induce any acute responses. Therefore, further studies are needed to assess the impact of DEP exposure on pulmonary immune responses, including the susceptibility to pathogenic infections where no particle-induced inflammation exists.

Listeria monocytogenes (Listeria), a gram-positive, facultative intracellular bacterium that induces both innate and cell-mediated immune responses, has been used extensively in our laboratory as a pulmonary infection model (Yin *et al.*, 2002, 2003, 2004a, 2004b). In previous studies we have shown that the effect of DEP exposure on *Listeria* infection in rats varies with varying exposure protocols. When rats were exposed to a single dose of DEP at 100 mg/m^3 for 4 h and infected 2 h later with *Listeria*, DEP aggravated bacterial infection at day 3 post-infection but triggered a strong T-cell–mediated immunity resulting in complete clearance of bacteria within 7 days post-infection (Yin *et al.*, 2002, 2003). In a short-term DEP exposure experiment (20 mg/m^3, 4 h/day, for 5 days) followed by infection, DEP prolonged bacterial survival beyond 7 days post-infection and downregulated T-cell production of interferon-γ (IFN-γ) (Yin *et al.*, 2004a).

The present study was carried out using the same short-term DEP exposure protocol, but the infection with *Listeria* was carried out following a 7-day resting period after DEP exposure. The objective was to study the delayed effect of pre-exposure to DEP on the susceptibility of the lung to bacterial infection occurring later and its correlation with DEP-mediated changes in cytokine secretion by AM and T lymphocytes and lymphocyte populations in the DEP- and *Listeria*-exposed lungs.

MATERIALS AND METHODS

Animals. Male Brown Norway (BN) rats [BN/CrlBR] weighing 200–250 g were purchased from Charles River Laboratories (Wilmington, MA). They were housed in a clean-air room with restricted access, given a conventional laboratory diet and tap water *ad libitum*, and allowed to acclimate for 1 week before use. The animal facility is monitored free of pathogens and approved by the Association for Assessment and Accreditation of Laboratory Animal Care. During the week before inhalation exposure, the animals were conditioned to the exposure unit. Animals were placed in the tubes of the exposure unit for increasing time periods from 1–4 h/day for 4 successive days.

Inhalation exposure of rats to DEP. A standardized DEP sample (standard reference material 2975) was purchased from the National Institute of Standards and Technology (Gaithersburg, MD). The inhalation exposure system and DEP exposure procedure used in this study have been previously described and characterized (Yin *et al.*, 2002). Briefly, rats were exposed to either filtered air or DEP (21.2 ± 2.3 mg/m^3) for 4 h/day for 5 consecutive days using a nose-only directed flow exposure unit (CH Technologies, Inc., Westwood, NJ). Diesel exhaust particle concentrations in the exposure unit were monitored by both gravimetric sampling of dust collected on a polycarbonate membrane filter (37 mm, 0.45 μm, Poretics Corporation, Livermore, CA) at a sampling rate of 1 l/min, and a Grimm Model 1.108 portable dust monitor (GRIMM Technologies, Inc., Douglasville, GA), which allows continuous measurement of the particle concentration in the exposure unit in real time. The estimated mean lung deposition of DEP for the inhalation exposure (4 h × 5 days), according to the calculating method of Leong *et al.* (1998), was estimated to be 406 ± 29 μg/rat.

Listeria culture and intratracheal instillation. The *Listeria* used was strain 10403S, serotype 1, routinely cultured in our laboratory (Yin *et al.*, 2002). *Listeria* was cultured overnight in brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI) at 37°C in a shaking incubator. Diluted solution of the *Listeria* culture was further cultured for 3 h to achieve log growth. Following incubation, the bacterial concentration was determined spectrophotometrically at 600 nm. For the preparation of heat-killed *Listeria* (HKLM), cultured bacteria were incubated at 80°C for 1 h, washed, and resuspended in sterile phosphate-buffered solution (PBS). An aliquot of the HKLM was plated overnight on BHI agar plates (Becton Dickinson Co., Cockeysville, MD) to ensure that there were no viable bacteria. For animal infection, the culture was diluted with sterile saline to the desired concentration; 7 days after the last DEP exposure (day 0), rats were lightly anesthetized with methohexital sodium (25 mg/kg, ip; Eli Lilly Co., Indianapolis, IN) and inoculated intratracheally with 100,000 of *Listeria* in 500 μl of sterile saline or 500 μl of the vehicle alone, as previously described (Antonini *et al.*, 2000). To ensure that the number of *Listeria* given to the rats was suitable, the bacterial sample used for animal infection was diluted and plated on BHI agar plates (Becton Dickinson Co.) and the number of bacteria was counted after being cultured overnight at 37°C.

Bronchoalveolar lavage (BAL) and evaluation of BAL fluid and cells. At 3 and 7 days after bacterial inoculation, rats were deeply anesthetized with an overdose of sodium pentobarbital (200 mg/kg, ip; Butler, Columbus, OH) and euthanized by exsanguination *via* the abdominal aorta. The lungs were lavaged with Ca^{2+}/Mg^{2+}-free PBS (pH 7.4) at a volume of 6 ml for the first lavage and 8 ml for subsequent lavages until a total of 80 ml of BAL fluid was collected. The BAL fluid samples were centrifuged at 500 × *g* for 10 min at 4°C, and the cell-free supernatant from the first lavage was analyzed for various biochemical parameters. The cell pellets from all washes for each rat were combined, washed, and resuspended in 1 ml PBS. The numbers of AM and neutrophils in the BAL cell suspension were determined according to their unique cell diameters using an electronic cell counter equipped with a cell-sizing unit (Coulter Electronics, Hialeah, FL).

Albumin content, a measure to quantify increased permeability of the bronchoalveolar-capillary barrier, and LDH activity, an indicator of general cytotoxicity, were determined in the acellular BAL fluid from the first lavage. Measurements were performed with a COBAS MIRA auto-analyzer (Roche Diagnostic Systems, Montclair, NJ). Albumin content was determined colorimetrically at 628 nm based on albumin binding to bromcresol green using an albumin BCG diagnostic kit (Sigma, St. Louis, MO). Lactate dehydrogenase activity was determined by measuring the oxidation of lactate

to pyruvate coupled with the formation of reduced form of nicotinamide adenine dinucleotide at 340 nm using Roche Diagnostic reagents and procedures (Roche Diagnostic Systems, Indianapolis, IN).

Isolation and differential counts of lymphocytes. All LDLN from each rat were collected, and a single cell suspension was prepared as described previously (Yin *et al.*, 2003). The cells were washed twice with PBS and lymphocytes were isolated by Histopaque gradient centrifugation (density, 1.083; Sigma). Briefly, the samples were centrifuged for 30 min at 2500 rpm and lymphocytes were collected, washed twice, and resuspended in 1 ml of PBS. The number of lymphocytes was counted by a standard hemocytometer, and the cell viability was assessed by the trypan blue dye exclusion technique. The cell samples thus prepared showed both the lymphocyte content and viability of greater than 98%.

The numbers of T cells and $CD4^+$ and $CD8^+$ T-cell subsets in lymphocytes recovered at 7 days post-infection were determined by flow cytometry as described previously (Yin *et al.*, 2003). Briefly, lymphocytes were stained with the addition of fluorescein-5-isothiocyanate (FITC)-labeled CD3, CD4, or CD8 monoclonal antibody (BD Pharmingen, San Diego, CA) for 30 min on ice in the dark. The flow cytometric data were collected with a Becton-Dickinson FACScan using FACScan Research Software (version B; Becton-Dickinson Immunocytometry System, San Jose, CA) and analyzed using the PC-LYSYS (version 1.0) software (Becton-Dickinson). The absolute numbers of cells in each lymphocyte subpopulation were calculated by multiplying the total number of cells by the percentage of the total within each phenotype as determined by flow cytometry.

Pulmonary clearance of Listeria. The colony forming units (CFU), an index of viable bacteria, were determined as described previously (Yin *et al.*, 2002, 2004a). Briefly, the lungs were removed from all rats following BAL and homogenized in sterile water. The tissue homogenates or their dilutions were quantitatively plated in triplicate on BHI agar plates (Becton Dickinson Co.). After incubation at 37°C overnight, the CFU in each plate were counted. The counts were averaged and corrected for dilution to yield the CFU/ml, through which the CFU per lung from each treatment group were determined.

Cell culture and cytokine determination. The BAL cells and lymphocytes were suspended in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) containing 2 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, and 10% heat-inactivated fetal bovine serum. Before the stimulation, the BAL cells were incubated in a humidified incubator (37°C and 5% CO_2) for 2 h to allow cell attachment to the culture plate. The nonadherent BAL cells were then removed by rinsing the monolayer three times with culture medium. The remaining AM-enriched cells or lymphocytes were then treated with either lipopolysacchride (LPS, 1 μg/ml, Sigma), concanavalin A (ConA, 2 μg/ml, Sigma), or HKLM (10^7/ml) for 24 or 48 h. The AM- and lymphocyte-conditioned media were collected, centrifuged (1200 × *g* for 4 min), and aliquots of the supernatants were stored at −70°C until assayed.

The amounts of TNF-α, IL-1β, and IL-10 produced by AM and the production of IL-2, IL-6, IL-10, and IFN-γ produced by lymphocytes in cell cultures under various exposure conditions were quantified by the enzyme linked immunosorbent assay (ELISA) using the OptEIA ELISA sets according to the manufacturer's instructions (BD PharMingen). Briefly, a 96-well ELISA plate (Corning, Corning, NY) was coated with a purified antirat monoclonal antibody and blocked with an assay diluent (BD PharMingen) before use. Recombinant standards (BD PharMingen) and samples were added to the plate and incubated for 2 h at room temperature. The plate was then incubated with biotinylated antibody for 1 h and avidin-horseradish peroxidase conjugate for 30 min at room temperature. The plate was developed with tetramethylbenzidine with 50% H_2O_2 in the dark, and color reaction was stopped with 2 N H_2SO_4 and then analyzed at 450 nm with a SpectraMax 250 plate spectrophotometer using Softmax Pro 2.6 software (Molecular Devices Co., Sunnyvale, CA). The levels of IL-12 in the culture media were quantified by ELISA using a commercial ELISA kit (BioSource International, Inc., Camarillo, CA). The range of detection was: 31.3–2000 pg/ml for IL-1β, IL-2, IL-6, and IFN-γ; 15.6–1000 pg/ml for IL-10 and TNF-α; and 7.8–500 pg/ml for IL-12.

Statistical analysis. The experimental results are expressed as means ± standard error (SE). Statistical analyses were carried out with the JMP IN statistical program (SAS Institute, Inc., Cary, NC). The significance of difference between treatment groups was analyzed using the Tukey-Kramer's Honestly Significant Different (HSD) Test. For all analyses, the criterion of significance was set at $p < 0.05$.

RESULTS

Effects of DEP on Pulmonary Responses to Listeria and Bacterial Clearance

The numbers of recovered AM and neutrophils in the BAL fluid were counted, and LDH activity and albumin content in the first fraction of acellular BAL fluid were determined for the characterization of inflammatory lung injury for each exposure group (Fig. 1). Compared to non-infected controls, *Listeria* infection caused an increase in all parameters measured at days 3 and 7, except for albumin, which showed no significant change at 7 days post-infection. Diesel exhaust particle exposure, however, was found to slightly increase numbers of AM and neutrophils at day 3 (not statistically significant) but to significantly decrease both cell counts at day 7 in *Listeria*-infected rats, even though the cell counts were still higher than that of non-infected controls.

The pulmonary handling of *Listeria* infection by air- and DEP-treated rats was determined at 3 and 7 days post-infection (Fig. 2). Intratracheal instillation of 10^5 *Listeria* into the rat lung resulted in a strong bacterial growth, with a similar number of CFU in both air-exposed and DEP-exposed rats at 3 days post-infection. At day 7, *Listeria* infection in the lungs of air-exposed rats was largely resolved, as indicated by the greatly diminished lung CFU (0.32×10^5), which was less than the initial infection dose (10^5). In comparison, the lung bacterial counts for the DEP-exposed rats (4.09×10^5) were significantly higher than that of the air-exposed controls (~ 13-fold) and the initial infection dose (~ 4-fold).

Effects of DEP on AM Responses to Listeria Infection

Figure 3 shows the changes in cytokine secretion by AM from various exposure groups. The basal production of cytokines by AM in culture was low, but it followed the same pattern as when they were primed by LPS; hence, LPS was used in AM culture for enhanced cytokine secretion. Compared to the non-infected controls, AM from *Listeria*-infected rats showed increased production of IL-1β, TNF-α, IL-12, and IL-10, in which TNF-α and IL-12 was highly elevated at day 3 but declined at day 7, whereas the secretions of IL-1β and IL-10 were higher at day 7 than at day 3. Diesel exhaust particle exposure was found to inhibit *Listeria*-induced IL-1β secretion by AM at day 7 and the production of TNF-α and IL-12 at both time points. The production of IL-10 by AM in response to *Listeria*, however, was found to be augmented in rats also exposed to DEP at day 7.

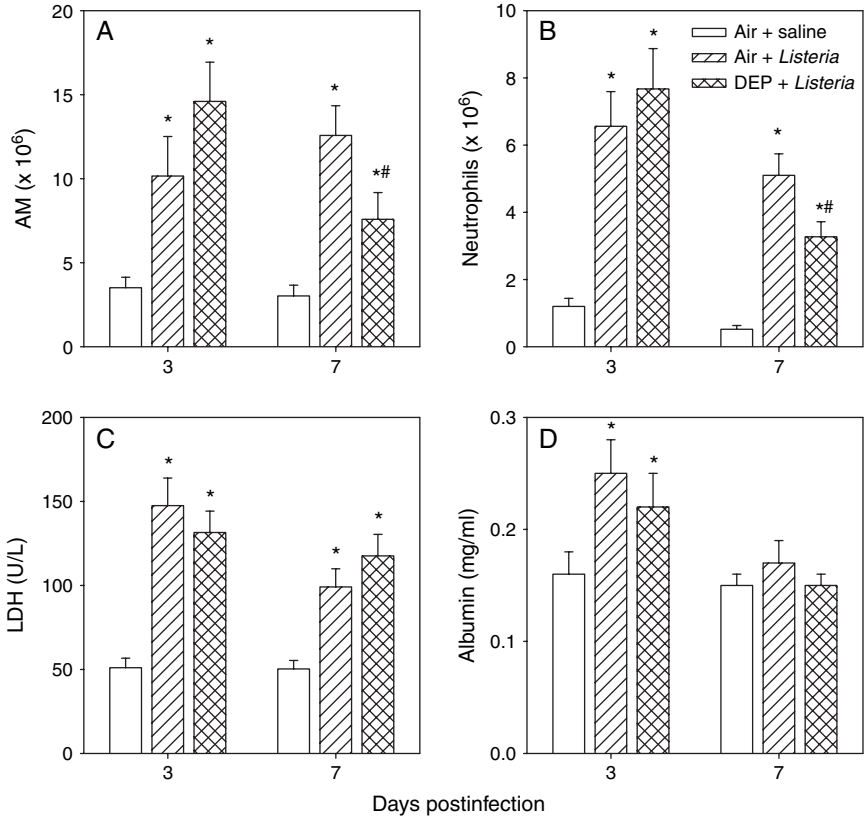

FIG. 1. Yield of AM (A) and neutrophils (B), LDH activity (C), and albumin content (D) in the first fraction of bronchoalveolar lavage fluid. Values are expressed as the means ± SE ($n = 5$). *Significantly different from non-infected controls, $p < 0.05$; #Significantly different from air-exposed and *Listeria*-infected controls, $p < 0.05$.

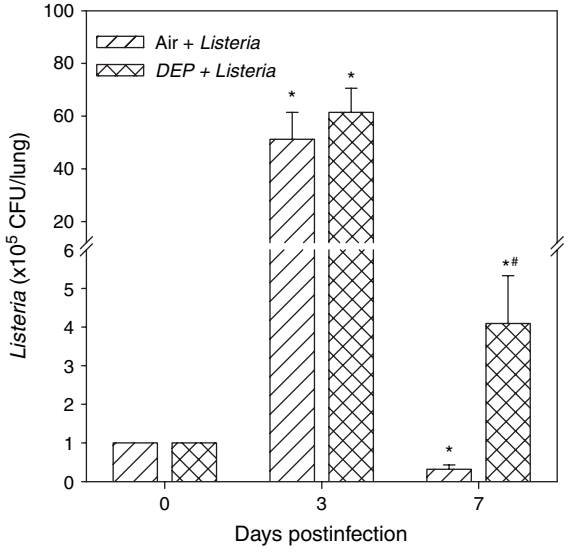

FIG. 2. Effects of DEP exposure on pulmonary clearance of *Listeria*. Rats were inoculated intratracheally with 10^5 *Listeria* at 7 days after the final air or DEP exposure (day 0). At 3 and 7 days postinfection the colony-forming units (CFU) per lung were determined. Values are expressed as the means ± SE ($n = 5$). *Significantly different from the initial infection dose, $p < 0.05$; #Significantly different from air-exposed and *Listeria*-infected controls, $p < 0.05$.

Effects of DEP on T Lymphocyte Responses to Listeria Infection

Animals with *Listeria* infection, as compared to non-infected controls, had an associated increase in lymphocytes in LDLN at 7 days post-infection (Fig. 4). Approximately 70% of lymphocytes from the infected rats were T cells, with a $CD4^+/CD8^+$ ratio of 3.7. The development of T cells through *Listeria* infection in rats pre-exposed to DEP was significantly different from that in those exposed to *Listeria* alone. Diesel exhaust particle exposure reduced the total lymphocyte and T cell counts by 26% and 35%, respectively. The T cells, which accounted for 70% of total lymphocytes, had a $CD4^+/CD8^+$ ratio of 5.3, reflecting a 31% reduction in $CD4^+$ and a 52% reduction of $CD8^+$ cells due to the presence of DEP.

The lymphocyte-mediated immune responses were assessed by examining cellular production of key cytokines in response to either ConA, which activates lymphocytes in cytokine secretion, or HKLM, which activates *Listeria*-specific lymphocytes. Figure 5 shows that lymphocytes from *Listeria*-infected rats at 3 days post-infection were highly responsive to activation by HKLM in the production of IL-6 (Fig. 5A) and IL-10 (Fig. 5B) compared to the non-infected controls. Cells obtained

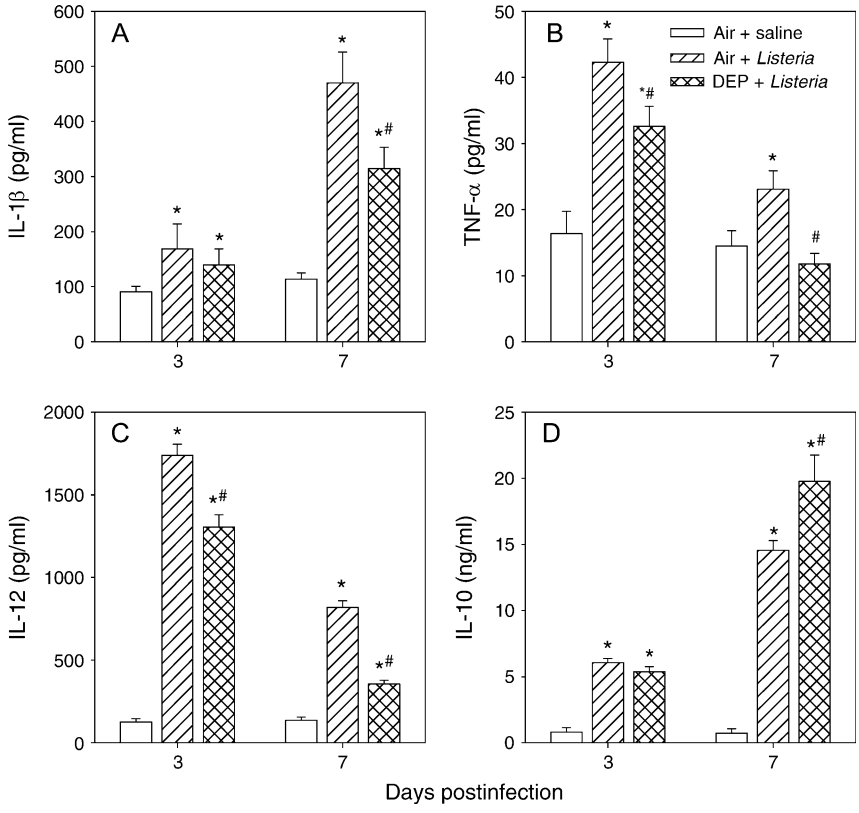

FIG. 3. Production of IL-1β (A), TNF-α (B), IL-12 (C), and IL-10 (D) by AM in responses to *ex vivo* stimulation with LPS (1 μg/ml, 24 h). Concentrations of the cytokines in the culture media were quantified by ELISA. Values are expressed as the means ± SE ($n = 5$). *Significantly different from non-infected controls, $p < 0.05$; #Significantly different from air-exposed and *Listeria*-infected controls, $p < 0.05$.

at 7 days post-infection also secreted elevated levels of both cytokines, but at much lower levels than those obtained at 3 days post-infection. From rats pre-exposed to DEP, lymphocytes showed similar secretion of IL-6 at day 3 but increased production of this cytokine at day 7, compared to cells obtained from air-exposed and *Listeria*-infected rats. These lymphocytes were also noted to secrete significantly diminished levels of IL-10 at both 3 and 7 days post-infection, compared to those obtained from rats exposed to *Listeria* alone. Figure 6 shows the production of IL-2 and IFN-γ by cultured lymphocytes stimulated with ConA. The *Listeria*-infected lymphocytes, at both 3 and 7 days post-infection, were highly primed to secrete IL-2 (Fig. 6A). In contrast, significant production of IFN-γ, which is critical for T-cell mediated immunity, was observed only in cells obtained at 7 days post-infection (Fig. 6B), indicating that *Listeria* induces a time-dependent response in the development of lymphocyte-mediate immunity. Both IL-2 and IL-6 produced by cells at 3 days post-infection enhance lymphocyte proliferation, leading to the development of T lymphocytes at 7 days post-infection that secrete elevated levels of IFN-γ. The action of inhaled DEP was found to inhibit *Listeria*-induced lymphocyte production of IL-2 at day 3 and day 7 and of IFN-γ at 7 days post-infection (Fig. 6).

DISCUSSION

Alveolar macrophages are the principal cell type in pulmonary host defense and are responsible for the clearance of inhaled particles or microorganisms through phagocytosis and the initiation of innate and T-cell–mediated immune responses against bacteria through release of cytokines, enzymes, and reactive intermediates. Studies from our laboratory have shown that direct interaction of DEP with AM in cell culture inhibited phagocytosis and bacterial killing in a dose- and time-dependent manner (unpublished data). Diesel exhaust particles inhibit AM production of IL-1β and TNF-α in response to *Listeria*, which may account for their inhibition of bacterial killing and the initial spread of bacterial growth. Inhibition of phagocytosis by DEP can significantly retard the pulmonary clearance mechanism. This, together with the fact that DEP are fine particles with diameters less than 1 μm, suggests that inhaled DEP may stay in the lung for a long periods of time, allowing an accumulation of DEP to toxic levels.

The present study was designed to investigate whether inhaled DEP exert a delayed effect on the pulmonary immune system against *Listeria* infection administered 7 days after DEP exposure. We employed a short-term DEP exposure protocol

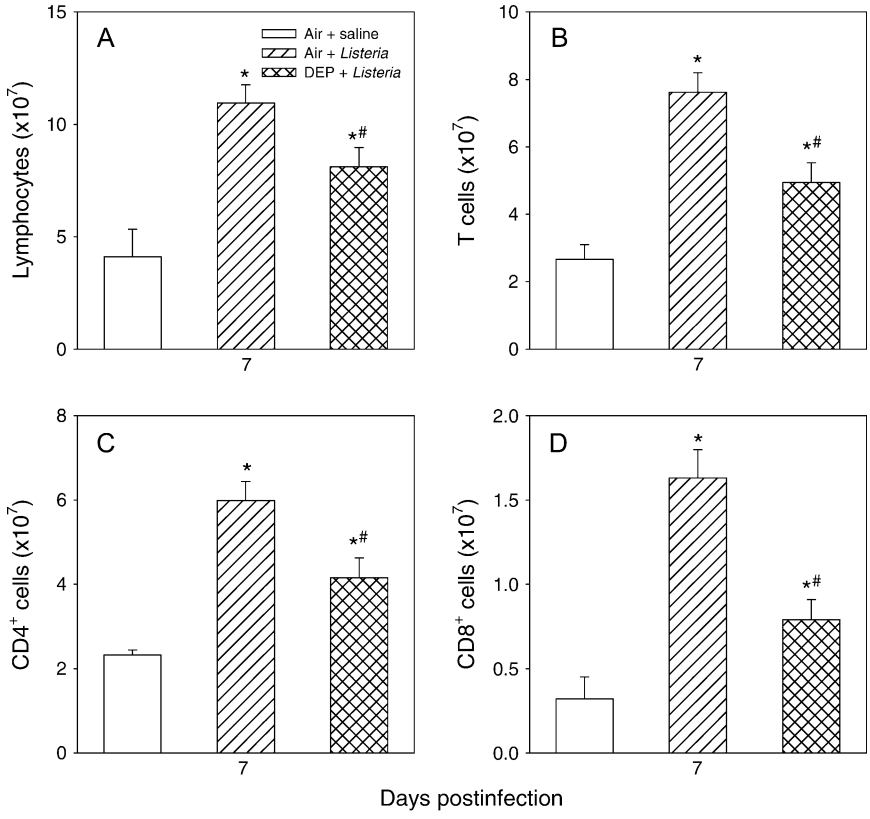

FIG. 4. Differentials of lymphocyte from lung-draining lymph nodes recovered at 7 days postinfection. Number of lymphocytes (A) was counted by a standard hemocytometer. Numbers of T cells (B) and $CD4^+$ (C) and $CD8^+$ T cell subsets (D) in lymphocytes were determined by flow cytometry. Values are expressed as the means ± SE ($n = 5$). *Significantly different from non-infected controls, $p < 0.05$; #Significantly different from air-exposed and *Listeria*-infected controls, $p < 0.05$.

(20.62 ± 1.31 mg/m^3, 4 h/day × 5 days), which yielded an estimated lung burden of 406 μg/rat of DEP, but did not augment the *Listeria*-induced inflammatory responses at 10 or 14 days after the last DEP exposure (Yin *et al.*, 2004a). The dose we used for animal exposure in this study may appear to be high in comparison to reported environmental and occupational concentrations, but, in fact, it results in a lung deposition that is relevant to both non-occupational and occupational exposure settings, as discussed previously (Yin *et al.*, 2002, 2004a).

A number of studies have shown that acute exposure to DEP at high doses, through either intratracheal instillation (5 or 35 mg/kg) or inhalation (50 or 100 mg/m^3), significantly induced

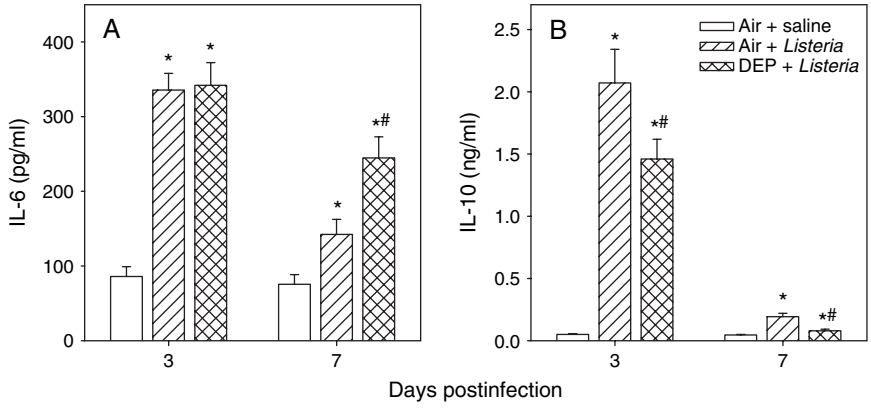

FIG. 5. Production of IL-6 (A) and IL-10 (B) by lymphocytes in responses to *ex vivo* stimulation with HKLM (10^7 HKLM/ml, 48 h). Concentrations of the cytokines in the culture media were quantified by ELISA. Values are expressed as the means ± SE ($n = 5$). *Significantly different from non-infected controls, $p < 0.05$; #Significantly different from air-exposed and *Listeria*-infected controls, $p < 0.05$.

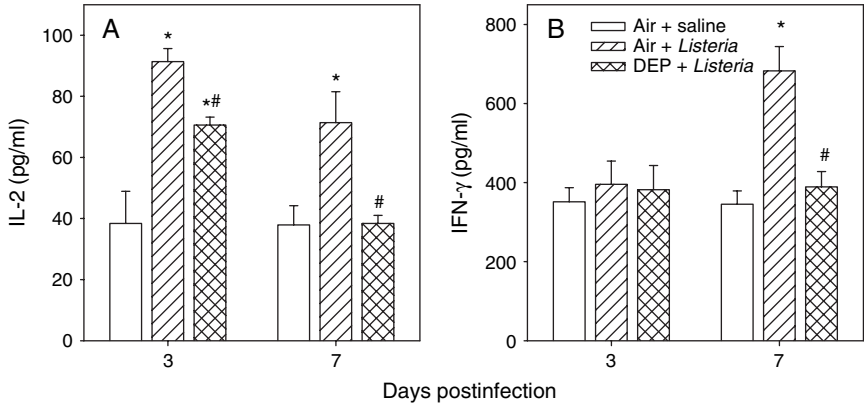

FIG. 6. Production of IL-2 (A) and IFN-γ (B) by lymphocytes in responses to *ex vivo* stimulation with ConA (2 μg/ml, 24 h). Concentrations of the cytokines in the culture media were quantified by ELISA. Values are expressed as the means ± SE ($n = 5$). *Significantly different from non-infected controls, $p < 0.05$; #Significantly different from air-exposed and *Listeria*-infected controls, $p < 0.05$.

dose-dependent lung injury and inflammation in rats, persisting up to 7 days post-exposure (Yang *et al.*, 1999, 2001; Yin *et al.*, 2002). Histochemical observations of mice intratracheally instilled with DEP (0.4 or 0.8 mg/mouse) also revealed a dose-dependent lung toxicity that included damage to capillary endothelial cells and type I pneumocytes at 6 h, and subsequent alveolar edema and infiltration of inflammatory cells at 18–24 h post-DEP exposure (Ichinose *et al.*, 1995). In human volunteers, short-term exposure to DEP at 0.1–0.3 mg/m^3 significantly increased neutrophils and B lymphocytes in BAL fluid, and bronchial biopsies obtained 6 h after exposure showed increased numbers of mast cells and CD4$^+$ and CD8$^+$ T lymphocytes (Salvi *et al.*, 1999; Stenfors *et al.*, 2004). These studies showed that acute pulmonary responses to high doses of DEP are characterized by inflammatory lung injury, but they gave little or no information on effects of low-dose DEP exposure or of how DEP alter the pulmonary immune system. Indeed, we have shown previously that the effect of DEP exposure on *Listeria* infection in the BN rat varies with varying exposure protocols. When rats were exposed to a single dose of DEP at 100 mg/m^3 for 4 h and were infected 2 h later with *Listeria*, DEP strongly aggravated bacterial infection at 3 days post-infection but also triggered a strong T-cell–mediated immunity, resulting in complete clearance of bacteria within 7 days post-infection (Yin *et al.*, 2002, 2003). In a short-term DEP exposure (20 mg/m^3, 4 h/day, for 5 days) followed by an immediate infection, although with a similar lung DEP burden, aggravation of bacterial growth by DEP at 3 days after infection was less severe, but DEP caused a prolonged bacterial survival beyond 7 days and down-regulated T-cell production of IFN-γ (Yin *et al.*, 2004a). The present study further demonstrates that inhaled DEP in a delayed infection protocol suppresses T-cell–mediated immunity against *Listeria* infection occurring 7 days after DEP exposure.

Alveolar macrophages play a major role in the clearance of bacteria, and they secrete cytokines that are critical to the innate immune response (Bancroft *et al.*, 1989; Czuprynski *et al.*, 1992). Both IL-1β and TNF-α secreted by AM are known to activate NK cells to release IFN-γ, which, along with activated AM, kills the bacteria. The anti-inflammatory cytokine IL-10, on the other hand, is a potent immunosuppressive factor that downregulates the bactericidal activity of AM (Fleming *et al.*, 1999). The fact that DEP enhance *Listeria*-induced AM production of IL-10 is of interest, because some intracellular pathogens including *Listeria* specifically target macrophages for infection and use IL-10 to dampen the host immune response (Redpath *et al.*, 2001). The innate immunity is efficient in limiting the initial spread of infection, but sterilization of *Listeria* infection depends on the later development of acquired T cell responses involving CD4$^+$, Th1, and CD8$^+$ cells (Shen *et al.*, 1998; Unanue, 1997). Interleukin-12 plays a key role in linking AM to T-cell–mediated immunity (Trinchieri, 1995, 1998). This cytokine is produced rapidly by AM following infection, and it initiates the development of Th1 responses (Park and Scott, 2001). In addition, IL-1β, which acts in concert with IL-6, induces lymphocyte production of IL-2 and IL-2–mediated T cell proliferation (Akira *et al.* 1990; Ford *et al.* 1991; Renauld *et al.* 1989). Diesel exhaust particles, which inhibit AM secretion of IL-1β and TNF-α, along with diminished production of IL-12 and enhanced production of IL-10 in response to *Listeria*, exhibit a potent immunosuppressive effect on immune response and may result in failure of the host to mount a T-cell–mediated response to bacterial infection.

There is, however, a time-dependent effect of DEP on the immune system, depending on both the severity of DEP exposure and the time of infection. The AM-orchestrated innate immunity, which attenuates the initial spread of infection, is likely highly affected by acute DEP exposure, whereas in delayed infection, the effect of DEP on innate immune responses would be low. Indeed, as seen in the present study, inhaled DEP did not significantly enhance bacterial growth at 3 days post-infection compared to rats exposed to *Listeria* alone. This also corresponds to a significant but moderate

inhibitory effect of DEP on cytokine production by AM (IL-12, TNF-α) and lymphocytes (IL-2) at 3 days post-infection. In comparison, the same short-term DEP exposure, as reported in a previous study (Yin *et al.*, 2004a), suppressed the innate immune responses when *Listeria* was given 2 h after DEP exposure and examined at 3 days post-infection, and although the short-term exposure did not induce inflammation, it augmented *Listeria*-induced neutrophil infiltration, LDH activity, and albumin content in the BAL fluid. The apparent lesser effect on the innate immune responses by inhaled DEP, however, was countered by their marked effect on the development of T-cell–mediated immune responses. The current study shows that at 7 days post-infection, animals pre-exposed to DEP clearly exhibit a lymphocyte population that was fewer in number and in specific $CD4^+$ and $CD8^+$ T lymphocytes than that of the air-exposed, *Listeria*-infected rats. Pre-exposure of rats to DEP also significantly changed the cytokine secretion pattern by lymphocytes obtained at 7 days post-*Listeria* infection, which was consistent with the results of T cell analysis that reflected a change in cell population. *Listeria* infection alone resulted in cells that secret elevated levels of IL-2, IL-6, IL-10, and IFN-γ. The *Listeria* infection in rats pre-exposed to DEP, on the other hand, resulted in a cell population that produces a higher level of IL-6 but significantly lowered levels of IL-2, IL-10, and IFN-γ, indicating that inhaled DEP suppress the development of T-cell–mediated immune responses against *Listeria* infection. Although these cells showed increased secretion of IL-6, which may enhance IL-2 responsiveness and IL-2 secretion by lymphocytes (Akira *et al.*, 1990; Ford *et al.*, 1991), they did not indicate a T cell population that was primed against *Listeria*. The effect of inhaled DEP on AM secretion of IL-12 and lymphocyte production of IL-2, although moderate at 3 days post infection, persisted up to 7 days post-infection, suggesting that as residents in these cell types, DEP may have a effect on modifying cellular responses to infectious agents. As the T cell development requires time, inhaled DEP also have a delayed or sustained effect on the immune system. The current study shows that inhaled DEP suppress T-cell–mediated immunity against *Listeria* infection administered 7 days after DEP exposure in rats.

In conclusion, this study demonstrates that inhaled DEP, after a 7 day resting period, increase the susceptibility of the lung to bacterial infection occurring at a later time by inhibiting macrophage immune function and suppressing the development of T-cell–mediated immune responses. The results support the epidemiological observations that exposure to DEP may be responsible for the pulmonary health effects on humans.

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