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 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+CD44hiCD62Llo), 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 uncertain.

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...
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 CO2 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/PRI/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 1% (v/v) of erythrocytes is being counted.

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 assessments 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 51Cr-release assay, as described by Nonacs et al. (1992). Briefly, BAL cells or ex vivo re-stimulated MLN cells were incubated with 51Cr-labeled, influenza virus-infected MC57G fibroblasts at E/T ratios from 100:1 to 62.5:1. Released radioactivity was measured after a 5-h incubation at 37°C. Total releasable 51Cr 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, 51Cr-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-Vß8.3 from Caltag Laboratories (Burlingame, CA); and FITC-labeled anti-CD4, FITC-labeled anti-Vß8.3, 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 (×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.
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 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; 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...
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, IgG1, IgG2a, IgG2b, 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, IgG1, IgG2a, and IgG2b 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 IFN-γ 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 (\(<2 \times 10^3\)) 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,
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 IFN\(\gamma\) (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, IFN\(\gamma\) 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 IFN\(\gamma\) 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 V\(\beta8.3^{+}\) T cell receptor (TCR) recognize a 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-V\(\beta8.3\) antibody to assess the recruitment of an influenza virus-specific subset. The results obtained from analysis of CD8\(^{+}\) V\(\beta8.3^{+}\) cells were analogous to the results of the total CD8\(^{+}\) T cells: exposure to TCDD decreased the percent (and number) of CD8\(^{+}\) V\(\beta8.3^{+}\) 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 CD62 L (Doherty et al., 1996; Hou and Doherty, 1993). Based on the expression of CD44 and CD62 L, 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...
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 IFN\(\gamma\) 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 IFN\(\gamma\) 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 IFN\(\gamma\) 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 IFN\(\gamma\) 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).
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.

Another T cell-dependent response to infection with influenza virus is the generation of antibodies (Gerhard et al., 1997). IgM, IgG1, IgG2a, and IgG2b, 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 (Mbuwuike 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 la-
vage 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 mecha-
nism is responsible for the enhanced 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 influ-
enza 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 out-
comes following infection, often resulting in increased mortal-
ity (Nguyen and Biron, 1999; Zhang et al., 1996). Thus,
overproduction of TNFα in the lung would provide an expla-
nation for both virus clearance and increased mortality. Infec-
tion 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 undetect-
able after about 72 h (our unpublished observations). There-
fore, 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 expla-
nation for the destruction of virus-infected cells and enhanced
mortality. The pivotal role of IFNγ in host resistance to intra-
cellular pathogens is well-documented (Farrar and Schreiber,
1993; Young and Hardy, 1995). In the case of anti-viral im-
munity, IFNγ enhances T-cell expansion and differentiation,
influences antibody isotype switching, and induces proteins
and enzymes that alter viral replication. Moreover, via aug-
mentation 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 relation-
ship 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 Ilbak, 1992) NK-cell activity. Never-
theless, in addition to promoting viral clearance and compen-
sating 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 neu-
rophils 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 patho-
logical 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 syn-
hasis (NOS) and NOS2-deficient mice, they found NOS2 in-
fluences antibody isotype switching, and induces proteins
and enzymes that alter viral replication. Moreover, via aug-
mentation 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 relation-
ship 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 Ilbak, 1992) NK-cell activity. Never-
theless, in addition to promoting viral clearance and compen-
sating 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 neu-
rophils 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
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virus clearance appears unlikely. Instead, one possible relation-
ship is that exposure to TCDD enhances the activity of NK
cells or increases the recruitment of NK cells to the lung.
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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