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Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) Suppresses the Humoral and Cell-Mediated Immune Responses to Influenza A Virus without Affecting Cytolytic Activity in the Lung

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The immune response to influenza virus is exquisitely sensitive to suppression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); however, the cellular mechanisms underlying the suppressive effects of TCDD are unknown. Mice exposed to TCDD exhibited a dose-responsive increase in mortality following an otherwise non-lethal influenza virus infection. Given that cytotoxic T lymphocytes (CTL) are generally thought to resolve primary infections in the lung, we tested the hypothesis that exposure to TCDD suppresses T-cell responsiveness, leading to decreased CTL in the lung. After infection with influenza virus, naive $CD8^+$ lymphocytes are activated and differentiate in the mediastinal lymph node (MLN). In mice exposed to TCDD and infected with influenza virus, the number of $CD8^+$ MLN cells was reduced 60% compared to vehicle-treated mice. Moreover, MLN cells from TCDD-treated mice failed to develop cytolytic activity, and the production of interleukin (IL)-2 and interferon (IFN)-γ was suppressed. Exposure to TCDD also altered the production of virus-specific antibodies, decreased the recruitment of $CD8^+$ cells to the lung, reduced the percentage and number of bronchoalveolar lavage cells bearing a CTL phenotype ($CD8^+CD44^{hi}CD62L^{lo}$), and suppressed IL-12 levels in the lung. Despite our findings that exposure to TCDD suppressed T cell-dependent functions, the cytolytic activity of lung lavage cells from TCDD and vehicle treated mice was equivalent, and IFNγ levels in the lungs of mice treated with TCDD were enhanced 10-fold. Thus, while exposure to TCDD suppressed a number of responses associated with the development of adaptive immunity to influenza virus, a direct link between these effects and enhanced susceptibility to influenza remains unclear.

Key Words: **dioxin; lymphocyte; mouse; pulmonary; lymph node; immune suppression; host resistance; anti-viral immunity; cytokine.**

The immune system is recognized as one of the most sensitive targets for the toxicity of the environmental contaminant TCDD. In fact, the decreased host resistance observed in mice following influenza virus infection and exposure to TCDD represents the most sensitive adverse effect of TCDD reported to date (Burleson *et al.*, 1996; House *et al*. 1990). However, few studies have been performed to determine the mechanism by which exposure to TCDD causes this enhanced mortality, and the effects of TCDD on the development of protective immunity following *in vivo* infection with influenza virus have not been determined. While the direct cellular targets and specific immunotoxic mechanisms of TCDD are unknown, the toxicity of TCDD is initiated when it binds to an intracellular, ligand-dependent transcription factor, the aryl hydrocarbon receptor (AhR). The toxicity of individual AhR ligands correlates directly with AhR binding affinity (Goldstein and Safe, 1989; Whitlock, 1993). Of the known AhR ligands, TCDD exhibits the highest binding affinity, making it the prototype and most toxic ligand of the AhR.

While the precise mechanism of immunotoxicity remains unclear, exposure of mice to TCDD suppresses both humoral and cell-mediated immune responses to a variety of antigens (reviewed by Kerkvliet 1998). Numerous studies have shown that *in vivo* exposure to TCDD specifically leads to the dose-dependent suppression of T lymphocyte function, including proliferation, differentiation, cytokine production, and T cell-dependent B-cell responses (Kerkvliet *et al.*, 1990, 1996; Lundberg *et al*., 1992; Neumann *et al.*, 1993; Prell *et al*., 1995; Tomar and Kerkvliet, 1991). The immune response to influenza virus is T-cell dependent, relying on the activation of both $CD4^+$ and $CD8^+$ T lymphocytes. Following virus entry and infection of lung epithelial cells, antigen-presenting cells (APC) migrate to the regional lymph nodes, where they present viral antigens and activate virus-specific T lymphocytes. $CD4^+$ T cells produce cytokines such as interleukin (IL)-2 and interferon (IFN)-γ, which drive both B-cell activation and the clonal expansion and differentiation of $CD8^+$ T cells, leading to the creation of virus-specific cytotoxic T lymphocytes (CTL) and neutralizing antibodies. Following activation and differentiation in the lymph node, CTL migrate to the lung and kill virus-infected cells. Based on numerous studies using athymic mice, MHC class I-deficient mice, and neutralizing antibodies, it is generally accepted that a primary infection with influenza virus is resolved by the contact-dependent cytolytic activity of

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$CD8^+$ T lymphocytes (Allan *et al.*, 1990; Baumgarth *et al.*, 1994; Eichelberger *et al.*, 1991; Lukacher *et al.*, 1984; Topham *et al.*, 1997; Yap *et al.*, 1978).

Given the importance of T cells in the clearance of influenza virus and the sensitivity of T cells to perturbation by exposure to TCDD, we hypothesized that decreased host resistance to influenza virus is due, at least in part, to suppression of T cell-dependent adaptive immunity. To test this, we examined whether exposure to TCDD suppresses the expansion and cytolytic activity of T cells in the regional lymph node, and we measured the production of influenza virus-specific antibodies and cytokines that are important for the differentiation and proliferation of T cells. Additionally, we examined TCDD-induced alterations in the pulmonary immune response to infection with influenza virus, including cellular recruitment to the lung, cytolytic activity, and production of IFNγ and IL-12.

MATERIALS AND METHODS

Animals. C57Bl/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in microisolators in a specified pathogen-free facility at Washington State University and were provided food and water *ad libitum*. Female mice were used in experimental studies at 7 to 9 weeks-of-age. Mice were sacrificed, either by an ip dose of Avertin (2,2,2-tribromoethanol) or CO_2 asphyxiation, at various times after infection with influenza virus.

TCDD exposure. TCDD (Cambridge Isotope Laboratories, Inc., Woburn, MA) was dissolved in anisole and diluted in peanut oil to 1 μg/ml. Mice were given a single oral dose of 1, 5, or 10 μg/kg body weight by gavage one day prior to infection with influenza virus. Control mice received peanut oil-anisole vehicle in the same manner as described above.

Influenza virus. Influenza virus (A/HKx31; H3N2) was received as a gift from Dr. Michael Coppola (Argonex, Charlottesville, VA). A/HKx31 is a murine-adapted recombinant strain that bears the internal components of A/PR8/34 (H1N1) and the external components of A/Aichi (H3N2). A/HKx31 was propagated according to methods described by Barrett and Inglis (1985). Briefly, viable fertilized chicken eggs (Spafas, Preston, CT) were inoculated with 0.05 hemagglutinating units (HAU) influenza virus in 100 μl of Hanks balanced salt solution, 10 mM HEPES, on gestational day 10. Infected eggs were incubated for 48 h at 37°C followed by refrigeration overnight at 4°C. Under aseptic conditions, allantoic fluid was harvested, centrifuged, and immediately frozen at –80°C until just prior to use. The titer of the allantoic fluid was determined by hemagglutination of avian erythrocytes. Mice were intranasally infected under anesthesia (Avertin) with 120 HAU influenza virus in 31 μl of allantoic fluid. One HAU of influenza virus is defined as the amount of virus that agglutinates 50% of the erythrocytes when a solution containing virus and a 0.5% solution of erythrocytes are combined in equal volumes.

Collection and preparation of bronchoalveolar lavage (BAL) cells. A catheter attached to a 1-ml syringe was inserted into an incision in the trachea immediately posterior to the larynx. The respiratory tract was washed with RPMI 1640 containing 1% BSA and 10 mM HEPES using 3 separate 1-ml aliquots, each of which was infused and withdrawn 3 times. Cells were enumerated using a Coulter Counter (Beckman Coulter Corp., Miami, FL). Isolated BAL cells were used immediately for assessment of cytolytic activity, differential cell staining (Leukostat; Fisher Scientific, Santa Clara, CA), or were stained for flow cytometric analysis.

Collection and preparation of mediastinal lymph node (MLN) cells. Single-cell suspensions were prepared under aseptic conditions by pressing both MLN from a single animal between the frosted ends of 2 microscope slides. MLN cells were suspended in cold RPMI 1640 containing 2.5% FBS (Hyclone, Logan, UT) and 10 mM HEPES. Cellular debris was removed by sedimentation and the cells were enumerated using a Coulter Counter. MLN cells were either used immediately for analysis by flow cytometry or were transferred to cultures for re-stimulation. For *ex vivo* re-stimulation, MLN cells (2×10^6) were suspended in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μg/ml gentamicin, and 50 μM 2-mercaptoethanol, and were incubated at 37°C for 24 h or 5 days with 1×10^6 influenza virus-infected, irradiated antigen-presenting cells (DC2.4 dendritic cells; a gift from Dr. Ken Rock, Dana Farber Cancer Institute, Boston, MA).

CTL assay. Influenza virus-specific cytolytic activity was assessed using a standard 5-h ^{51}Cr-release assay, as described by Nonacs *et al.* (1992). Briefly, BAL cells or *ex vivo* re-stimulated MLN cells were incubated with ^{51}Cr-labeled, influenza virus-infected MC57G fibroblasts at E:T ratios from 100:1 to 6.25:1. Released radioactivity was measured after a 5-h incubation at 37°C. Total releasable ^{51}Cr was determined by lysing target cells with 0.5% SDS, and spontaneous release was obtained by incubating target cells in media only. Specific lytic activity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{experimental release} - \text{naive release}}{\text{maximum release} - \text{spontaneous release}} \times 100.$$

Control experiments were performed to validate that the observed cytolytic activity is specific for cells infected with influenza virus. No killing of mock-infected, ^{51}Cr-labeled MC57G cells was observed.

Immunophenotypic analyses. Freshly isolated cells were incubated with previously determined optimal concentrations of fluorochrome-conjugated antibodies. Appropriately labeled, isotype-matched immunoglobulins were used as controls for non-specific fluorescence. The following were used as primary antibodies: Tricolor-labeled anti-CD8a, FITC-labeled anti-CD44, PE-labeled anti-Vβ8.3 from Caltag Laboratories (Burlingame, CA); and FITC-labeled anti-CD4, FITC-labeled anti-Vβ8.3, and PE-labeled anti-CD62L from PharMingen (San Diego, CA). Data were collected from 25,000 (MLN) or 100,000 (BAL) cells by listmode acquisition, using a FACScan flow cytometer (Becton Dickenson, San Jose, CA). Dead cells, clumps, and debris were excluded from analysis, using a combination of forward angle and 90° light scatter and propidium iodide exclusion. Data were analyzed using WinList software (Verity Software, Topsham, ME).

Differential cell analyses. BAL cells from individual animals were transferred to microscope slides using a cytological centrifuge, and were stained with hematoxylin and eosin (LeukoStat; Fisher Scientific, Pittsburgh, PA). Monocytes/macrophages, neutrophils, or lymphocytes were enumerated by differential counts of 200 cells on coded slides.

Cytokine analyses. Cytokines were analyzed using matched antibody pairs in a sandwich enzyme-linked immunosorbant assay (ELISA). ELISA reagents were supplied by the following sources: IL-2 and IFNγ, PharMingen (San Diego, CA); IL-12, R&D Systems (Minneapolis, MN), and Genzyme Diagnostics (Cambridge, MA). ELISAs were conducted according to the manufacturers' recommended protocols.

Antibody analyses. Influenza virus-specific antibody levels were analyzed using a stacking ELISA. Purified influenza virus ($\times$31, Spafas, Preston, CT) was bound to 96-well plates. A volume of 100 μl of each sample (e.g., plasma) was added to the ELISA in 4-fold serial dilutions from 1:25 to 1:25,600. Vehicle- and TCDD-treatment groups were compared at a plasma dilution in which both groups were in the linear range of absorbance. Anti-isotype-specific antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL) were used to assess specific isotypes, according to the manufacturer's recommended procedure.

Statistical analyses. The results presented are representative of at least 3 to 5 independent experiments. Statistical analyses were performed using Statview (version 4.01, Abacus Concepts, Berkeley, CA). Using a one-way ANOVA, followed by *post hoc* tests (Fisher PLSD), differences between independent variables were compared over time and between each treatment group. Differences were considered significant when p was less than 0.05.

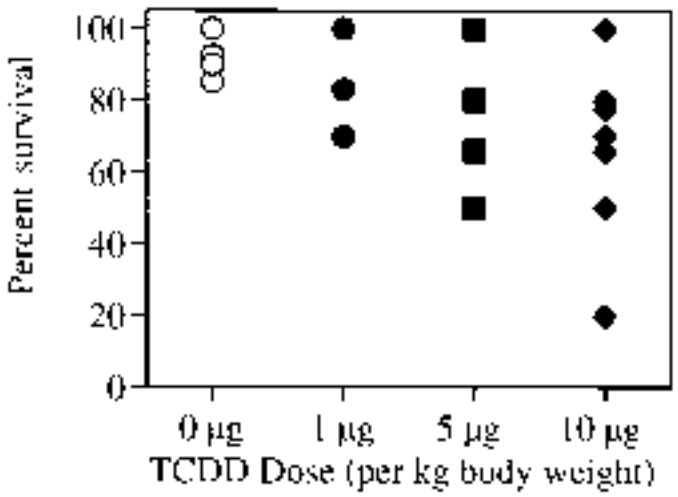

FIG. 1. Exposure to TCDD dose-dependently increases mortality following infection with influenza virus (A/HKx31). Female C57Bl/6 mice were gavaged with TCDD at the indicated dose one day prior to intranasal infection with influenza virus (120 HAU). Symbols represent individual experiments (initial treatment size for each experiment was 6–10 mice per group). The following number of experiments are shown for each treatment group: 0 μg TCDD, 10; 1 μg TCDD, 3; 5 μg TCDD, 4; and 10 μg TCDD, 8 experiments.

RESULTS

Exposure to TCDD dose-dependently increases mortality after infection with influenza virus. Previous studies of the effects of TCDD on host resistance to influenza virus used a lethal challenge of influenza A (Burleson *et al.*, 1996). In the present study, we challenged mice with a dose and strain of influenza virus, A/HKx31, which typically does not cause mortality in infected animals. Nevertheless, we found that infection with influenza and exposure to TCDD (1–10-μg/kg body weight) resulted in a dose-dependent increase in mortality following intranasal infection (Fig. 1). When observed, mortality varied widely between experiments at equivalent doses of TCDD. For example, in 2 out of 8 experiments, no mortality was observed in infected mice exposed to 10 μg/kg TCDD, while in another experiment, 80% of the mice died. It is important to note that the immunological data we present were derived only from mice that survived infection with influenza virus. In these studies, survival in the vehicle treatment group was 100% while survival in infected mice treated with TCDD was 75–80%. When mortality occurred, mice consistently died on days 5 through 8 after infection.

TCDD treatment decreases CD8$^+$ and CD4$^+$ T cell expansion and cytokine production in the MLN. Following a primary infection with influenza virus, the activation, proliferation, and differentiation of naive T cells can be measured in the MLN (Baumgarth *et al.*, 1997; Hamilton-Easton and Eichelberger, 1995; Tripp *et al.*, 1995). Specifically, compared to uninfected controls, there is a well-documented expansion in the number of CD4$^+$ and CD8$^+$ T cells in the MLN following infection with influenza virus. Furthermore, following *ex vivo* re-stimulation, isolated MLN cells from infected animals produce IL-2 and IFNγ (Allan *et al.*, 1990; Baumgarth *et al.*, 1997; Flynn *et al.*, 1998; Hennet *et al.*, 1992). Using these indicators, we tested the hypothesis that exposure to TCDD suppresses T-cell responsiveness to influenza virus. In vehicle-treated mice, no significant changes in T-cell number or cytokine production were detected in the MLN prior to day 5 post-infection. However, after 5 days, there was an increase in the number of CD4$^+$ and CD8$^+$ MLN cells (Figs. 2A and 2B), and both IL-2 and IFNγ were detected in supernatants of re-stimulated MLN cells (Figs. 2C and 2D). These changes were much less pronounced in MLN cells from TCDD-treated mice. On day 9 post-infection, TCDD-treated mice exhibited a 2.5-fold decrease in the number of CD4$^+$ and CD8$^+$ MLN cells, a 2-fold decrease in IL-2, and a 3-fold decrease in IFNγ production by MLN cells, as compared to cells from vehicle-treated mice.

Exposure to TCDD decreases CTL activity of MLN cells and alters the plasma antibody profile. Consistent with the idea that TCDD causes an overall suppression of T-cell function, we tested whether exposure to TCDD impairs virus-specific

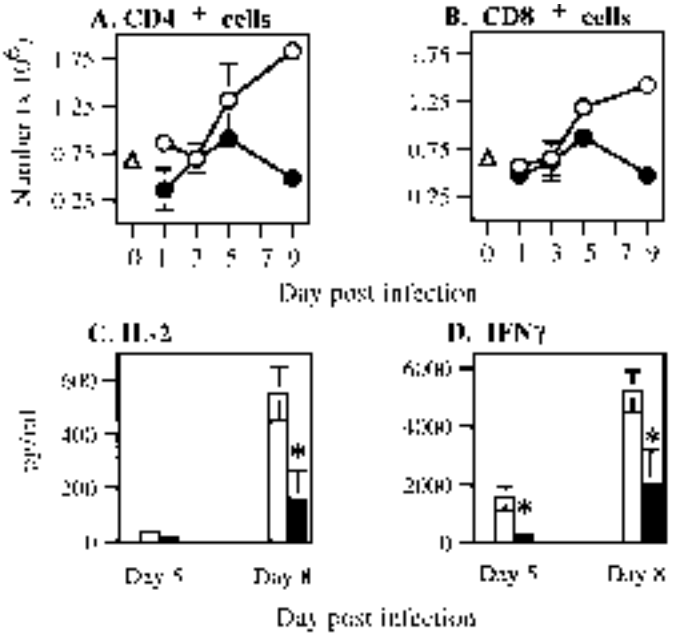

FIG. 2. Exposure to TCDD suppresses T-cell expansion and cytokine production in mediastinal lymph nodes (MLN). Female C57Bl/6 ($n = 10$) mice were exposed orally to TCDD (10 μg/kg) one day prior to intranasal infection with 120 HAU influenza virus. Animals were sacrificed on the indicated day post-infection and MLN cells were collected. Immunophenotypic analysis was conducted by flow cytometry to determine the number of CD4$^+$ (A) and CD8$^+$ (B) T cells in MLN from naive (triangle), vehicle- (circle) and TCDD-treated (filled circle) mice. Cytokine production by *ex vivo* re-stimulated MLN cells was determined using IL-2- (C) and IFNγ- (D) specific ELISA. Data points represent the mean $\pm$ SEM, and asterisks indicate a significant difference compared to vehicle group ($p \leq 0.05$). No IL-2 or IFNγ was produced by MLN cells isolated from naive mice.

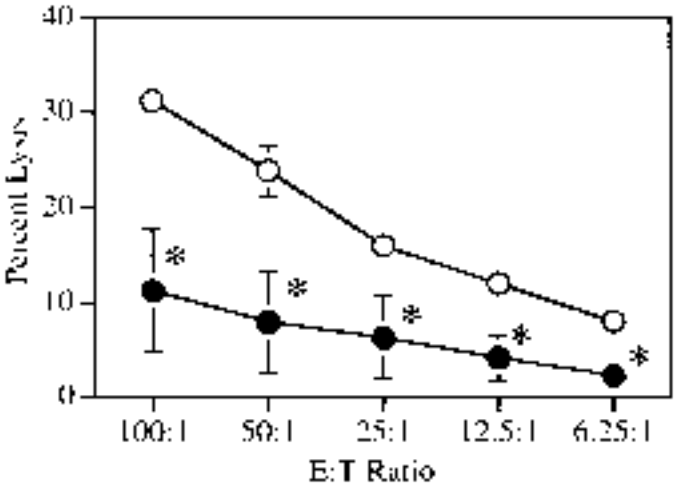

FIG. 3. Exposure to TCDD decreases MLN cytolytic activity. Mice (n = 10) were treated as described in Figure 2 and sacrificed 9 days after infection with influenza virus. CTL activity of MLN cells isolated from vehicle-treated (circle) and TCDD-treated (filled circle) mice was assessed using a standard ^{51}Cr-release assay at the indicated effector:target (E:T) ratios, as described in Materials and Methods. Data points represent the mean ± SEM. Asterisks indicate a significant difference compared to the vehicle group ($p \leq 0.05$).

CTL and antibody responses. We examined the generation of virus-specific CTL activity by comparing the lytic activity of MLN cells isolated from vehicle- and TCDD-treated mice against A/HKx31-infected target cells. While *ex vivo* re-stimulated MLN cells isolated from vehicle-treated mice generated virus-specific CTL activity, MLN cells from TCDD-treated mice failed to develop activity (Fig. 3).

Using virus-specific ELISA, we determined plasma antibody levels of IgM, IgG_1, IgG_{2a}, IgG_{2b}, and IgA. All 5 of these immunoglobulin isotypes have been detected in mice responding to infection with influenza virus. All isotypes were detected in plasma from vehicle-treated mice 9 days after intranasal infection with A/HKx31 (Fig. 4). IgM, IgG_1, IgG_{2a}, and IgG_{2b} levels in plasma obtained from TCDD-treated mice were decreased 2-fold. However, in contrast to IgM and IgG isotypes, there was a 4-fold increase in plasma IgA levels in mice treated with TCDD. In BAL fluid, total IgG levels were suppressed in mice exposed to TCDD, but there was no difference in the amount of IgA in BAL fluid from vehicle- and TCDD-treated mice (data not shown).

TCDD alters cellular recruitment to the lung. Given that we observed a decrease in T-cell expansion, IL-2 and IFNγ production, and CTL activity in the MLN, and that CTL are considered the principal means for viral clearance in a primary influenza-virus infection, we examined the effects of TCDD on the pulmonary immune response. Cellular recruitment to the lung in response to infection was assessed in two ways: by measuring the total number of cells obtained by lavage and by differential cell counts of BAL cells. Relatively few cells ($<2 \times 10^5$) were recovered in the BAL fluid from uninfected animals. Within 48 h, intranasal infection with influenza virus resulted in a 10-fold increase in the total number of BAL cells (Fig. 5A). Exposure to TCDD did not alter the total number of BAL cells collected on post-infection days 1–5. However, exposure to TCDD resulted in a 40% reduction in BAL cell number on day 9, the time point at which the peak number of BAL cells was observed in vehicle-treated mice.

To assess whether this decrease in cell number reflects the reduction of a specific cell population or diminution of multiple cell types, BAL cells were collected over the course of infection and the percentage of macrophages, lymphocytes, and neutrophils was determined by differential cell counts. In uninfected animals, macrophages comprised 95% of the total BAL cells (Fig. 5B). Within 24 h after infection with influenza virus, the total number of macrophages increased in vehicle-treated mice, but the relative percentage of macrophages decreased to roughly 60% as neutrophils began entering the lung. After infection, neutrophils made up about 30% of all lavage cells. In contrast, an increase in lymphocytes was not detected until about 5 days after infection, and continued to rise through day 9. Exposure to TCDD did not affect the overall percentage or number of monocytes/macrophages. However, the percentage and absolute number of neutrophils in the TCDD-treated group continued to increase, rising to 50% of all BAL cells on days 7 and 8 post-infection. In contrast to the increase in neutrophils, exposure to TCDD caused a 2-fold decrease in the percentage and number of lymphocytes.

TCDD alters cytokine production and decreases the influx of CTL cells into the lung. Based on our observations that exposure to TCDD diminishes T-cell expansion and CTL activity in the MLN and suppresses lymphocyte recruitment to the lung, we hypothesized that CTL recruitment to the lung would likewise be suppressed. If this were true, we would expect to find alterations in the pulmonary cytokine profile,

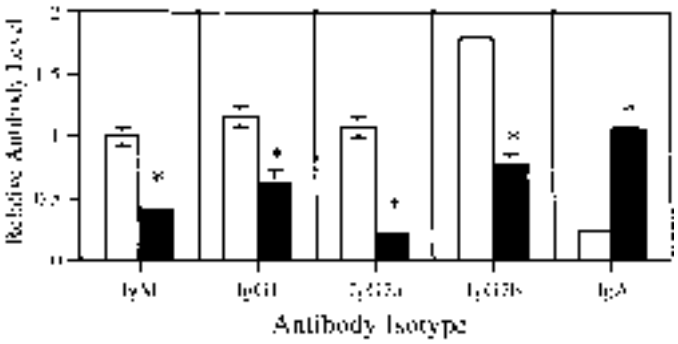

FIG. 4. Exposure to TCDD alters plasma antibody profile. Mice (n = 10) were treated as described in Figure 2 and sacrificed on day 9 post-infection. Relative levels of virus-specific plasma antibodies were determined by ELISA, as described in Materials and Methods. The mean (± SEM) antibody level is shown for vehicle (white bars) and TCDD (black bars) groups at a plasma dilution of 1:6400 for all isotypes except IgA, which is shown at a plasma dilution of 1:400. Asterisks indicate a significant difference from the vehicle group ($p \leq 0.05$).

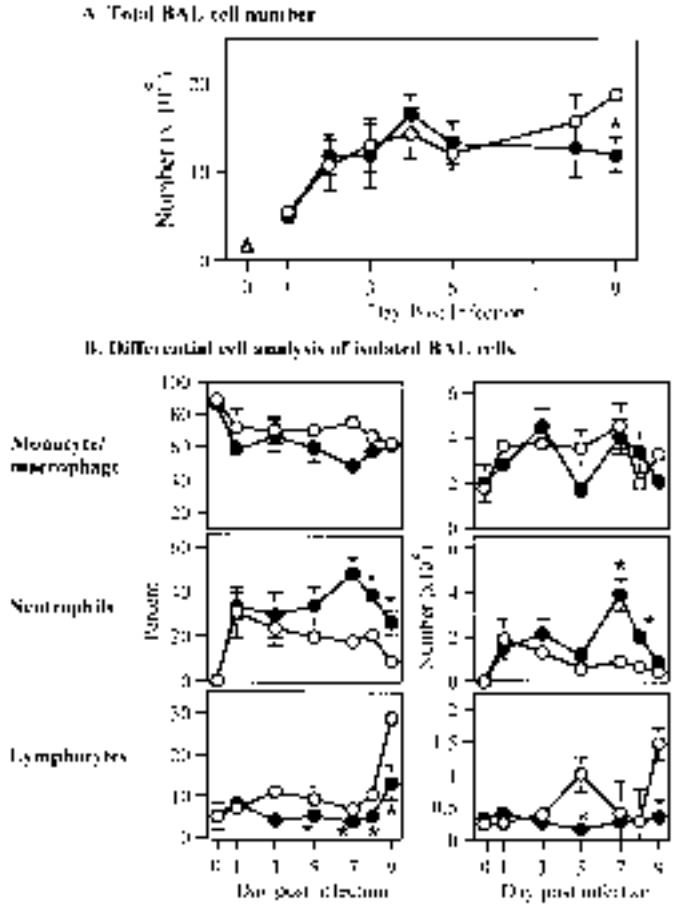

FIG. 5. Exposure to TCDD alters recruitment of cells to the lung. BAL cells were collected from uninfected (triangle), vehicle-treated (circle), or TCDD-treated mice (filled circle) on the indicated days after infection with influenza virus. (A) Total number of BAL cells was determined using a Coulter counter. (B) Differential cell analysis was performed by counting 200 random Leukostat-stained BAL cells per animal ($n = 4–8$ per day). Asterisks indicate a significant difference compared to vehicle-treated mice ($p \leq 0.05$).

reduced numbers of $CD8^+$ T cells, and diminished CTL activity in lungs from mice exposed to TCDD. Two cytokines that are particularly important for a CTL response are IL-12 and IFNγ (Kos and Engleman, 1996; Monteiro *et al.*, 1998). We examined the levels of these two cytokines in the lung over the course of infection. In vehicle-treated mice, IFNγ was first detected in the BAL fluid 5 days after infection, but rapidly decreased and was undetectable 3 days later, while IL-12 increased steadily throughout the course of infection (Fig. 6, open circles). In mice treated with TCDD, the concentration of IFNγ in BAL fluid reached a maximum on post-infection day 7, 2 days after the vehicle-treated group, and was nearly 10-fold greater at that time point. In contrast, IL-12 in lung lavage fluid from the TCDD-treated group declined steadily beginning on day 4 (Fig. 6B). By day 9 post infection, the concentration of IL-12 in lung lavage fluid from vehicle-treated mice was nearly 10-fold greater than the level in TCDD-treated mice.

Having found that exposure to TCDD alters the profile of cytokines important for a CTL response, we then focused on the effects of TCDD on T cells and T-cell function in the lung. As shown in Figure 7A, $CD8^+$ cells were detected in the lung beginning 5 days after infection with influenza virus, with both the percentage and absolute number (numerical data not shown) increasing steadily through day 9. Exposure to TCDD decreased the percentage (and number) of $CD8^+$ cells in the lung on days 8 and 9 by more than 30% when compared to vehicle controls.

On day 9, the time point at which $CD8^+$ T cells are found in greatest number and percentage, we conducted a more detailed immunophenotypic analysis to identify the influenza virus-specific subset of $CD8^+$ T cells. $CD8^+$ T cells that bear the Vβ8.3$^+$ T cell receptor (TCR) recognize an immunodominant antigenic peptide of A/HKx31, and represent the majority of virus-specific $CD8^+$ T cells (Deckhut *et al*., 1993; Townsend *et al*., 1986). Therefore, in addition to examining all $CD8^+$ T cells, we used an anti-Vβ8.3 antibody to assess the recruitment of an influenza virus-specific subset. The results obtained from analysis of $CD8^+$Vβ8.3$^+$ cells were analogous to the results of total $CD8^+$ T cells: exposure to TCDD decreased the percent (and number) of $CD8^+$Vβ8.3$^+$ cells to about 60% of the level found in the vehicle-treated group on day 9 post infection (Fig. 7B).

We further characterized $CD8^+$ T cells and identified CTL in the lung. Virus-specific CTL have been characterized as a subset of $CD8^+$ T cells that express high levels of CD44 and low levels of CD62L (Doherty *et al.*, 1996; Hou and Doherty, 1993). Based on the expression of CD44 and CD62L, we assessed the presence of an effector population of $CD8^+$ T cells in the lung. In vehicle-treated mice, 95% of the $CD8^+$ BAL cells bear the CTL phenotype (Fig. 8A). Measuring CTL over time revealed that these cells comprise about 20% of the total population of BAL cells on day 8 post infection, and 30% on day 9 post infection (Fig. 8C). In virus-infected mice treated with TCDD, there was a 20% decrease in the mean channel

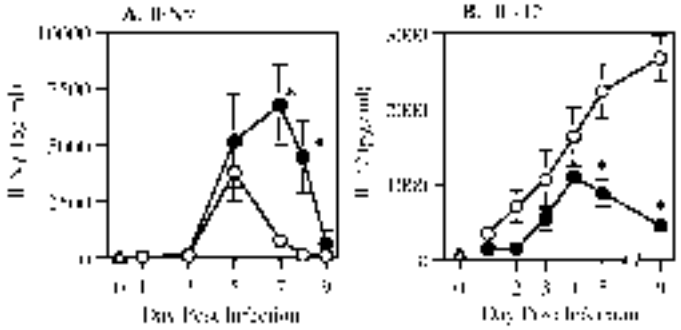

FIG. 6. TCDD treatment suppresses IL-12 but enhances IFNγ levels in bronchoalveolar lavage (BAL) fluid. BAL fluid was collected from uninfected (triangle), vehicle-treated (circle), or TCDD-treated mice (filled circle) on the indicated day after infection. The level of IFNγ (A, $n = 8$) and IL-12 (B, $n = 10$) were measured by ELISA. Data points represent the mean ($\pm$ SEM), and asterisks indicate a significant difference compared to vehicle-treated mice ($p \leq 0.05$).

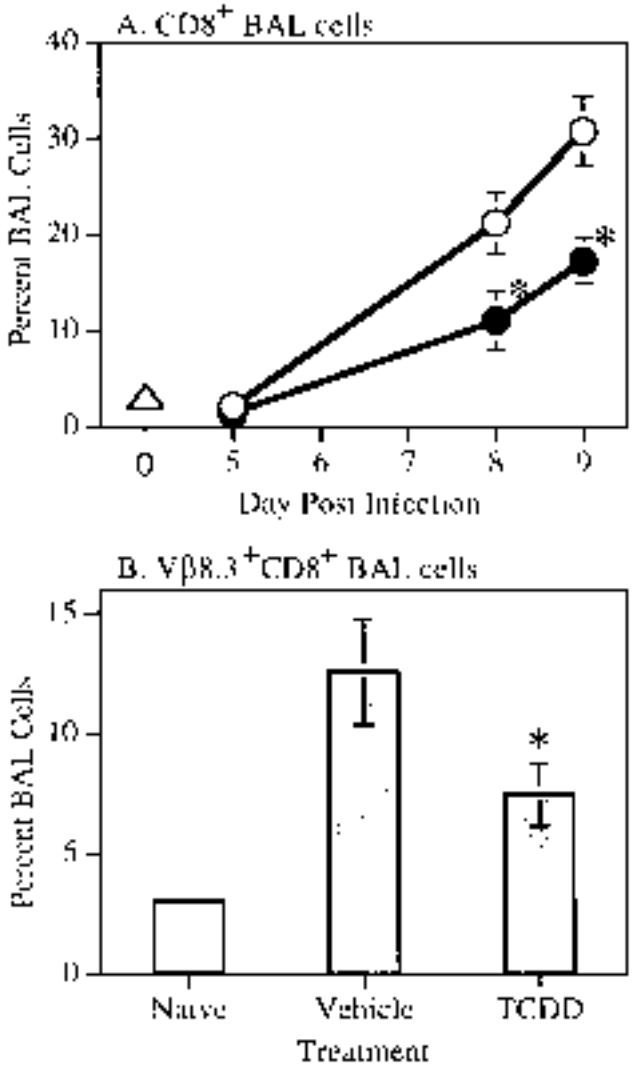

FIG. 7. TCDD treatment decreases the percentage of $CD8^+$ and $V\beta8.3^+CD8^+$ BAL cells. Mice ($n = 8$) were treated as described in Figure 2 and were sacrificed on the indicated day (A) or on day 9 (B) following infection with influenza virus. Percentage of all BAL cells bearing the indicated phenotype was assessed by examining cells from uninfected (triangle), vehicle-treated (circle), or TCDD-treated mice (filled circle). Cells were stained with fluorochrome-conjugated antibodies against CD8 and Vβ8.3, and immunophenotypic analysis was conducted by flow cytometry. Data points represent the mean (± SEM), and asterisks indicate a significant difference compared to vehicle-treated mice ($p \leq 0.05$).

fluorescence of CD44 on $CD8^+$ BAL cells on day 9 post infection (Fig. 8B) and the percentage of all BAL cells bearing a CTL phenotype was reduced more than 2-fold compared to vehicle-treated mice.

BAL cells from vehicle-treated mice and TCDD-treated mice have equivalent lytic activity. Having observed that exposure to TCDD suppresses the recruitment of CTL to the lung, we expected that TCDD treatment would also decrease cytolytic activity. In contrast to this expectation, we found that BAL cells from vehicle- and TCDD-treated mice exhibited equivalent cytolytic activity against influenza virus-infected target cells (Fig. 9). Moreover, in both treatment groups, cytolytic activity in the lung is first detected 5 days after infection and obtains a maximum level on day 9. When we examined cytolytic activity on days 5, 8, 9, and 10 we also observed no differences between cells isolated from vehicle- and TCDD-exposed mice (Lawrence *et al.*, 2000).

DISCUSSION

While trying to understand the mechanisms by which exposure to TCDD affects the susceptibility of mice to infection with influenza virus, we have found evidence that TCDD has differential effects on cellular immune responses in the lung and lymph node. This evidence includes the observation that TCDD treatment suppresses IL-2 and IFNγ production, T cell expansion, and CTL generation in the MLN, yet lung lavage cells isolated from vehicle- and TCDD-treated mice have the same cytolytic activity against virus-infected target cells. Moreover, lung lavage fluid from TCDD-treated mice has ten times more IFNγ than lavage fluid from vehicle-treated mice. These findings establish a relationship between exposure to TCDD and the impaired generation of T cell-dependent immunity to infection with influenza virus, and raise intriguing questions about the nature of the cytolytic activity in the lung and the underlying cause of mortality.

Several laboratories have reported that *in vivo* treatment of rats and mice with TCDD increases mortality following respiratory infection with influenza A virus (Burleson *et al.*, 1996; House *et al.*, 1990; Yang *et al.*, 1994). However, few studies have been performed to determine the mechanisms by which exposure to TCDD decreases host resistance to influenza virus. In particular, the effects of TCDD on T-cell function following *in vivo* infection with influenza virus have not been ascertained. Thus, one goal of this study was to determine whether exposure to TCDD suppresses T-cell function following respiratory viral infection. The effects of TCDD on T-cell responses reported here are consistent with numerous reports that exposure to TCDD suppresses T cell-dependent immunity. For example, our results are quite similar to findings of Kerkvliet *et al.* (1996), who reported a diminished percentage of $CD4^+$ and $CD8^+$ T cells and suppression of *ex vivo* splenic IL-2 and IFNγ in a tumor allograft model. In this same model, exposure to TCDD also decreased the expansion and differentiation of $CD8^+$ T cells, as defined by the altered expression of CD44 and CD62L, phenotypic markers of CTL (Kerkvliet *et al.*, 1996). Our findings concur with other studies, in which activation-induced T cell expansion and the production of IL-2 and IFNγ were reduced following *in vivo* exposure to TCDD (Lundberg *et al.*, 1992; Prell *et al.*, 1995). Likewise, treatment with TCDD suppresses splenic CTL responses to tumor cell challenge in mice (De Krey and Kerkvliet, 1995; Kerkvliet *et al.*, 1990) and disrupts cell-mediated immunity to cytomegalovirus infection in rats (Ross *et al.*, 1997).

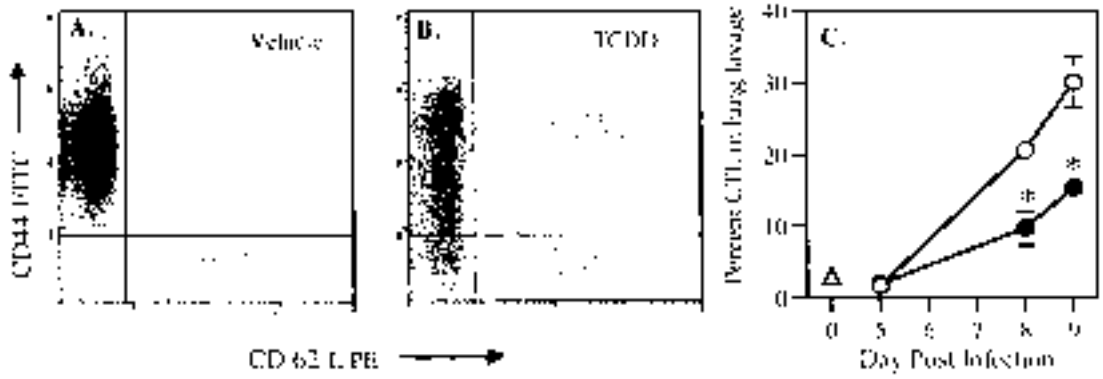

FIG. 8. Exposure to TCDD decreases the percentage of $CD8^+$ BAL cells bearing a CTL phenotype. Mice ($n = 10$) were treated as described in Figure 2. Representative histograms of $CD8^+$ cells from vehicle- (A) and TCDD-treated (B) mice on day 9 following infection with influenza virus are shown. (C) The percent of all BAL cells bearing a CTL phenotype in uninfected (triangle), vehicle-treated (circle) or TCDD-treated mice (filled circle) over the course of infection with influenza virus. Cells were stained with fluorochrome-conjugated antibodies against CD8, CD44, and CD62L. Data points represent the mean (± SEM), and asterisks indicate a significant difference compared to vehicle-treated mice ($p \leq 0.05$). The vehicle-treated group was significantly different from the naive group on days 8 and 9.

Further evidence that exposure to TCDD disrupts cell-mediated immunity is provided by our observation that TCDD treatment suppresses IL-12 levels. Perhaps more than any other cytokine studied to date, IL-12 plays an obligatory role in the activation and maintenance of cell-mediated immunity to infectious diseases (Seder *et al.*, 1996; Trinchieri, 1997). IL-12 induces the expression of co-stimulatory molecules on APC and directly drives the generation of CTL (Trinchieri, 1997). The specific role of IL-12 in cell-mediated immunity to influenza A virus was recently examined (Monteiro *et al.*, 1998). *In vivo* depletion of IL-12 in BALB/c mice decreased virus-specific CTL activity and viral clearance from the lung, confirming that IL-12 contributes to protective immunity to influenza virus. Consistent with a role for altered IL-12 levels in TCDD-mediated suppression of cell-mediated immunity, decreased IL-12 levels were reported in mice exposed to TCDD and challenged with P815 mastocytoma cells (Shepherd *et al.*, manuscript submitted). However, a causal relationship between altered IL-12 and TCDD-mediated immunotoxicity remains to be formally examined.

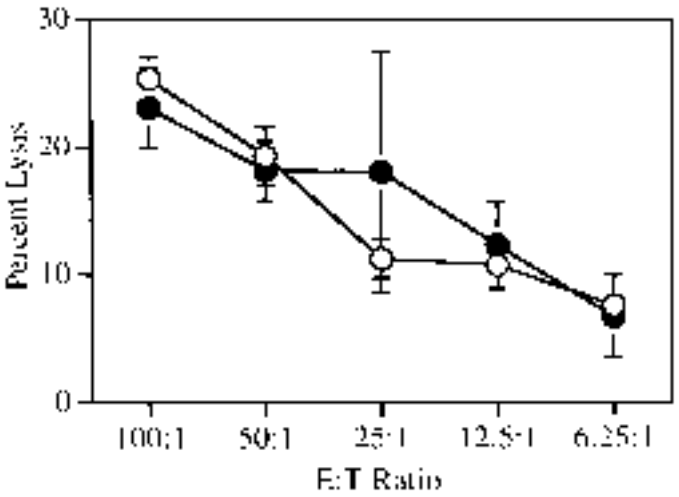

FIG. 9. Lung cells isolated from vehicle (circle) and TCDD-treated (filled circle) mice exhibit equivalent lytic activity. Mice were treated as described in Figure 2 and sacrificed on day 9 following infection with influenza virus. Average (± SEM) cytolytic activity of BAL cells was determined in a 5-h ^{51}Cr-release assay using influenza virus-infected MC57G fibroblasts. There were 8 mice per treatment group.

Another T cell-dependent response to infection with influenza virus is the generation of antibodies (Gerhard *et al.*, 1997). IgM, IgG_1, IgG_{2a} and IgG_{2b}, and IgA isotypes have been implicated as important effectors of viral clearance and protective immunity in mice (Hocart *et al.*, 1989; Jones and Ada, 1986; Justewicz *et al.*, 1995). Consistent with an earlier report, we found that exposure to TCDD suppresses the level of influenza virus-specific IgM and IgG in plasma and lung lavage fluid (House *et al.*, 1990). In contrast to IgM and IgG levels, TCDD treatment enhances the amount of influenza virus-specific IgA in plasma, a finding that is consistent with reports that exposure to TCDD increases IgA in rats and humans (Moran *et al.*, 1986; USAF, 1991). This effect of TCDD on IgA is quite interesting and may reveal an effect of TCDD on cytokines that regulate isotype switching to IgA. However, it is worth noting that recent studies using IgA-deficient mice have demonstrated that IgA is not required to prevent infection, generate neutralizing antibodies, or effectively clear virus and virally infected cells (Mbawuike *et al.*, 1999).

While the significance of increased IgA remains unclear, our findings that exposure to TCDD suppresses influenza-specific IgM and IgG, impairs cytokine production and CTL activity in the MLN, and decreases $CD8^+$ cells and IL-12 levels in the lung indicate that TCDD impairs both the cell-mediated and humoral immune responses to influenza A virus. This interpretation is consistent with the deleterious effects of TCDD observed in other experimental systems and supports the conclusion that TCDD suppresses the generation of an adaptive immune response. However, the idea that exposure to TCDD

prevents adequate development of adaptive immunity and that this defect underlies the impaired host resistance to infection with influenza virus is likely to be overly simplistic. While exposure to TCDD clearly impairs T-cell responses, lung lavage cells isolated from TCDD-treated mice are able to lyse virus-infected target cells as effectively as BAL cells from vehicle-treated controls. Furthermore, when we compared the virus titer of lungs obtained on post infection day 9 from vehicle- and TCDD-treated mice, we detected no infectious virus in either group (Lawrence *et al.*, in press). Thus, in addition to equivalent cytolytic activity, this demonstrated that the TCDD-treated mice that survived to day 9 were able to clear the virus from their lungs.

The ability of TCDD to suppress adaptive immunity and enhance mortality following an otherwise non-lethal infection, yet at the same time not to diminish lung cell cytolytic activity or the clearance of virus from the lung, was surprising to us. This finding raises several very interesting questions: what cell or molecule is responsible for the cytolytic activity and viral clearance in lungs of mice treated with TCDD, and, in the absence of an increased pulmonary virus burden, what mechanism is responsible for the enhanced mortality observed in mice exposed to TCDD? Possible explanations include the activation of another population of cells that compensates for the diminished number and activity of $CD8^+$ T cells in the lung, or the excessive production of cytokines such as IFNγ or tumor necrosis factor (TNF)-α. Alternatively, it is possible that the fewer CTL in the lung are simply more efficient killers. This last scenario seems unlikely. The overwhelming evidence that exposure to TCDD suppresses T-cell function, makes it difficult to put forth a plausible explanation for how fewer T cells could have much greater cytolytic activity than T cells from vehicle-treated mice. Instead, it seems more probable that another mechanism is responsible.

In addition to contact-mediated lysis, cells infected with influenza virus can be killed by TNFα-induced apoptosis (Liu *et al.*, 1999), and TNFα is found in the lung following influenza virus infection (Hennet *et al.*, 1992; our unpublished observations). Furthermore, in other model systems, exposure to TCDD has been reported to increase TNFα levels (Clark and Taylor, 1994; Fan *et al.*, 1997; Moos *et al.*, 1994). Excessive production of TNFα has been correlated with adverse outcomes following infection, often resulting in increased mortality (Nguyen and Biron, 1999; Zhang *et al*., 1996). Thus, overproduction of TNFα in the lung would provide an explanation for both virus clearance and increased mortality. Infection with influenza virus stimulates the rapid production of TNFα in the lung. However, in both vehicle- and TCDD-treated mice, TNFα levels declined rapidly and were undetectable after about 72 h (our unpublished observations). Therefore, it seems unlikely that excessive pulmonary TNFα is a mechanism for cytolytic activity, virus clearance, or enhanced mortality observed in mice exposed to TCDD and infected with influenza virus.

On the other hand, the elevated level of IFNγ in lung lavage fluid from mice exposed to TCDD provides a possible explanation for the destruction of virus-infected cells and enhanced mortality. The pivotal role of IFNγ in host resistance to intracellular pathogens is well-documented (Farrar and Schreiber, 1993; Young and Hardy, 1995). In the case of anti-viral immunity, IFNγ enhances T-cell expansion and differentiation, influences antibody isotype switching, and induces proteins and enzymes that alter viral replication. Moreover, via augmentation of the killing activity of macrophages, neutrophils, and NK cells, IFNγ facilitates the destruction of virus-infected cells. A cause-and-effect relationship between excessive IFNγ in the lung, cytolytic activity, and the immunotoxic effects of TCDD is not yet clear. When specifically considering a role for IFNγ in the context of host resistance to influenza virus, it is important to point out that IFNγ has no known direct activity against influenza virus (Baumgarth and Kelso, 1996; Graham *et al.*, 1993). Thus, a direct IFNγ-mediated mechanism for virus clearance appears unlikely. Instead, one possible relationship is that exposure to TCDD enhances the activity of NK cells or increases the recruitment of NK cells to the lung.

NK cells are very effective at recognizing and destroying lung epithelial cells infected with influenza virus, and NK cells and IFNγ produced by NK cells are necessary for the clearance of a primary infection with influenza virus (Kos and Engleman, 1996; Monteiro *et al.*, 1998). Reports of the effects of TCDD on NK cells are contradictory. Exposure of rats and mice to TCDD has been found to have no effect (House *et al.*, 1990; Montovani *et al.*, 1980), decrease (Yang *et al.*, 1994), and increase (Funseth and Ilback, 1992) NK-cell activity. Nevertheless, in addition to promoting viral clearance and compensating for diminished CTL in mice exposed to TCDD, overly activated NK cells, via the production of IFNγ, may damage healthy tissue and cause mortality.

In addition to enhancing NK-cell activity, increased IFNγ may also lead to the over-activation of macrophages and neutrophils in the lung. In fact, the increased percentage and number of neutrophils in BAL cells from TCDD-treated mice provides circumstantial evidence for excessive inflammation as a mechanism for tissue damage. Interestingly, when observed, mortality in the TCDD-treated group consistently occurred between days 5 to 8 after infection with influenza virus: the same days in which increased IFNγ and increased neutrophils are found. Evidence for an IFNγ-mediated increase in pathological effects following infection with influenza virus has recently been demonstrated by Karupiah *et al.* (1998). They reported an association between elevated pulmonary nitric oxide following infection of mice with influenza A virus. Furthermore, using metabolic inhibitors of nitric oxide synthase (NOS) and NOS2-deficient mice, they found NOS2 induction by IFNγ is detrimental to host survival following infection with influenza A virus. Thus, one possible reason for mortality is that exposure to TCDD increases IFNγ levels in the lung, leading to excessive inflammation and tissue damage.

The relationship between increased IFNγ in the lung and TCDD-mediated immunotoxicity has yet to be established. One possible mechanism is that exposure to TCDD increases IFNγ by a direct AhR-mediated mechanism. In support of this theory, a putative dioxin response element has been identified in the murine IFNγ gene (Lai *et al.*, 1996). Further study is needed to fully understand how exposure to TCDD enhances pulmonary IFNγ levels, while IFNγ production by MLN cells is suppressed. Nevertheless, increased IFNγ in the lung offers a mechanistic explanation for the enigmatic finding that lung cells from mice exposed to TCDD and infected with influenza virus exhibit equivalent cytolytic activity. Moreover, through the over-activation of inflammatory mediators, IFNγ-induced tissue damage may explain the enhanced mortality observed in mice treated with TCDD.

In summary, we have found that IFNγ levels in the MLN are suppressed while IFNγ levels in the lung are greatly enhanced, indicating that exposure to TCDD affects cytokine production in a tissue-specific manner. We have also presented evidence that treatment with TCDD suppresses the adaptive immune response to infection with influenza virus. However, it is not clear whether this effect of TCDD on T cell-dependent immunity underlies the enhanced mortality because lung cells isolated from mice exposed to TCDD have cytolytic activity against influenza virus-infected cells. While the mechanisms for the presence of cytolytic activity and decreased host resistance to influenza virus remain to be determined, it is clear that there are differences in the effects of TCDD on immune cells and cytokine production in the lymph node and lung.

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