Alteration of Pulmonary Immunity to *Listeria monocytogenes* by Diesel Exhaust Particles (DEPs). II. Effects of DEPs on T-Cell–Mediated Immune Responses in Rats

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Previously, we showed that diesel exhaust particles (DEPs) suppressed pulmonary clearance of *Listeria monocytogenes* (*Listeria*) and inhibited the phagocytosis of alveolar macrophages and their response to *Listeria* in the secretion of interleukin (IL)-1β, tumor necrosis factor α, and IL-12. In this report we examined the effects of DEPs and/or *Listeria* on T-cell development and secretion of IL-2, IL-6, and interferon (IFN)-γ. We exposed Brown Norway rats to clean air or DEPs at 50 or 100 mg/m^3 for 4 hr by nose-only inhalation and inoculated with 100,000 *Listeria*. Lymphocytes in the lung-draining lymph nodes were isolated at 3 and 7 days postexposure, analyzed for CD4$^+$ and CD8$^+$ cells, and measured for cytokine production in response to concanavalin A or heat-killed *L. monocytogenes*. *Listeria* infection induced lymphocyte production of IL-6. At 7 days postinfection, lymphocytes from *Listeria*-infected rats showed significant increases in CD4$^+$ and CD8$^+$ cell counts and the CD8$^+$/CD4$^+$ ratio and exhibited increased production of IFN-γ and IL-2 receptor expression compared with the noninfected control. These results suggest an immune response that involves the action of IL-6 on T-cell activation, yielding *Listeria*-specific CD8$^+$ cells. DEP exposure alone enhanced lymphocyte production of both IL-2 and IL-6 but inhibited lymphocyte secretion of IFN-γ. In rats exposed to 100 mg/m^3 DEPs and *Listeria*, a 10-fold increase occurred in pulmonary bacterial count at 3 days postinfection when compared with the *Listeria*-only exposure group. The isolated lymphocytes showed a significant increase in the CD4$^+$ and CD8$^+$ cell counts and the CD8$^+$/CD4$^+$ ratio and exhibited increased IL-2 responsiveness and increased capacity in the secretion of IL-2, IL-6, and IFN-γ. This T-cell immune response was sufficient to allow the Brown Norway rats to clear the bacteria at 7 days postinfection and overcome the down-regulation of the innate immunity by the acute DEP exposure. *Key words:* diesel exhaust particles, IL-2 receptor, interferon-γ, interleukin-2, interleukin-6, *Listeria monocytogenes*, T-cell responses. *Environ Health Perspect* 111:524–530 (2003). doi:10.1289/ehp.5709 available via *http://dx.doi.org/* [Online 17 January 2003]

In addition to their occurrences at workplaces where heavy-duty diesel engines are used, diesel exhaust particles (DEPs) are a major component of the environmental particulate pollutants that have a diameter of less than 2.5 μm ($PM_{2.5}$). These particles trigger adverse biologic responses within the lungs (Diaz-Sanchez et al. 1994; Fujimaki et al. 1997; Yang et al. 1997, 1999; Yoshino and Sagai 1999). Indeed, in a previous study in which rats were infected with *Listeria monocytogenes* (*Listeria*) by intratracheal instillation, we demonstrated that acute exposure to DEPs retarded bacterial clearance from the lung. This DEP effect correlated with an inhibition of the phagocytic activity of alveolar macrophages (AMs) and a diminished response of AMs to *Listeria* [or heat-killed *L. monocytogenes* (HKLM)] in the secretion of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-12 (Yin et al. 2002).

Listeria is a ubiquitous species of gram-positive, facultative intracellular bacterium used in recent studies as a pulmonary infection model in rodents (Antonini et al. 2000; Reasor et al. 1996; Yang et al. 2001). Our studies showed that in *Listeria*-infected rats, AMs and neutrophil counts in the bronchoalveolar lavage (BAL) fluid were elevated, and the isolated AMs exhibited increased production of IL-1, IL-12, and TNF-α (Yin et al. 2002). Although the innate immune response is efficient in limiting the initial spread of infection, a successful host defense, on the other hand, requires specific T-cell–mediated immunity (Kaufmann 1993; Twigg 1998). These responses provide a cooperative action between the specific (T lymphocyte) and nonspecific (macrophage) elements of the immune system in defense against *Listeria*. The release of proinflammatory cytokines by macrophages, for example, is pivotal in specific T-cell development, whereas interferon (IFN)-γ produced by T cells, which induces AM secretion of TNF-α (Dery and Bissonnette 1999), may clear *Listeria* by enhancing the antimicrobial and antigen-presenting properties of macrophages (Kaufmann and Flesch 1990; McCormack et al. 1992).

Listeria induces both major histocompatibility complex (MHC) class I–restricted CD8$^+$ and MHC class II–restricted CD4$^+$ T-cell responses (Mielke et al. 1997; North et al. 1997). A number of studies have shown that *Listeria* as a live vector induces CD8$^+$-mediated immune responses in various viral and tumoral experimental models (Goossens et al. 1995; Weiskirch and Paterson 1997). *Listeria* has also been used to induce the development of IFN-γ–secreting T-helper (Th)-1 CD4$^+$ T lymphocytes (Soussi et al. 2000). Studies have shown that *Listeria* efficiently induces the production of IL-12 by macrophages, either in the noninfectious form of heat-killed bacteria or as live infectious organisms. This cytokine regulates the development of appropriate CD4$^+$ Th subset during the immune response to *Listeria* (Hsieh et al. 1993). IL-12 also plays an important role in maintaining the Th1 response (Park and Scott 2001) and provides protection from *Listeria* by inducing IFN-γ production (Trinchieri 1995, 1998). The fact that AMs from *Listeria*-infected rats show increased secretion of IL-12 (Yin et al. 2002) indicates that pulmonary host defense against *Listeria* may involve CD4$^+$ Th1 immune responses. The induction of CD8$^+$-mediated immune responses by *Listeria* may involve bacteria-induced lymphocyte production of IL-6. This cytokine, which is originally known to induce B-cell differentiation, regulates both acute-phase responses to injury (Hirano et al. 1990) and the activation of T cells (Lotz et al. 1988). In the latter function, IL-6, in concert with IL-1, controls the initial steps of the T-cell activation, with CD8$^+$ being the target cells. IL-6 enhances IL-2 responsiveness, and IL-6 and IL-1 act in synergy to stimulate T-cell production of IL-2 (Akira et al. 1990; Ford et al. 1991; Renauld et al. 1989). The subsequent interaction of IL-2 with its high-affinity receptor promotes the rapid clonal expansion of the T-cell population originally activated by the bacteria (Smith 1992).

Despite the fact that exposure of rats to DEPs resulted in particle distribution in the

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alveolar region as well as in the lung-draining lymph nodes (LDLNs) through particle translocation in the local lymphoid system (Chan et al. 1981; Yu and Yoon 1991), the potential effect of DEPs on T-cell–mediated immune responses has not been fully characterized. Lymphocytes are intimately involved in defense against bacterial, fungal, viral, and toxic assaults. Ample evidence suggests that macrophage-derived cytokines such as IL-1, TNF-α, and IL-12 are T-cell activators (Akira et al. 1990; Hsieh et al. 1993). As the production of these cytokines by AMs in response to *Listeria* (or HKLM) stimulation is inhibited by the DEP exposure, it is likely that DEPs may also alter lymphocyte-mediated host defense mechanism. For this reason, we have studied the effects of acute DEP exposure on T-cell–mediated immune responses in the lungs of *Listeria*-infected rats. This article describes the responses of the Brown Norway rats to DEPs and *Listeria* exposures in the development of $CD4^+$ and $CD8^+$ cells and the ability of these lymphocytes to secrete IL-2, IL-6, and IFN-γ.

Materials and Methods

Materials. A standardized DEP sample (standard reference material 1650a), representative of heavy-duty engine emissions, was obtained from the National Institute of Standards and Technology (Gaithersburg, MD). This sample had a mass median aerodynamic diameter of approximately 0.5 μm. *Listeria* was of strain 10403s and serotype 1. Male Brown Norway rats (200–250 g) were purchased from Harlan Laboratories (Indianapolis, IN). They were housed in a clean-air and viral-free room with restricted access, given a conventional laboratory diet and tap water *ad libitum*, and allowed to acclimate in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved animal facility for 1 week before use.

DEP *inhalation and intratracheal bacterial inoculation.* Groups of rats were exposed to either purified air or DEP-containing air (50 and 100 mg/m^3) for 4 hr using a nose-only inhalation system that consisted of a TSI model 3076 constant output atomizer (TSI, Inc., St. Paul, MN), a diffusion dryer, and an inhalation exposure system (CH Technologies, Inc., Westwood, NJ) with 12 ports for animal-holding tubes. Prior to the inhalation experiments, the animals were placed in the holding tube 4 hr per day for 5 days to allow the animals to adapt to the inhalation conditions. DEP suspensions in water were sonicated for 10 min, aerosolized using the TSI nebulizer, and carried by generated air (carrier air) through the diffusion dryer. The carrier air was mixed with clean humidified air (dilution air) from a different air source before entering the inhalation chamber. Effluent from the inhalation chamber was passed through a HEPA filter to remove particles. DEP concentrations in chamber were monitored by gravimetric sampling of dust collected on a polycarbonate membrane filter (37 mm, 0.45 mm; Poretics Corp., Livermore, CA) at a sampling rate of 1 L/min. The estimated lung deposits of DEPs for the 4-hr inhalation exposure, according to the method of Leong et al. (1998), were 192 and 384 μg/rat for 50 and 100 mg/m^3 dose groups, respectively.

Listeria was cultured overnight in brain heart infusion broth (Difco Laboratories, Detroit, MI) at 37°C in a shaking incubator. Diluted solution of the *Listeria* culture was further cultured for 3 hr to achieve log growth. After incubation, the bacteria concentration was determined spectrophotometrically at 600 nm and diluted with sterile saline to the desired concentrations. Two hours after DEP exposure, rats were lightly anesthetized with methohexital sodium (35 mg/kg body weight, intraperitoneal injection; Eli Lilly Co., Indianapolis, IN) and inoculated intratracheally with 500 μL sterile saline or 500 μL saline containing approximately 100,000 *Listeria*, according to the method of Antonini et al. (2000).

After *Listeria* inoculation, the animals were returned to the AAALAC-approved animal facility and housed in a separate room completely isolated from noninfected animals. They received normal care until the time for subsequent experiments. The effect of DEP exposure on AM-mediated responses to *Listeria* infection based on the above exposure experiments has been reported (Yin et al. 2002). This study characterizes T-lymphocyte–mediated immune responses to *Listeria* and DEP exposures using data obtained from the same experiments.

Cell isolation and differential counts of T-cell subsets. At 3 and 7 days after *Listeria* instillation, the rats were deeply anesthetized with an overdose of sodium pentobarbital (50 mg/kg intraperitoneal injection; Butler, Columbus, OH) and exsanguinated by severing the abdominal aorta. The LDLNs from each rat were excised and teased apart with forceps, and single-cell suspensions were obtained by expressing the cells into RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) through a nylon mesh bag (Tetko, Inc., Briarcliff Manor, NY). Repeated passes of cell clumps through a 22-gauge needle attached to a 10-mL syringe were performed to disperse the cells. The cell suspension was then washed twice with the medium and counted using standard hemocytometer methods. The viability of harvested cells through this procedure was assessed by trypan blue exclusion and found to be above 98% for all cell preparations.

To enumerate $CD4^+$ and $CD8^+$ T-cell subsets in lymphocytes recovered from rats at 7 days after bacteria inoculation, each of the respective cell types was stained with the addition of an appropriate monoclonal antibody conjugated with a fluorescent probe for visualization (BD Pharmingen, San Diego, CA). After a 30-min incubation on ice in the dark, cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) containing 2% fetal bovine serum (FBS) and 0.02% NaN_3 and fixed by suspending the cells in 0.4% paraformaldehyde in PBS. Flow cytometric analysis was performed the following day. 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). Live lymphocytes were analyzed based on forward versus 90° scatter set to exclude dead cells and contaminating red blood cells, which are smaller than live lymphocytes. The absolute numbers of cells in each lymphocyte subpopulation were calculated by multiplying the total number of viable cells by the percentage of the total within each phenotype, determined by flow cytometry.

Determination of cytokines. Lymphocytes recovered from rats at 3 and 7 days postinfection were suspended in the RPMI-1640 medium (Gibco BRL) containing 2 mM glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin, 5×10^{-5} M 2-β-mercaptoethanol, 5 mM HEPES, and 10% heat-inactivated FBS (Hyclone, Logan, UT). Aliquots of 1 mL cell suspensions, adjusted to 4×10^6 lymphocytes, were added to each well of 24-well tissue culture plates (Costar, Cambridge, MA) and incubated in a humidified incubator (37°C and 5% CO_2) for 24 or 48 hr with or without either concanavalin A (ConA) 2 μg/mL (Sigma Chemical Co., St. Louis, MO) or HKLM (10^5–10^7/mL). The lymphocyte-conditioned media were collected and centrifuged (1,200 × *g* for 4 min), and aliquots of the supernatants were stored at –70°C until assayed. To ensure that the number of cells was the same in various culture samples, studies were carried out to determine the cellular protein levels after incubation. The cells were treated with 0.5% Triton X100 at 37°C for 30 min, and the media were collected and centrifuged. The protein contents in supernatants were determined using Sigma Diagnostic reagents and procedures (Sigma Chemical Co.) on a Cobas Fara II analyzer (Roche Diagnostic System, Montclair, NJ). The results did not show a significant difference in protein content among the samples from various treatment groups. The levels of IFN-γ, IL-2, and IL-6 in the culture supernatants were determined using the commercially developed enzyme-linked

immunosorbent assays (ELISA) (BioSource International, Inc., Camarillo, CA) and a spectrophotometric microplate reader (SpectraMax 250; Molecular Devices Co., Sunnyvale, CA). The concentration range for accurate determination of each cytokine using the ELISA kit was 21.8–1,400 pg/mL for IFN-γ, 23.4–1,500 pg/mL for IL-2, and 31.2–2,000 pg/mL for IL-6.

The direct effect of DEPs on lymphocyte secretion of IFN-γ through particle–cell interactions was determined. Lymphocytes in all LDLNs were isolated from rats treated with 100,000 *Listeria* or saline at 7 days postinfection. Cells (4×10^6) were treated with varying concentrations of DEPs (0–50 μg/mL) in a humidified incubator (37°C and 5% CO_2) and incubated with HKLM (10^7/mL) for 48 hr. After treatment, the lymphocyte-conditioned media were collected, centrifuged, and stored at –70°C until assayed. The levels of IFN-γ in the culture supernatants were quantified by ELISA.

Determination of IL-2 receptor α on lymphocytes. Animals were lightly anesthetized by intraperitoneal inoculation of methohexital sodium (35 mg/kg body weight; Eli Lilly Co.) and intratracheally instilled with 300 μL sterile saline or 384 μg/rat DEPs presuspended in 300 μL saline by sonication for 2 min. This intratracheal DEP dose was selected because the estimated lung deposit in rats exposed to 100 mg/m^3 DEPs for 4 hr by nose-only inhalation was 384 μg (Yin et al. 2002). Two hours after DEP exposure, rats were inoculated intratracheally with either saline or approximately 100,000 *Listeria* according to the method previously described. At 7 days postinfection, rats were sacrificed, and the lymph node cells were harvested and counted as previously described. The isolated lymphocytes, adjusted to 4×10^6 cells/mL/well, were incubated in a humidified incubator for 0–72 hr with ConA (2 μg/mL). The lymphocyte-conditioned media were collected and measured for IL-2 production by ELISA. The ConA-stimulated lymphocytes were stained with appropriate antibody for the CD4$^+$ and CD8$^+$ T-cell subsets and with R-phycoerythrin–conjugated monoclonal mouse antirat IL-2 receptor α (IL-2Rα) antibody (BD Pharmingen), and analyzed using a Becton-Dickinson FACScan, according the method described in the previous section.

Statistical analysis. Results are expressed as mean ± SE of multiple measurements. Statistical analyses were carried out with the JMP IN statistical program (SAS, Inc., Cary, NC). The significance of the interaction among the different treatment groups for the different parameters at each time point was assessed using an analysis of variance (ANOVA). The significance of difference between individual groups was analyzed using the Tukey-Kramer's honestly significant difference (HSD) test and chi-square test. For all analyses, the criterion of significance was set at $p < 0.05$.

Results

Effects of DEPs and listeria exposures on lymphocyte population in LDLNs. The numbers of total lymphocytes and the CD4$^+$ and CD8$^+$ T-cell subsets recovered from LDLNs for each exposure group at 7 days post-*Listeria* infection are given in Table 1. Rats treated with *Listeria* (1×10^5 bacteria/rat on day 0) showed increased counts in total lymphocytes, T cells, and the CD4$^+$ and CD8$^+$ T-cell subsets in LDLNs at day 7, as well as a slight increase in the CD8$^+$/CD4$^+$ ratio compared with that of the noninfected control. Exposure to DEPs alone also resulted in increased total lymphocytes, T cells, and T-cell subsets. In the combined exposure, elevation of cell counts appeared to be DEP dose-dependent. At 50 mg/m^3, the number of total lymphocytes or CD4$^+$ cells recovered from LDLNs corresponded to the added total of the DEP-exposure alone and *Listeria*-treated alone. There was, however, a significant increase in the CD8$^+$ subset. At the higher DEP dose (100 mg/m^3), a significant increase occurred in both the cell number and the CD8$^+$/CD4$^+$ ratio by the combined DEP and *Listeria* exposures, indicating that this acute, high-dose DEP exposure augments the effects of *Listeria* on promoting T-lymphocyte development. At 3 days postinfection, neither DEP exposure nor *Listeria* infection resulted in significant changes in T lymphocytes when compared with the air-exposed, noninfected control (data not shown).

***Effects of DEP and* Listeria *exposures on cytokine production by lymphocytes.* Interleukin-2.** The production of IL-2 by lymphocytes isolated from rats exposed to DEPs and/or *Listeria* at 3 and 7 days postexposure was determined following incubation of cells with ConA for 24 and 48 hr. Figure 1A shows the *Listeria*-mediated changes in IL-2 secretion by lymphocytes from air-exposed rats. Compared with the noninfected control, lymphocytes from rats treated with *Listeria* for 7 days showed a decreased secretion of IL-2. Figure 1B compares the IL-2 levels in

Table 1. Effects of DEP exposure and/or *Listeria* infection on lymphocyte differentiation in LDLNs of Brown Norway rats.[a]

DEP dose (mg/m^3)	Lymphocytes (↔10^6)	T cells (↔10^6)	CD4$^+$ T cells (↔10^6)	CD8$^+$ T cells (↔10^6)	CD8$^+$/CD4$^+$
Without *Listeria*					
Air	19.25 ± 3.06	10.55 ± 1.60	9.67 ± 1.47	0.87 ± 0.13	0.09 ± 0.01
50	32.88 ± 10.69*	18.98 ± 6.43*	17.33 ± 5.84*	1.65 ± 0.59*	0.10 ± 0.02
100	48.66 ± 8.80*	28.11 ± 5.30*	25.74 ± 4.92*	2.37 ± 0.39*	0.09 ± 0.01
With *Listeria*					
Air	73.16 ± 9.30*	37.75 ± 4.84*	33.38 ± 4.57*	4.37 ± 0.41*	0.13 ± 0.02
50	108.44 ± 17.53*,**,#	58.31 ± 10.71*,**,#	50.48 ± 9.67*,**,#	7.91 ± 1.04*,**,#,##	0.16 ± 0.02*
100	143.72 ± 18.47*,**,#	80.99 ± 9.30*,**,#,##	66.85 ± 7.75*,**,#	14.15 ± 2.20*,**,#,##	0.22 ± 0.03*,**,#

[a]See "Materials and Methods" for details. Values are expressed as mean ± SE ($n = 5$). Data were analyzed by one-way ANOVA followed by Tukey-Kramer's HSD test for multiple mean comparisons for each treatment group at the same dose and exposure time. The viability of all cell preparations was above 98%. *Significantly different from the noninfected air controls, $p < 0.05$. **Significantly different from the *Listeria*-only group, $p < 0.05$. #Significantly different from the DEP-only groups, $p < 0.05$. ##Significantly different from the sum of DEP-only and *Listeria*-only groups, $p < 0.05$.

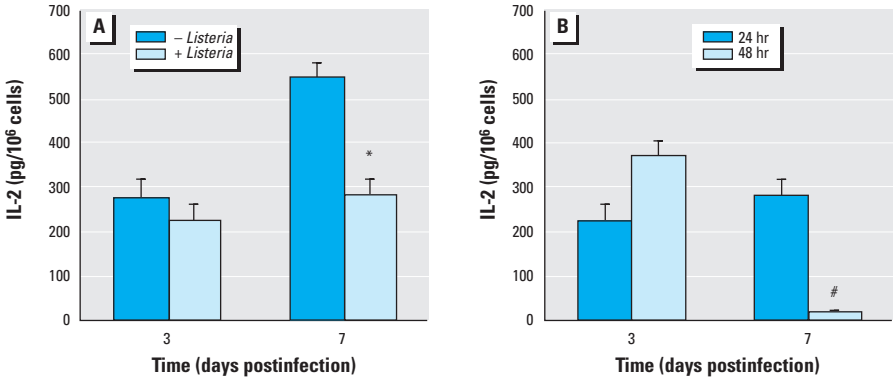

Figure 1. Effect of *Listeria* infection on the production of IL-2 by lymphocytes: (*A*) Comparison of IL-2 production by lymphocytes from *Listeria*-infected rats (gray bar) and noninfected controls in 24-hr culture with ConA (2 μg/mL); (*B*) Comparison of IL-2 production by lymphocytes from *Listeria*-infected rats in 24-hr and 48-hr cultures with ConA (2 μg/mL) stimulation. Rats were exposed to clean air for 4 hr. *Listeria* was given 2 hr postexposure. Lymphocytes were obtained from rats at 3 and 7 days postexposure. Values are expressed as mean ± SE ($n = 3$–5).
*Significantly different from that of noninfected control, $p < 0.05$. #Significantly lower than that of the 24-hr culture, $p < 0.05$.

the 24- and 48-hr cultures secreted by lymphocytes isolated from air-exposed, *Listeria*-infected rats. For cells obtained at 3 days postinfection, the IL-2 level in the 48-hr culture was greater than that of the 24-hr culture. However, lymphocytes isolated at 7 days postinfection produced significantly diminished levels of IL-2 in the 48-hr culture when compared with the IL-2 levels in the 24-hr culture.

Figure 2A, B shows the effects of DEP exposure on lymphocyte production of IL-2 in noninfected and *Listeria*-infected rats, respectively. In noninfected rats, DEP exposure enhanced the production of IL-2 in the 24-hr culture by lymphocytes obtained at both 3 and 7 days postexposure, and there was a marked decrease in IL-2 levels in the 48-hr cultures. In *Listeria*-infected rats, the DEP exposure at 100 mg/m^3 enhanced the production of IL-2 in the 24-hr culture by lymphocytes obtained at 7 days postinfection compared with the air-exposed control. In the 48-hr culture, however, the IL-2 levels produced by the same cells were sharply diminished. In comparison, lymphocytes obtained from rats exposed to DEPs and *Listeria* at 3 days postinfection did not show a significant increase in IL-2 production in the 24-hr culture compared with that of the air control. The DEP exposure had only a moderate effect on lowering IL-2 levels in the 48-hr culture. The isolated lymphocytes from various exposure groups did not respond to stimulation by HKLM in IL-2 secretion (data not shown).

IL-2 promotes T-cell proliferation during the immune response. The diminished levels of IL-2 in the 48-hr culture compared with levels of the 24-hr culture suggest that the secreted IL-2 may be reutilized by the lymphocytes for cell proliferation. This would require the lymphocytes to exhibit increased expression of the inducible IL-2Rα. To ascertain that the IL-2 levels measured in cell cultures signal concurrent events of IL-2 secretion and utilization by lymphocytes, we conducted separate experiments to characterize the expression of inducible IL-2Rα on lymphocytes isolated from DEP- and *Listeria*-exposed rats and their time-dependent secretion of IL-2. Figure 3 shows the flow cytometric analysis of the percent of IL-2Rα$^+$, CD4$^+$/IL-2Rα$^+$, and CD8$^+$/IL-2Rα$^+$ cells in lymphocytes isolated from various exposure groups at 7 days postinfection. Lymphocytes from saline- or DEP-treated rats (384 μg/rat, intratracheally instilled) showed a moderate but time-dependent increase in IL-2Rα in response to ConA stimulation. In comparison, both *Listeria* and the combined DEP and *Listeria* exposures produced lymphocytes that show increased expression of ConA-inducible CD4$^+$/IL-2Rα$^+$ and CD8$^+$/IL-2Rα$^+$ cells. This increase in IL-2Rα became significant when cells were incubated with ConA for 24 hr or longer. Figure 4 shows the corresponding IL-2 levels measured in the cell culture media. The combined DEP and *Listeria* exposure and *Listeria* infection alone resulted in lymphocytes that secreted the highest levels of IL-2 in cell cultures incubated for up to 24 hr. At longer incubation times, when these cells exhibited increased expression of IL-2Rα (Figure 3), the IL-2 levels in the incubation media sharply declined (Figure 4).

The above results show that lymphocytes obtained at 7 days postinfection from rats exposed to *Listeria* or to *Listeria* and DEPs exhibit increased IL-2Rα and ability to reutilize the secreted IL-2. In comparison, cells obtained at 3 days postinfection did not show a significant decrease in IL-2 level in the 48-hr culture. This is consistent with the fact that the total number of CD4$^+$ and CD8$^+$ cells and the CD8$^+$/CD4$^+$ ratio in *Listeria*-infected animals, especially under DEP exposure, were significantly increased at 7 days postinfection but not at 3 days postinfection.

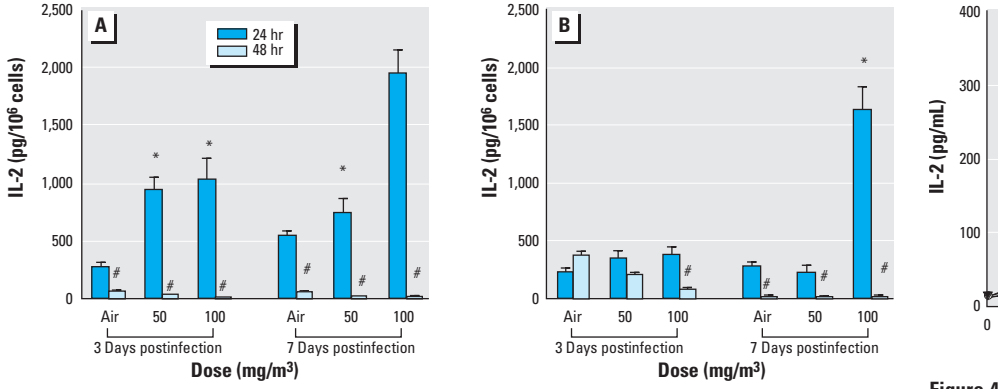

Figure 2. Effect of DEP exposure on the production of IL-2 by lymphocytes from (*A*) noninfected and (*B*) *Listeria*-infected rats in 24- and 48-hr cultures with ConA (2 μg/mL) stimulation. Rats were exposed to clean air or DEPs at 50 or 100 mg/m^3 for 4 hr. *Listeria* was given 2 hr postexposure. Lymphocytes were obtained from rats at 3 and 7 days postexposure. Values are expressed as mean ± SE (n = 3–5).
*Significantly different from the air-exposed control, $p < 0.05$. #Significantly lower than that of the 24-hr culture, $p < 0.05$.

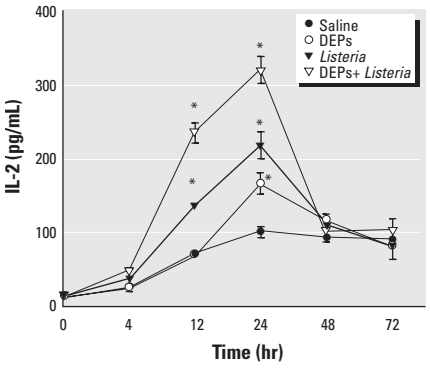

Figure 4. The time-dependent production of IL-2 in response to ConA stimulation by lymphocytes isolated from rats exposed to saline, 384 μg DEPs, 10^5 *Listeria*, or both *Listeria* and DEPs at 7 days postinfection. Values are expressed as mean ± SE (n = 3–5).
*Significantly different from the saline control, $p < 0.05$.

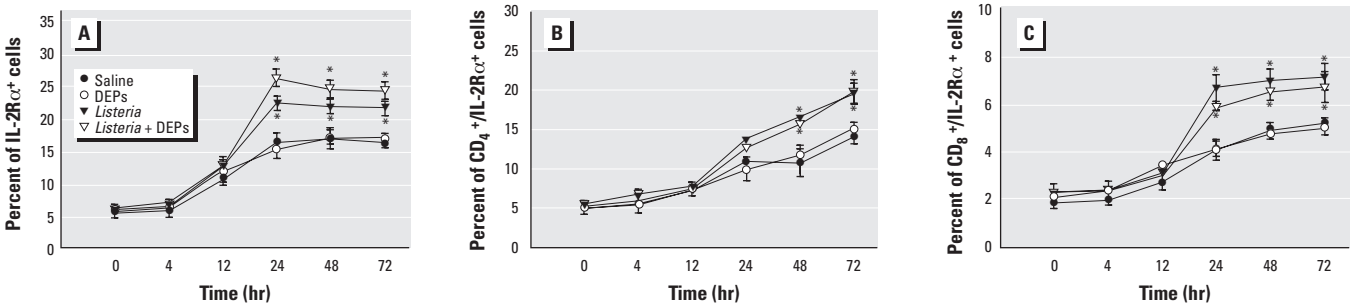

Figure 3. Flow cytometric analysis of the percentage of ConA-induced (*A*) IL-2Rα$^+$, (*B*) CD4$^+$/IL-2Rα$^+$, and (*C*) CD8$^+$/IL-2Rα$^+$ cells in lymphocytes isolated from rats exposed to saline, 384 μg DEPs, 10^5 *Listeria*, or both *Listeria* and DEPs at 7 days postinfection. Values are expressed as mean ± SE (n = 3–5).
*Significantly different from the saline control, $p < 0.05$.

Interleukin-6. Figure 5 shows the secretion of IL-6 by lymphocytes isolated from various exposure groups in response to HKLM stimulation. DEP exposure showed a moderate but dose-dependent enhancement of the production of IL-6 by lymphocytes at 3 days postexposure. In comparison, lymphocytes from *Listeria*-treated rats at 3 days postinfection were easily inducible by HKLM to secrete IL-6. Lymphocytes obtained from rats exposed to both DEPs and *Listeria* showed a significant increase in their ability to produce IL-6 compared with cells from DEP- or *Listeria*-exposed rats. The combined DEP and *Listeria* effect on IL-6 production was DEP dose–dependent. These results show that DEP exposure augments *Listeria*-induced lymphocyte production of IL-6.

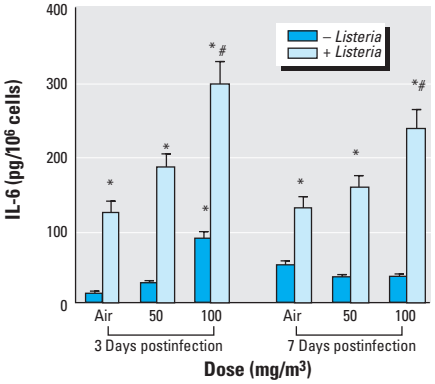

Figure 5. Production of IL-6 by lymphocytes from various exposure groups at 3 and 7 days postexposure in response to 10^7/mL HKLM. Rats were exposed to clean air or DEPs at 50 or 100 mg/m^3 for 4 hr. *Listeria* was given 2 hr after air or DEP exposure. Values are expressed as mean ± SE (n = 3–5). *Significantly different from the air-exposed control, $p < 0.05$. #Significantly different from the sum of *Listeria*-only and DEP-only groups, $p < 0.05$.

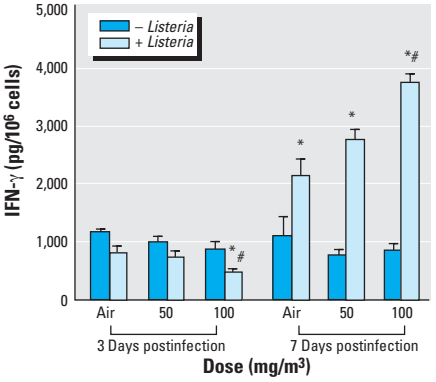

Figure 6. Production of IFN-γ by lymphocytes from various exposure groups in response to ConA (2 μg/mL) stimulation. Rats were exposed to clean air or DEPs at 50 or 100 mg/m^3 for 4 hr. *Listeria* was given 2 hr after air or DEP exposure. Lymphocytes were harvested from rats at 3 and 7 days postexposure. Values are expressed as mean ± SE (n = 3–5). *Significantly different from the air-exposed, noninfected control, $p < 0.05$.#Significantly different from the air-exposed, *Listeria*-infected group, $p < 0.05$.

Interferon-γ. The secretion of IFN-γ by lymphocytes from various exposure groups in response to *ex vivo* ConA stimulation is shown in Figure 6. Lymphocytes from all exposure groups responded to the nonspecific stimulation by ConA. At 3 days postexposure, both *Listeria* infection and DEP exposure appeared to decrease the production of IFN-γ by lymphocytes in response to ConA stimulation, when compared with cells from the air-exposed, noninfected groups. DEP exposure at 100 mg/m^3 also resulted in a decrease in IFN-γ production by cells from the *Listeria*-infected groups. At 7 days postexposure, however, *Listeria*-infected cells showed a significant increase in IFN-γ production compared with cells from the corresponding noninfected group. There was also an increase in IFN-γ production by cells from DEP-exposed and *Listeria*-infected rats. This increase was associated with an increase in DEP exposure dose. Figure 7 shows that the production of IFN-γ by lymphocytes from *Listeria*-infected rats was also inducible by HKLM in a dose-dependent relationship. In comparison, cells from air- or DEP-exposed rats without *Listeria* infection did not show substantial response to HKLM. Figure 7 also shows that DEP exposure attenuated the HKLM-induced secretion of IFN-γ by lymphocytes obtained at 3 days postinfection but not the secretion of IFN-γ by lymphocytes obtained at 7 days postinfection. In fact, the secretion of IFN-γ by lymphocytes from DEP- and *Listeria*-exposed rats at 7 days postinfection increased with increasing DEP dose and was significantly higher than the added secretion by cells from DEP-exposed and *Listeria*-exposed rats. These results show that at 7 days postinfection, T lymphocytes were developed specifically in response to *Listeria* infection. The DEP exposure augmented the T-cell immune responses.

To ascertain whether DEPs have a direct effect on lymphocyte secretion of IFN-γ, cells from rats treated with saline or 100,000 *Listeria* for 7 days were isolated and *ex vivo* challenged with DEPs (0–50 μg/mL). Figure 8 shows that lymphocytes from noninfected rats are not stimulated by HKLM in IFN-γ production, with or without DEPs. In contrast, lymphocytes from *Listeria*-infected rats secrete a significantly elevated level of IFN-γ in response to HKLM, but this secretion is strongly inhibited by DEPs at all dose levels tested.

Discussion

The effect of acute DEP exposure (50 or 100 mg/m^3, 4 hr) on lung clearance of *Listeria* under the current experimental conditions has been reported in a previous article (Yin et al. 2002). Rats exposed to clean air followed by inoculation of 1×10^5 *Listeria* on day 0 showed an increased bacteria count in the lungs (4.3×10^5) on day 3 but a substantially declined bacteria count (0.2×10^5) at day 7. Rats exposed to 50 and 100 mg/m^3 DEPs for 4 hr followed by inoculation of *Listeria* exhibited bacteria counts of 9.5×10^5 and 42.2×10^5, respectively, at day 3 and a decline of bacteria count to nearly the level of the air-exposed control at day 7. The current study shows a strong correlation between the bacterial counts mentioned above and the lymphocyte differentials measured at 7 days postexposure. At 100 mg/m^3, DEP exposure resulted in a marked increase in total lymphocytes in *Listeria*-infected lungs (Table 1). The combined effects of *Listeria* and DEP exposure were characterized by an increase in CD4$^+$ and CD8$^+$ cell counts and, in particular, a clear elevation of the CD8$^+$ subset population, as indicated by the significant increase in the CD8$^+$/CD4$^+$ ratio. This change in lymphocyte population was not observed on day 3, suggesting that DEPs, through their initial

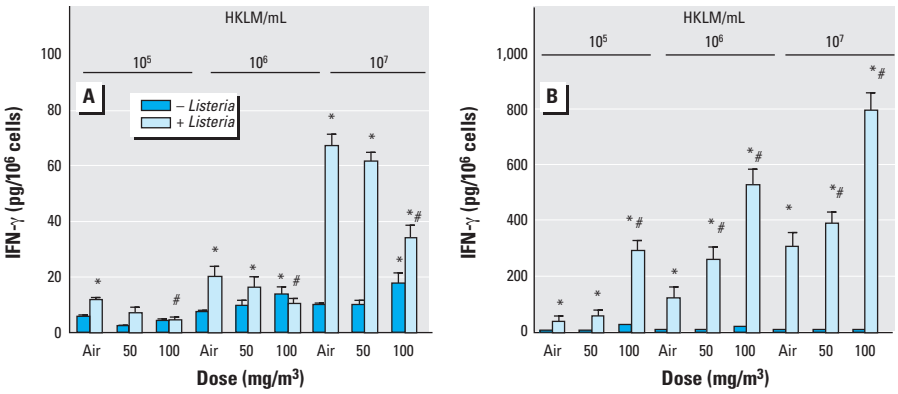

Figure 7. Production of IFN-γ by lymphocytes from various exposure groups in response to varying concentrations of HKLM. Rats were exposed to clean air or DEPs at 50 or 100 mg/m^3 for 4 hr. *Listeria* was given 2 hr after air or DEP exposure. Lymphocytes were harvested from rats at (*A*) 3 and (*B*) 7 days postexposure and measured for IFN-γ in response to 10^5–10^7/mL HKLM. Values are expressed as mean ± SE (n = 3–5).
*Significantly different from the air-exposed, noninfected control, $p < 0.05$. #Significantly different from the air-exposed, *Listeria*-infected group, $p < 0.05$.

down-regulation of AM functions, aggravated *Listeria* infection and facilitated later development of T-cell–mediated immunity. This immune response involves, in part, the development of *Listeria*-specific $CD8^+$ T cells.

The pattern of lymphocyte secretion of IL-2, IL-6, and IFN-γ offers a plausible mechanism for the effect of DEPs on the development of T-cell–mediated immune responses in *Listeria*-infected rats. *Listeria* infection elicits the development of lymphocytes that exhibit increased IL-2 responsiveness. This is indicated by the fact that lymphocytes obtained at 7 days postinfection are more inducible in the expression of IL-2Rα than cells from noninfected rats. They are also capable of diminishing the secreted IL-2 level at the time when elevated IL-2Rα expression is produced, suggesting that these lymphocytes can indeed utilize the secreted IL-2 through their inducible IL-2Rα for cell proliferation. During the immune response, the ability of lymphocytes to respond to IL-2 is completely dependent upon the induced expression of the high-affinity IL-2Rα on the cell surface (Kaempfer 1994). One of the inducers for IL-2 responsiveness is IL-6, a multifunctional cytokine originally discovered as a growth factor stimulating, among other cell types, B-cell differentiation (Akira et al. 1990). Studies have shown that IL-6 also regulates acute-phase responses to injury (Hirano et al. 1990) and the activation of T cells (Lotz et al. 1988). The latter function has been described to involve a synergistic interaction between IL-1 and IL-6 on the induction of cytolytic T-lymphocyte responses (Akira et al. 1990; Ford et al. 1991; Renauld et al. 1989).

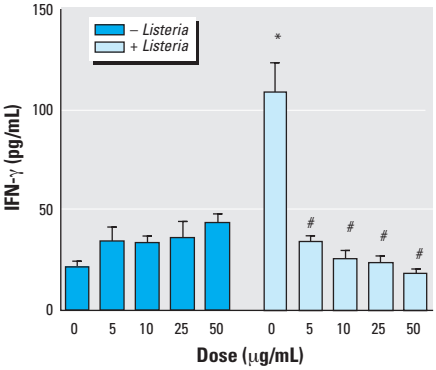

Figure 8. Effect of *ex vivo* DEP exposure on the secretion of IFN-γ by lymphocytes. Rats were inoculated intratracheally with approximately 100,000 *Listeria* or sterile saline (control). Lymphocytes were isolated from rats at 7 days postinfection and measured for INF-γ production in response to HKLM (10^7/mL, 48 hr) stimulation, in the presence and absence of varying concentrations of DEPs (0–50 μg/mL). Values are expressed as mean ± SE (n = 3–5).
*Significantly different from the noninfected control, $p < 0.05$. #Significantly different from the *Listeria*-infected group, $p < 0.05$.

Whereas the effect of IL-6 on B-cell differentiation occurs at later stages of cell maturation, IL-6, in concert with IL-1, controls the initial steps in T-cell activation, with $CD8^+$ being the target cells. IL-6 increases IL-2 responsiveness, and IL-6 and IL-1, acting together, stimulate T-cell production of IL-2. *Listeria* infection induces lymphocyte production of IL-6 (Figure 5). Previously, we have shown that in rats exposed to *Listeria* only, there was an elevated level of IL-1β in the BAL fluid, and that AMs obtained at 3 days after infection showed a strong increase in IL-1β production with or without *ex vivo* challenge by lipopolysaccharide (Yin et al. 2002). The fact that *Listeria* also induces the production of IL-6 by lymphocytes obtained at day 3 indicates that these two cytokines are probably involved in the observed T-cell differentiation at day 7. This IL-1– and IL-6–mediated T-cell activation, although moderate in magnitude in rats exposed to *Listeria* only, is consistent with the data that at day 7, lymphocytes from these animals showed increased responsiveness to IL-2 and secreted higher levels of IFN-γ than cells from the air-exposed, noninfected control.

DEP exposure alone resulted in a moderate elevation of $CD4^+$ and $CD8^+$ cell counts, but the ratio of $CD8^+/CD4^+$ was not different from that of the air-exposed control. Lymphocytes from rats exposed to 100 mg/m^3 DEPs at 3 days postexposure secreted higher levels of IL-2 and IL-6 and showed greater utilization of IL-2 in response to ConA stimulation than cells from air-exposed controls. DEP exposure at 100 mg/m^3 also enhanced the production of IL-1 by AMs obtained 3 days after exposure (Yin et al. 2002). Taken together, these results show that lymphocytes may be activated by DEP exposure through the inflammatory responses involving IL-1, IL-2, and IL-6. The multifunctional IL-6 probably plays a coordinated role in pulmonary responses to particle-induced lung injury. Although lymphocytes from all exposure groups responded to ConA stimulation in the production of IFN-γ, cells from DEP-exposed rats were not activated to produce more IFN-γ than cells from the air-exposed control in response to either ConA or HKLM. This suggests that in the absence of *Listeria*, there is a lack of antigen-specific responses in T-cell differentiation. Consequently, these lymphocytes are not primed to secrete IFN-γ.

In DEP- and *Listeria*-exposed rats, there is synergistic action between DEPs and *Listeria* on T-cell activation. The development of T lymphocytes with increased ability to secrete and reutilize IL-2 was observed even at 3 days postexposure. At 7 days postexposure, there was a clear increase in IL-2 production and IL-2 responsiveness by lymphocytes obtained from rats exposed to *Listeria* and 100 mg/m^3 DEPs. This change in IL-2 secretion and responsiveness can be attributed to their effect on lymphocyte production of IL-6. Both DEP exposure and *Listeria* infection produce lymphocytes that secrete elevated levels of IL-6. Lymphocytes from rats exposed to both *Listeria* and 100 mg/m^3 DEPs showed even greater production of IL-6 at both 3 and 7 days postinfection. The pulmonary bacterial count in rats exposed to *Listeria* and 100 mg/m^3 DEPs at 3 days postinfection was about 10-fold of the value observed for rats exposed to *Listeria* only. This overwhelming increase in bacterial number is conducive for a strong T-cell activation through the IL-6/IL-2 action. The significant increase in the $CD8^+/CD4^+$ ratio at 7 days postinfection suggests that $CD8^+$ cells capable of secreting IL-2 and IFN-γ are involved in the T-cell–mediated immune responses. The specific effect of DEP exposure on the Th1 $CD4^+$ responses is not yet clear. Studies by Hsieh et al (1993) have shown that the mechanism of *Listeria*-induced Th1 $CD4^+$ T-cell development involves an interaction between *Listeria* and host macrophages that results in IL-12 production. Because DEPs inhibit *Listeria*-induced IL-12 production by AMs and the IL-12 level in the BAL fluid (Yin et al. 2002), it is possible that DEPs may inhibit the development of Th1 $CD4^+$ responses.

The secretion of IFN-γ by lymphocytes from *Listeria*-infected rats responded to stimulation by both ConA and HKLM. At 3 days postexposure, DEPs had a moderate but significant inhibitory effect on lymphocyte production of IFN-γ. At 7 days postexposure, however, cells from rats exposed to DEPs and *Listeria* showed highly elevated production of IFN-γ in response to stimulation by HKLM or ConA. This is despite the fact that DEPs, through direct contact with the cells, inhibit lymphocyte production of IFN-γ. One explanation of the strong development of *Listeria*-specific, IFN-γ-secreting T cells by rats exposed to DEPs and *Listeria* is that DEP exposure strongly inhibited the AM-orchestrated immune responses, resulting in a large increase in pulmonary bacterial count at 3 days postinfection. The Brown Norway rats were able to respond to the aggravated infection with a T-cell immunity that involves $CD8^+$ cells.

In summary, this study shows that *Listeria* elicited a T-cell–mediated immune response characterized by an increase in $CD8^+$ cells in the Brown Norway rats. The mechanism involves an elevated lymphocyte production of IL-6, which acts in concert with IL-1 to induce the IL-2 secretion and responsiveness by lymphocytes for rapid clonal expansion of the effector T-cell population. DEPs exhibited multiple effects on the T-cell–mediated immune responses. Exposure of rats to DEPs increased lymphocyte differentials in LDLNs

and their production of IL-2 and IL-6 but inhibited the secretion of IFN-γ by lymphocytes. The acute DEP exposure, which aggravates *Listeria* infection by down regulating the innate immunity, augments lymphocyte production of IL-2 and IL-6 by *Listeria*, resulting in increased development of $CD8^+$ cells capable of secreting IL-2 and IFN-γ. This mechanism allowed the Brown Norway rats to efficiently clear the bacteria at 7 days post-DEP exposure, even though the bacteria infection was severely worsened by DEPs at 3 days post-exposure. It is not clear, however, whether DEPs inhibit the development of T-cell responses under chronic or subchronic inhalation exposure. DEPs, when incubated with lymphocytes from *Listeria*-infected rats, inhibit the cellular production of IFN-γ. The fact that DEPs may also attenuate $CD4^+$ Th1 responses through inhibition of macrophage production of IL-12 suggests that both the $CD4^+$ Th1 and $CD8^+$ immune responses to *Listeria* may be impaired by long-term DEP exposure.

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