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Mechanistic exploration of AhR-mediated host protection against *Streptococcus pneumoniae* infection

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Abstract

Streptococcus pneumoniae is a primary cause of invasive bacterial infection and pneumonia and is one of the leading causes of death worldwide. In prior studies we showed that pre-treating mice with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a potent agonist of the aryl hydrocarbon receptor (AhR), protects against *S. pneumoniae*-induced mortality and reduces pulmonary bacterial burden. The current studies were conducted to help elucidate the mechanism for this protective effect, and to characterize the response in the lung during the first 10 hours following infection. C57Bl/6 mice were treated with TCDD one day prior to intranasal infection with serotype 3 *S. pneumoniae*. Monitoring of bacteria in the lung airways revealed that bacterial growth was inhibited in the TCDD-treated animals within 10 hours of infection. To address the mechanism of this rapid protective response, macrophages, neutrophils, and invariant Natural Killer T (iNKT) cells were quantified, and levels of natural antibodies produced by B-1 B cells were evaluated. Functional assays addressed whether AhR activation reduced the capacity of lung epithelial cells to bind bacteria, and whether TCDD treatment enhanced production of antimicrobial agents in the lung or blood. None of the hypothesized mechanisms was able to explain the protective effect. Finally, the exposure paradigm was manipulated to test whether administration of TCDD after instillation of the bacteria was also protective. Results showed that TCDD must be administered in advance of exposure to bacteria, suggesting that the lung environment is rendered inhospitable to the pathogens.

Keywords

Aryl hydrocarbon receptor; *Streptococcus pneumoniae*; Natural antibodies; iNKT cells; Host-protection; TCDD

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1. Introduction

Streptococcus pneumoniae is a common commensal bacterium of the upper respiratory tract, but when it spreads beyond the nasopharynx can cause pneumonia, otitis media, meningitis and bacteremia [1]. Infection with *S. pneumoniae* is an important cause of human disease and, together with influenza virus, ranks among the leading causes of death in the US and worldwide [2, 3]. Although significant success has been achieved in preventing pneumococcal diseases using vaccines and antibiotics, the large number of serotypes, potential for mutation, and continuing emergence of antibiotic-resistant strains underscore the need for continued research into mechanisms that influence host resistance to this pathogen [4].

In previous studies we tested the effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a potent agonist of the aryl hydrocarbon receptor (AhR), in mice given a lethal infection of type 3 *S. pneumoniae* [5]. We found that TCDD pre-treatment for 1 day reduced bacterial burden in the lungs and typically doubled the percentage of animals that survived the infection. Furthermore, mice that survived never showed signs of morbidity, suggesting that AhR activation blocked the bacteria from ever becoming productively established. Additional studies conducted using AhR-null mice demonstrated that TCDD was not directly toxic to the *S. pneumoniae* bacteria, but rather that the protective effect resulted from AhR activation within the infected animal.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that likely has endogenous ligands and physiologic functions, including roles in organogenesis and regulation of the cell cycle [6–8]. The receptor is well-known for its role in metabolism of many exogenous chemicals, including some natural compounds in fruits and vegetables, and certain toxic environmental pollutants such as dioxins [6]. Environmental toxicants that activate AhR exhibit a diverse array of adverse health effects, including toxicity to liver, skin, thymus, and the developing fetus [9]. The immune system is one of the most sensitive targets for AhR agonists, and adaptive immune responses involving T cells and conventional B-2 B cells are generally suppressed by treatment with TCDD. Consistent with impaired adaptive immunity, TCDD-exposed mice are more susceptible to infection and morbidity caused by numerous pathogens, including influenza virus, *Salmonella*, herpesviruses and *Listeria monocytogenes* [10, 11]. However, in contrast with suppressed adaptive immune responses, innate immune responses are often enhanced following exposure to AhR agonists. For example, TCDD treatment causes substantial increases in recruitment of macrophages, neutrophils, and NK cells to sites of antigen challenge, and exacerbates production of inflammatory cytokines including TNF α , IL-1 and IFN γ [12–18]. Such enhanced innate immune responses could be beneficial in combating certain infections, such as *S. pneumoniae*.

When *S. pneumoniae* bacteria escape the protective barriers of the upper respiratory tract, they spread to the lung and adhere to epithelial cells in the airways [19, 20]. Innate immune cells are poised to respond very rapidly to the presence of bacteria in the lung, and are critical for killing the invading bacteria and controlling the infection in the early stages. Neutrophils and macrophages are activated by the invading pathogens, and phagocytose the bacteria and kill them by producing cytotoxic agents [21, 22]. Invariant Natural Killer T (iNKT) cells are another population of innate cells that contribute to reducing bacterial burden and protect against *S. pneumoniae*-induced mortality [23, 24]. Other host-protective mechanisms include natural antibodies produced by B-1 B cells, which kill the bacteria through activation of the complement system and by acting as opsonins [25–27]. In addition to immune cells, non-hematopoietic cells of the lung and liver contribute to protection against invading bacteria. For example, lung epithelial cells release antimicrobial peptides

such as defensins, and the liver produces many proteins necessary for complement-mediated destruction and opsonization of pathogens [2, 26, 28–30].

In light of our prior studies demonstrating a host-protective effect of TCDD against *S. pneumoniae* infection, we were interested in exploring potential mechanisms to explain suppressed bacterial growth. Therefore, in the current studies we tested the effect of TCDD treatment on innate immune cell responses, including influx of phagocytes, recruitment of iNKT cells and production of natural antibodies by B-1 cells. We also addressed hypotheses that AhR activation in lung epithelium reduces adherence molecules necessary for bacterial invasion, and augments production of antimicrobial agents that kill invading pathogens. A final goal was to further characterize the protective effect of AhR activation, specifically by testing alternative times of TCDD treatment relative to infection, and monitoring the growth of bacteria in the lung over the initial hours of the infection.

2. Methods

2.1 Animal care and TCDD treatment

Female C57Bl/6 mice were obtained from commercial suppliers, or from breeding colonies maintained by the Washington State University Animal Reproduction Core facility. Animals were typically 7–9 weeks old when used for experiments. TCDD (>99% purity; Cambridge Isotope Laboratories, Woburn, MA) was dissolved in anisole and diluted in peanut oil. Mice were treated with 10 µg/kg body weight TCDD or vehicle control (peanut oil with 0.1% anisole) by oral gavage. TCDD was administered 1 day prior to infection (day -1), except as described for the time course administration study shown in Fig. 1. All animal treatments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee.

2.2 Infection

S. pneumoniae (Type 3, ATCC 6303) were prepared as described previously [5]. Briefly, bacteria were grown to mid-logarithmic phase in brain-heart infusion medium (BHI) containing 10% horse serum, washed, and diluted in PBS. Mice were anesthetized with avertin (2,2,2-tribromoethanol, Aldrich, Milwaukee, WI), given an intranasal infection of 10⁴ colony forming units (CFU) in 30 µl PBS, and held upright for 1 minute afterward.

2.3 Pulmonary macrophages and neutrophils, and bacterial burden

Lungs were perfused with three sequential washes of PBS via an incision in the trachea [10]. The first wash was retained as lung lavage fluid. For determining pulmonary bacterial burden, serial dilutions of lung lavage fluid were spread on blood agar plates. Colonies were counted after incubating plates overnight at 37°C. For analysis of macrophages and neutrophils from the lung airways, cytospin preparations were made from lung lavage cells pooled from all three washes. The slides were air dried, stained with Wright-Giemsa, and cells were quantified by a 100 nucleated cell differential analysis. Immune cells from lung parenchyma were prepared by digesting lungs with Type 2 collagenase (1 mg/ml; Worthington Biochemical, Lakewood, NJ) containing DNase (30 µg/ml; Roche Applied Science, Indianapolis, IN) [31]. Macrophages and neutrophils were identified by flow cytometry, using antibodies against Gr-1 and CD11c (eBiosciences, San Diego, CA). Appropriately-labeled isotype control antibodies were used to determine non-specific fluorescence. Neutrophils were defined as cells that stained positive for both Gr-1 and CD11b, and macrophages were defined as CD11b⁺, Gr-1⁻. Listmode data were collected on an Accuri C6 flow cytometer (BD Accuri, Ann Arbor, MI) and analyzed using FlowJo Analysis Software (TreeStar Inc, Ashland, OR).

2.4 Collection of plasma, and fluids from pleural cavity and peritoneal cavity

Blood was collected by cardiac puncture, or from the vena cava, using heparinized syringes. Plasma was separated by centrifugation. Pleural cavity lavage was performed using 1 ml of PBS inserted with a catheter through the diaphragm. Peritoneal cavity lavage was performed using 10 ml PBS.

2.5 Enumeration of iNKT cells

Immune cells from lung parenchyma were prepared by digesting lungs with Type 2 collagenase (1 mg/ml; Worthington Biochemical, Lakewood, NJ) containing DNase (30 µg/ml; Roche Applied Science, Indianapolis, IN) [31]. Spleen cells were released by pressing the organ between the frosted ends of microscope slides. White blood cells were enriched from whole blood obtained by cardiac puncture. Red blood cells were removed by water lysis or by using 0.15 M ammonium chloride. iNKT cells were identified by flow cytometry, using an antibody against TCRαβ (eBiosciences, San Diego, CA), and PE-mouse CD1d tetramers loaded with PBS57 (NIH Tetramer Facility, Emory University, Atlanta, GA). Appropriately-labeled isotype control antibodies and unloaded CD1d PE tetramers, were used to determine non-specific fluorescence. Listmode data were collected on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using FlowJo Analysis Software (TreeStar Inc, Ashland, OR).

2.6 Detection of Natural Antibodies by ELISA

High-binding EIA/RIA 96-well plates (Corning Costar, Corning, NY) were coated with 5 µg/ml of phosphorylcholine (PC-BSA high-binding; Biosearch Technologies, INC, Novato, CA) [32], or of heat-killed and sonicated *S. pneumoniae* (ATCC 6303) [5]. Serial dilutions of plasma, supernatant from homogenized lung, lung lavage fluid, pleural cavity lavage fluid, and peritoneal lavage fluid were added to the plates. Biotinylated antibodies for mouse IgM and IgA (Southern Biotech, Birmingham, AL) were used for detection of individual antibody isotypes.

2.7 Western blotting

Lungs were homogenized in RIPA buffer and debris separated by centrifugation. 40 µg of protein was separated by SDS-PAGE, and transferred to PVDF membranes. Primary antibodies included: E-cadherin (BD Biosciences, San Diego, CA); polymeric Ig receptor (a generous gift from Dr. Masanobu Nanno, Yakult Central Institute for Microbiological Research, Tokyo Japan; [33]); platelet-activating factor receptor, and actin (both from Santa Cruz Biotechnology, Santa Cruz, CA). Corresponding secondary antibodies included: IRDye 800CW-conjugated anti-rabbit IgG and anti-mouse IgG (LI-COR Biosciences, Lincoln, NE), and IRDye 700DX-conjugated anti-goat IgG (Rockland Inc. Gilbertsville, PA). Bands were visualized and quantified using the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

2.8 Bacterial adherence assay

Bacterial adherence to cultured lung epithelial cells was quantified using an *in vitro* assay [34]. *S. pneumoniae* bacteria (strain DS 2341-94) were added to confluent cultures of lung epithelial cells (A549 lung carcinoma) in multi-well plates. The Type 4 strain is used for this assay because Type 3 strains such as ATCC 6303 are heavily encapsulated and do not bind to epithelial cells *in vitro* (our observations, and S. Romero-Steiner, personal communication). Co-cultures were incubated for 2 hours at 37°C to allow the bacteria to adhere. Wells were washed 5 times to remove unbound bacteria, then overlaid with an agar solution (0.9% Bacto agar in brain-heart infusion media) that contained a 0.01% triphenyltetrazolium chloride (TTC; BD/Difco, Sparks, MD). Plates were incubated

overnight at 37°C, and resulting purple-stained colonies were counted using a dissecting microscope. For experiments testing direct effects of TCDD on lung epithelial cells, A549 cells were pre-treated with 1 nM TCDD, or 0.1% DMSO vehicle, for 24 hours prior to addition of bacteria. A549 cells are AhR-positive and are responsive to TCDD [35]. For experiments testing presence of soluble factors that block adherence, the bacteria were pre-incubated for 30 minutes with plasma isolated from vehicle- or TCDD-treated mice, then co-cultured with untreated A549 cells.

2.9 Ex vivo assay for bacterial survival and growth in lung

The potential for TCDD treatment to create an antimicrobial environment within the lung was assessed by monitoring growth of *S. pneumoniae* in samples of lung lavage fluid and of lung homogenate. For experiments testing anti-bacterial activity in lung lavage fluid, lungs were lavaged with 600µl sterile BHI. Lavage fluids collected from infected mice were filtered (0.45 µm UltraFree MC Centrifugal Filters; Millipore, Billerica, MA) to remove any *S. pneumoniae* washed out from the lung airways. Bacteria (ATCC 6303, 1×10^3 CFU in 10µl) were then added to 100µl aliquots of lavage fluid, mixed, and incubated at 37°C. Samples were collected after 0, 3, and 6 hours of culture, serially diluted, and plated on blood agar. Resulting colonies were counted after overnight incubation at 37°C. For experiments testing antibacterial activity in lung homogenate, lungs were homogenized in 500µl sterile PBS using a Tissue Tearer, and 500µl of the resulting homogenate was added to a sterile tube. Bacteria (ATCC 6303, 6×10^3 CFU in 50µl) were added to each homogenate sample, mixed, and incubated at 37°C. Samples were collected after 0, 3, and 6 hours of culture, and tested as described above for lavage fluid.

2.10 Statistical analysis

The difference in cell recoveries and bacterial colonies between experimental groups and controls was assessed by unpaired Student's *t* test using Prism software (GraphPad, San Diego, CA).

3. Results

3.1 TCDD must be administered prior to infection to confer protection against *S. pneumoniae*-induced mortality

In our previous studies we found that AhR activation significantly reduced *S. pneumoniae*-induced morbidity and mortality, specifically when the TCDD was administered either 1 or 2 days *prior* to instillation of bacteria ([5] and unpublished observations). In the current studies we sought to determine whether TCDD treatment was still protective if administered *after* bacterial challenge. The rationale for determining this was two-fold. First, to help elucidate possible mechanisms of action, and second, to determine the efficacy of AhR activation using a different therapeutically-relevant treatment paradigm. Survival of infected mice treated with TCDD one day prior to infection (referred to as “day -1”) was therefore compared with animals treated 12, 24, 36 or 48 hours after infection (Fig. 1). Consistent with prior studies, TCDD pre-treatment substantially improved survival compared to vehicle control mice, with 55% of TCDD-treated mice surviving and only 10% survival in vehicle-treated mice. In contrast, when administration of TCDD was delayed until 12 or more hours after infection, there was no protective effect and mortality was similar to the vehicle control group. In a separate study we also found that TCDD was not protective when administered at the time of infection (immediately prior to anesthetizing animals for bacterial administration; data not shown). These results demonstrate that it is necessary to administer TCDD in advance of bacterial infection in order to confer protection.

3.2 TCDD treatment suppresses pulmonary bacterial growth during early hours following infection

Another observation from our prior studies was that TCDD pre-treatment reduced pulmonary bacterial burden, which was assessed in homogenized whole lungs collected on days 1–4 following infection [5]. In the current studies these investigations were extended to measure bacteria recovered from the lung airways. The rationale being that because *S. pneumoniae* are instilled intranasally, the airways are the location at which bacteria first encounter barriers to invasion or growth. As shown in Fig. 2A, the number of bacteria recovered from the lung airways on day 1 was dramatically lower in the TCDD-treated animals, suggesting the bacteria are killed, or are otherwise rendered unable to grow, in the airways.

A time course study was also conducted to compare the kinetics of bacterial growth/survival in the lung airways during the first 10 hours following instillation (Fig. 2B). In vehicle-treated animals, increased bacterial growth was evident 8 – 10 hours following instillation of *S. pneumoniae*. TCDD-treated animals had variable elevation in colony numbers at 8 hours; however by 10 hours after infection the bacterial burdens in TCDD-treated mice were markedly suppressed. Taken together, these results indicate that diminished pulmonary bacterial burden evident in the TCDD-treated animals on day 1 reflects an inability of the bacteria to survive and become productively established after instillation.

3.3 Effect of TCDD on innate immune cells, including neutrophils, macrophages, B-1 cells, and iNKT cells

One hypothesis to explain the reduced bacterial load in the TCDD-treated mice is that AhR activation enhances activities of the innate immune system. More specifically, we hypothesized that TCDD treatment may reduce pulmonary bacterial burden by enhancing recruitment of protective phagocytes to the lung, by increasing levels of natural antibodies produced by B-1 cells, or by augmenting the number of iNKT cells.

To address the hypothesis that AhR activation causes a rapid increase in recruitment of phagocytes into the lung airways, macrophages and neutrophils were enumerated in lung lavage cells collected at intervals over the first 10 hours following instillation of bacteria. As shown in Fig. 3, the number of macrophages recovered from the lung airways generally increased over the time frame examined. The number of neutrophils was very low at the early time points, but also tended to increase during the 10 hour time frame. However, neither macrophages nor neutrophil numbers were increased in the TCDD-treated animals relative to vehicle controls, and in fact there was a general trend for TCDD to decrease phagocyte recovery. These results are consistent with our previous observations of suppressed neutrophils at later times following infection, which is likely a secondary consequence reflecting the diminished number of bacteria in the lung [5]. In a separate study, lungs of infected mice were digested with collagenase to enumerate pulmonary macrophages and neutrophils within the parenchymal tissue. No effect of TCDD treatment was observed at either 10 hours or on day 1 following infection (Table 1). Taken together, these results do not support the idea that decreased bacterial burdens in the TCDD-treated animals reflect an early increase in phagocyte recruitment to the lung.

“Natural antibodies”, which are produced by B-1 B cells, contribute to host defense against *S. pneumoniae* by binding bacterial polysaccharides to facilitate lysis and opsonization. To test the hypothesis that TCDD treatment increases levels of natural antibodies, IgM and IgA antibodies that recognize streptococcal antigens were evaluated on day 1 following infection and in uninfected mice. ELISAs were used to measure natural antibodies in plasma and in lung homogenate (Fig. 4, and Supplementary Fig. 1), and in lavage fluids collected from

lung airways, pleural cavity and peritoneal cavity (data not shown). Both IgM and IgA antibodies that recognized streptococcal antigens were present in all of the tested fluids, however there was no significant increase in either isotype in the TCDD-treated animals.

iNKT cells are another innate immune cell population that influences success of *S. pneumoniae* infection. To explore the possibility that these cells may be involved in TCDD-induced host protection, iNKT cells were quantified in lung, blood and spleen of both uninfected (day 0) and infected (day 1) mice. iNKT cells were present at all sites examined, but the recovery of these cells from vehicle- and TCDD-treated animals was similar (Fig. 5).

3.4 Effect of TCDD on bacterial adherence to lung epithelial cells

Successful bacterial colonization of the host is influenced by a number of factors unrelated to immune cells, such as whether or not the bacteria can physically adhere to lung epithelial cells in the airways. Therefore, in the next set of experiments we tested the hypothesis that TCDD treatment affects lung epithelial cells to inhibit their capacity to bind bacteria.

First, we examined pulmonary expression of E-cadherin, platelet activating factor receptor (PAFR), and polymeric Ig receptor (pIgR). These proteins are expressed on lung epithelial cells and provide sites for attachment and subsequent invasion of *S. pneumoniae* [20, 36, 37]. Levels of these adherence proteins were compared in lung homogenates from vehicle- and TCDD-treated mice, but we did not identify any difference in protein expression between treatment groups (Fig. 6A).

TCDD had no effect on these three adherence proteins, which were selected because of their known contribution to *S. pneumoniae* invasion; however there are numerous other adhesion molecules, as well as certain other soluble factors, that influence bacterial adherence. Because it would be difficult to define and examine each of these individually, we also tested the effect of TCDD on adherence using a functional assay that quantifies bacterial binding to cultured lung epithelial cells *in vitro*. First, we examined possible direct effects of TCDD on lung epithelial cells. A549 cells (which express AhR and are TCDD-responsive) were treated with 1 nM TCDD for 24 hours, and then co-cultured with *S. pneumoniae*. Bacteria that adhered to the epithelial cells were visualized by overlaying washed cultures with TTC-containing agar, which stains the resulting bacterial colonies purple. Results showed that the bacteria adhered well to the cultured lung epithelial cells, however TCDD treatment did not suppress the capacity of the epithelial cells to bind bacteria (Fig. 6B).

S. pneumoniae adherence is also inhibited by the presence of certain soluble factors, for example antibodies or C-reactive protein, that bind to the bacteria and block their physical association with lung epithelial cells [38]. Therefore, we also tested the hypothesis that TCDD treatment enhances levels of soluble factors that interfere with bacterial binding. In these assays, *S. pneumoniae* were pre-incubated with plasma isolated from vehicle- or TCDD-treated mice prior to co-culture with A549 cells. No difference in bacterial binding was detected between the treatment groups (Fig. 6C).

3.5 Testing whether TCDD augments antimicrobial activity in the lung

Antimicrobial peptides, particularly those synthesized by lung and liver, are directly toxic to invading pathogens and are important for protection against infection with *S. pneumoniae*. We hypothesized that AhR activation would enhance the production of these substances, to create an environment within the lung that would impede bacterial growth and survival. To test this hypothesis, *S. pneumoniae* were added to samples of lung lavage fluid or lung homogenate collected from vehicle- and TCDD-treated mice, and growth of the bacteria was monitored over time. As shown in Fig. 7, the bacteria grew equivalently well in lavage fluid and lung homogenate from TCDD-treated animals and vehicle controls. These results

therefore do not provide evidence that antimicrobial activity is augmented in TCDD-treated animals, at least as measured in this functional assay of bacterial growth.

4. Discussion

Infection with *S. pneumoniae* causes significant morbidity and mortality in the US and worldwide, and new therapeutic strategies to combat pneumococcal disease are needed. In 2006 we published findings that treatment with TCDD, a potent AhR agonist, protects against infection with a highly aggressive strain of *S. pneumoniae*, and significantly reduces morbidity and mortality. These results were very exciting, and suggested the possibility that AhR activation may be clinically useful to reduce the harmful effects caused by this common human pathogen. Furthermore, by understanding the cellular mechanism responsible for host-protection, other clinical strategies to control *S. pneumoniae* infection may be elucidated.

The purpose for conducting the studies presented in the current manuscript was therefore two-fold. One goal was to further characterize the protective effect, including testing alternative times of TCDD treatment relative to infection, and monitoring the growth of bacteria in the lung over the initial hours of the infection. The second goal was to test hypotheses directed at some of the logical cellular and chemical mechanisms that may explain the host-protection observed in the TCDD-treated mice.

With regard to timing of TCDD treatment, it was necessary to establish whether AhR activation could be delayed until after the bacteria were present and growing in the lung. This is important because therapeutic agents are often administered after an infection is established, and after physical symptoms of disease are clinically evident. For this particular strain and dose of *S. pneumoniae*, vehicle-treated mice typically show signs of morbidity beginning two or three days after infection, and die shortly thereafter. Therefore, in the current studies we tested whether AhR activation would still be protective if the agonist was administered up to 48 hours after bacterial exposure. The results showed that TCDD was not effective when administered at the time of infection or after the bacteria were instilled. This suggests that AhR activation preconditions the lung environment to be inhospitable to bacterial growth, but is not effective in combating the infection once it is established.

Therefore, from a therapeutic standpoint, AhR agonists are not likely to be of practical use for controlling *S. pneumoniae* infection, unless they were given prophylactically. Additionally, since TCDD and other strong AhR agonists are potent suppressors of adaptive immune responses, it is unlikely that such agents would be desirable for controlling pneumococcal infections, which often occur concomitantly with other infections such as influenza virus. Hence a more practical goal for these studies was to understand the mechanism of action for the host-protective effect, in hopes that those pathways could be specifically targeted with agents less likely to have immunosuppressive side effects.

When considering possible mechanistic explanations, it is important to note that TCDD treatment suppressed bacterial burden within 8–24 hours after infection. Therefore it was important to look for protective responses that could occur within a very short time following exposure to the bacteria, or even identify changes in the pulmonary environment in TCDD pre-treated mice before they were infected (the “day 0” treatment group). Therefore, it was logical to consider possible roles of innate immune cells and antimicrobial agents, but unreasonable to look for changes in adaptive immune responses that take many days for clonal expansion and development of effector functions.

One possible means by which AhR activation could improve host resistance is through enhancing neutrophil or macrophage recruitment to the site of infection. These phagocytes

are effective in killing and clearing pathogens such as *S. pneumoniae*, and are especially important in the early phases of the immune response before the adaptive immune system has been activated. In support of this hypothesis, there are a number of animal models wherein TCDD treatment results in a marked increase in the number of neutrophils and macrophages recruited to sites of antigen challenge [12, 13, 39, 40]. We did examine pulmonary neutrophils and macrophages in our previous studies, and did not find that TCDD increased their numbers when measured on day 1 or later. However it remained to be determined whether these cells could influence bacterial clearance in the early hours following infection. Thus in the current report we evaluated phagocytes during the first 10 hours after infection, when TCDD-induced decreases in bacterial burden first become evident. However, TCDD treatment did not increase the number of either cell type in the lung airways, and it does not appear that enhanced phagocyte recruitment to the lung airways explains the decrease in bacteria.

B-1 cells are the cellular source of “natural antibodies”, which bind bacterial antigens including polysaccharides and phosphorylcholine in the capsule and cell wall of *S. pneumoniae* [32, 41]. These innate immune cells are located mainly in the peritoneal and pleural cavities, providing rapid T-independent responses against pathogens such as bacteria invading from the lung and gut [42]. B-1 cells secrete IgM and IgA classes of natural antibodies, both in the absence of infection (“preexisting” antibodies), and in rapid response to infection [32]. Little information is available concerning effects of AhR activation on B-1 cells. TCDD treatment does not appear to affect the number of peritoneal B-1 cells in adult mice, nor alter their production of IgA natural antibodies [43, 44]. However, some evidence suggests that TCDD treatment changes the secretion or transport of IgA, which may affect the distribution of these antibodies at different locations in the body [43–45]. We therefore hypothesized that AhR activation would increase natural antibody levels in the lung to inhibit bacterial growth. However, examination of pulmonary IgM and IgA antibodies that specifically bound streptococcal antigens revealed no effect of TCDD treatment. Similar results were obtained for antibodies recovered from other sites, including plasma and the peritoneal and pleural cavities. To our knowledge this is the first report examining effects of AhR ligands on natural antibodies that recognize a specific pathogen, and thus it contributes new information concerning this aspect of innate immune function.

iNKT cells are another population of innate immune cells that exhibit a rapid response to *S. pneumoniae* infection. This sublineage of T cells has a restricted repertoire of T cell receptors that recognize lipid or glycolipid antigens presented in the context of CD1d [46]. Although the mechanism isn’t understood, growing evidence demonstrates that iNKT cells play an important role in host protection against microbes, including *S. pneumoniae* [23]. For example, mortality resulting from *S. pneumoniae* infection is higher in mice deficient in iNKT cells, and activation of iNKT cells in intact mice reduces bacterial burdens [23, 24]. We therefore tested whether AhR activation increased the number of iNKT cells recovered from lungs, blood, or spleen on day 0 or day 1 of infection. Although TCDD treatment did not alter iNKT cell numbers, future exploration of effects on iNKT cell effector functions may be warranted, once the role of these cells in *S. pneumoniae* resistance is understood. For example, a recent report using another AhR agonist, 6-formylindolo[3,2-b]carbazole (FICZ), demonstrated altered cytokine production by human iNKT cells treated *in vitro* [47]. Thus it remains possible that AhR activation may alter a yet to be identified antibacterial activity of these cells.

In addition to immune cells, other cell types including lung epithelial cells and even liver cells influence the success of bacterial invasion. Specifically, adherence molecules on the surface of lung epithelial cells provide essential binding sites for *S. pneumoniae*, without which the bacteria cannot successfully invade and colonize [19, 20]. Lung epithelial cells

also secrete antibacterial factors (e.g. surfactant and defensins) that have direct antimicrobial activity and also coat pathogens to promote phagocytosis [2, 28, 29]. Finally, liver-derived proteins such as complement and C-reactive protein kill pathogens by complement-mediated lysis and by facilitating uptake by phagocytes [26, 30]. Because it would be implausible to directly test the expanding list of possible adherence molecules [19] and the hundreds of possible antimicrobial peptides produced at different sites [48], we chose to test these mechanisms using functional assays. Unfortunately we did not find evidence that TCDD treatment diminished binding capacity of lung epithelium for *S. pneumoniae*, nor that it enhanced antimicrobial action measured in lung homogenate or in blood. However, these results should be interpreted cautiously, given that the functional assays may not be fully representative of host-pathogen interactions *in vivo*. For example, cultured lung epithelial cells may not accurately recapitulate the environment within intact lung. Additionally, the encapsulated strain of *S. pneumoniae* used for infecting mice does not bind to epithelial cells in culture; so use of a less pathogenic adherent strain is required for *in vitro* adherence assays.

In closing, the current studies have expanded our understanding of the treatment paradigm required for protection against *S. pneumoniae* infection conferred by an AhR agonist. Specifically, it was demonstrated that TCDD must be administered in advance of exposure to bacteria, suggesting that the lung environment is rendered inhospitable to the pathogens and they are unable to survive or grow. With regard to the mechanism of protection, we did not find evidence that phagocytic cells, natural antibodies, or iNKT cells were augmented in the TCDD-treated mice, nor that antimicrobial activity in lung or blood was increased. This unusual situation wherein TCDD protects against infection with a pathogenic organism is intriguing, but additional exploration will be required in order to elucidate the mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Mitchell TJ. The pathogenesis of streptococcal infections: from tooth decay to meningitis. *Nat Rev Microbiol.* 2003; 1:219–30. [PubMed: 15035026]
2. Kadioglu A, Andrew PW. The innate immune response to pneumococcal lung infection: the untold story. *Trends Immunol.* 2004; 25:143–9. [PubMed: 15036042]
3. Minino AM, Heron MP, Murphy SL, Kochanek KD. Deaths: final data for 2004. *Natl Vital Stat Rep.* 2007; 55:1–119. [PubMed: 17867520]
4. Quale J, Landman D, Ravishankar J, Flores C, Bratu S. *Streptococcus pneumoniae*, Brooklyn, New York: fluoroquinolone resistance at our doorstep. *Emerg Infect Dis.* 2002; 8:594–7. [PubMed: 12023915]

5. Vorderstrasse BA, Lawrence BP. Protection against lethal challenge with *Streptococcus pneumoniae* is conferred by aryl hydrocarbon receptor activation but is not associated with an enhanced inflammatory response. *Infect Immun*. 2006; 74:5679–86. [PubMed: 16988243]
6. Nguyen LP, Bradfield CA. The search for endogenous activators of the aryl hydrocarbon receptor. *Chem Res Toxicol*. 2008; 21:102–16. [PubMed: 18076143]
7. Marlowe JL, Puga A. Aryl hydrocarbon receptor, cell cycle regulation, toxicity, and tumorigenesis. *J Cell Biochem*. 2005; 96:1174–84. [PubMed: 16211578]
8. Bock KW, Kohle C. Ah receptor: dioxin-mediated toxic responses as hints to deregulated physiologic functions. *Biochem Pharmacol*. 2006; 72:393–404. [PubMed: 16545780]
9. Schecter A, Birnbaum L, Ryan JJ, Constable JD. Dioxins: an overview. *Environ Res*. 2006; 101:419–28. [PubMed: 16445906]
10. Vorderstrasse BA, Bohn AA, Lawrence BP. Examining the relationship between impaired host resistance and altered immune function in mice treated with TCDD. *Toxicology*. 2003; 188:15–28. [PubMed: 12748038]
11. Kerkvliet, NI. Immunotoxicology of dioxins and related chemicals. In: Schecter, A., editor. *Dioxins and Health*. New York: Plenum Press; 1994. p. 199-225.
12. Choi JY, Oughton JA, Kerkvliet NI. Functional alterations in CD11b(+)Gr-1(+) cells in mice injected with allogeneic tumor cells and treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Int Immunopharmacol*. 2003; 3:553–70. [PubMed: 12689660]
13. Moos AB, Baecher-Steppan L, Kerkvliet NI. Acute inflammatory response to sheep red blood cells in mice treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin: the role of proinflammatory cytokines, IL-1 and TNF. *Toxicology and Applied Pharmacology*. 1994; 127:331–5. [PubMed: 8048078]
14. Teske S, Bohn AA, Regal JF, Neumiller JJ, Lawrence BP. Activation of the aryl hydrocarbon receptor increases pulmonary neutrophilia and diminishes host resistance to influenza A virus. *Am J Physiol Lung Cell Mol Physiol*. 2005; 289:L111–L24. [PubMed: 15792965]
15. Neff-LaFord H, Teske S, Bushnell TP, Lawrence BP. Aryl hydrocarbon receptor activation during influenza virus infection unveils a novel pathway of IFN-gamma production by phagocytic cells. *J Immunol*. 2007; 179:247–55. [PubMed: 17579044]
16. Fan F, Yan B, Wood G, Viluksela M, Rozman KK. Cytokines (IL-1 beta and TNF alpha) in relation to biochemical and immunological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in rats. *Toxicology*. 1997; 116:9–16. [PubMed: 9020502]
17. Yin H, Li Y, Sutter TR. Dioxin-enhanced expression of interleukin-1 beta in human epidermal keratinocytes: potential role in the modulation of immune and inflammatory responses. *Exp Clin Immunogenet*. 1994; 11:128–35. [PubMed: 7826661]
18. Luebke RW, Copeland CB, Bishop LR, Daniels MJ, Gilmour MI. Mortality in Dioxin-Exposed Mice Infected with Influenza: Mitochondrial Toxicity (Reye's-Like Syndrome) Versus Enhanced Inflammation as the Mode of Action. *Toxicol Sci*. 2002; 69:109–16. [PubMed: 12215664]
19. Hammerschmidt S. Adherence molecules of pathogenic pneumococci. *Curr Opin Microbiol*. 2006; 9:12–20. [PubMed: 16338163]
20. Cundell DR, Gerard NP, Gerard C, Idanpaan-Heikkila I, Tuomanen EI. *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. *Nature*. 1995; 377:435–8. [PubMed: 7566121]
21. Kerr AR, Wei XQ, Andrew PW, Mitchell TJ. Nitric oxide exerts distinct effects in local and systemic infections with *Streptococcus pneumoniae*. *Microb Pathog*. 2004; 36:303–10. [PubMed: 15120156]
22. Kobrynski LJ, Sousa AO, Nahmias AJ, Lee FK. Cutting edge: antibody production to pneumococcal polysaccharides requires CD1 molecules and CD8+ T cells. *J Immunol*. 2005; 174:1787–90. [PubMed: 15699104]
23. Brigl M, Tatituri RV, Watts GF, Bhowruth V, Leadbetter EA, Barton N, et al. Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection. *J Exp Med*. 2011; 208:1163–77. [PubMed: 21555485]
24. Kawakami K, Yamamoto N, Kinjo Y, Miyagi K, Nakasone C, Uezu K, et al. Critical role of Valpha14+ natural killer T cells in the innate phase of host protection against *Streptococcus pneumoniae* infection. *Eur J Immunol*. 2003; 33:3322–30. [PubMed: 14635040]

25. Brown JS, Hussell T, Gilliland SM, Holden DW, Paton JC, Ehrenstein MR, et al. The classical pathway is the dominant complement pathway required for innate immunity to *Streptococcus pneumoniae* infection in mice. *Proc Natl Acad Sci U S A*. 2002; 99:16969–74. [PubMed: 12477926]
26. Casal J, Tarrago D. Immunity to *Streptococcus pneumoniae*: Factors affecting production and efficacy. *Curr Opin Infect Dis*. 2003; 16:219–24. [PubMed: 12821811]
27. Mold C, Rodic-Polic B, Du Clos TW. Protection from *Streptococcus pneumoniae* infection by C-reactive protein and natural antibody requires complement but not Fc gamma receptors. *J Immunol*. 2002; 168:6375–81. [PubMed: 12055255]
28. Wright JR. Pulmonary surfactant: a front line of lung host defense. *J Clin Invest*. 2003; 111:1453–5. [PubMed: 12750392]
29. Singh PK, Jia HP, Wiles K, Hesselberth J, Liu L, Conway BA, et al. Production of beta-defensins by human airway epithelia. *Proc Natl Acad Sci U S A*. 1998; 95:14961–6. [PubMed: 9843998]
30. Dong Q, Wright JR. Expression of C-reactive protein by alveolar macrophages. *J Immunol*. 1996; 156:4815–20. [PubMed: 8648129]
31. Jin GB, Moore AJ, Head JL, Neumiller JJ, Lawrence BP. Aryl hydrocarbon receptor activation reduces dendritic cell function during influenza virus infection. *Toxicol Sci*. 2010; 116:514–22. [PubMed: 20498003]
32. Haas KM, Poe JC, Steeber DA, Tedder TF. B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to *S. pneumoniae*. *Immunity*. 2005; 23:7–18. [PubMed: 16039575]
33. Shimada S, Kawaguchi-Miyashita M, Kushiro A, Sato T, Nanno M, Sako T, et al. Generation of polymeric immunoglobulin receptor-deficient mouse with marked reduction of secretory IgA. *J Immunol*. 1999; 163:5367–73. [PubMed: 10553061]
34. Rajam G, Jackson D, Pilishvili T, Whitney CG, Facklam RR, Carlone GM, et al. An in vitro model to assess pneumococcal adherence to nasopharyngeal cells under competition conditions. *J Microbiol Methods*. 2007; 70:219–26. [PubMed: 17512994]
35. Dohr O, Sinning R, Vogel C, Munzel P, Abel J. Effect of transforming growth factor-beta1 on expression of aryl hydrocarbon receptor and genes of Ah gene battery: clues for independent down-regulation in A549 cells. *Mol Pharmacol*. 1997; 51:703–10. [PubMed: 9145908]
36. Agarwal V, Hammerschmidt S. Cdc42 and the phosphatidylinositol 3-kinase-Akt pathway are essential for PspC-mediated internalization of pneumococci by respiratory epithelial cells. *J Biol Chem*. 2009; 284:19427–36. [PubMed: 19473971]
37. Anderton JM, Rajam G, Romero-Steiner S, Summer S, Kowalczyk AP, Carlone GM, et al. E-cadherin is a receptor for the common protein pneumococcal surface adhesin A (PsaA) of *Streptococcus pneumoniae*. *Microb Pathog*. 2007; 42:225–36. [PubMed: 17412553]
38. Gould JM, Weiser JN. The inhibitory effect of C-reactive protein on bacterial phosphorylcholine platelet-activating factor receptor-mediated adherence is blocked by surfactant. *J Infect Dis*. 2002; 186:361–71. [PubMed: 12134232]
39. Warren TK, Mitchell KA, Lawrence BP. 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. *Toxicol Sci*. 2000; 56:114–23. [PubMed: 10869459]
40. Kerkvliet NI, Oughton JA. Acute inflammatory response to sheep red blood cell challenge in mice treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): Phenotypic and functional analysis of peritoneal exudate cells. *Toxicology and Applied Pharmacology*. 1993; 119:248–57. [PubMed: 8480333]
41. Kaminski DA, Stavnezer J. Enhanced IgA class switching in marginal zone and B1 B cells relative to follicular/B2 B cells. *J Immunol*. 2006; 177:6025–9. [PubMed: 17056527]
42. Ansel KM, Harris RB, Cyster JG. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. *Immunity*. 2002; 16:67–76. [PubMed: 11825566]
43. Kinoshita H, Abe J, Akadegawa K, Yurino H, Uchida T, Ikeda S, et al. Breakdown of mucosal immunity in gut by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Environ Health Prev Med*. 2006; 11:256–63. [PubMed: 21432354]

44. Moran RA, Lee CW, Fujimoto JM, Calvanico NJ. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on IgA serum and bile levels in rats. *Immunopharmacol.* 1986; 12:245–50.
45. Ishikawa S. Children's immunology, what can we learn from animal studies (3): Impaired mucosal immunity in the gut by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): a possible role for allergic sensitization. *J Toxicol Sci.* 2009; 34(Suppl 2):SP349–61. [PubMed: 19571490]
46. Kronenberg M, Kinjo Y. Innate-like recognition of microbes by invariant natural killer T cells. *Curr Opin Immunol.* 2009; 21:391–6. [PubMed: 19646850]
47. Moreira-Teixeira L, Resende M, Coffre M, Devergne O, Herbeuval JP, Hermine O, et al. Proinflammatory Environment Dictates the IL-17-Producing Capacity of Human Invariant NKT Cells. *J Immunol.* 2011; 186:5758–65. [PubMed: 21478400]
48. Lai Y, Gallo RL. AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends Immunol.* 2009; 30:131–41. [PubMed: 19217824]

Highlights

AhR activation suppresses pulmonary *S. pneumoniae* burden within 10 hours of infection

Pre-treatment with TCDD is necessary to confer protection

TCDD does not elevate levels of natural antibodies, iNKT cells or phagocytes

Fig. 1. Time course of TCDD administration relative to infection, and effect on survival
C57Bl/6 mice (n = 9–10 per treatment group) were given an intranasal infection of 10^4 CFU *S. pneumoniae*. TCDD was administered by gavage either 1 day prior to infection, or 12, 24, 36, or 48 hours after infection. Survival was monitored daily for 16 days, at which time all remaining animals appeared healthy.

Fig. 2. Kinetics of pulmonary bacterial growth at early time points

Mice were treated with vehicle or TCDD 1 day prior to infection with *S. pneumoniae*. (A) Animals were sacrificed 1 day after infection ($n = 6 - 8$ per treatment group). Serial dilutions of lung lavage fluid were plated on blood agar. Bars represent the average number of colonies ($\pm$ SEM) in $10\mu\text{l}$ of a 10^{-1} dilution. * $p = 0.001$. (B) Animals were sacrificed at 4, 6, 8, and 10 hours after infection ($n = 4$ per treatment group at each time point). Bars represent the average number of colonies ($\pm$ SEM) that grew from $20\mu\text{l}$ of undiluted lung lavage fluid.

Fig. 3. TCDD treatment does not enhance infiltration of phagocytes in the lung

Mice were treated with vehicle or TCDD 1 day prior to infection with 10^4 CFU *S. pneumoniae*. Animals were sacrificed at 4, 6, 8, and 10 hours following infection ($n = 4$ per mice per time point). Immune cells from the lung airways were collected by lavage and quantified by differential cell analysis. Bars represent the average recovery ($\pm$ SEM) of macrophages and neutrophils, calculated by multiplying the percentage of each population by the total cell recovery for each sample. Uninfected mice were not tested in this study, because TCDD treatment in the absence of infection does not alter recoveries of pulmonary macrophages or neutrophils [39].

Fig. 4. Levels of natural antibodies that recognize streptococcal antigen

Vehicle- and TCDD-treated mice were sacrificed 1 day after infection with *S. pneumoniae* (n = 8 per treatment group). Levels of IgA and IgM natural antibodies were determined by ELISA, using plates coated with phosphorylcholine (PC). PC-specific antibodies were determined in indicated dilutions of (A) plasma separated from blood collected from the vena cava, and (B) extracted lungs homogenized in 500 μ l PBS. Levels of natural antibodies were also measured in uninfected vehicle- and TCDD-treated mice, with similar results (not shown). Analogous data collected using heat-killed *S. pneumoniae* as the coating antigen are shown in Supplementary Figure 1.

Fig. 5. iNKT cell populations in vehicle- and TCDD-treated mice

Vehicle- and TCDD-treated mice were sacrificed on day 0 (uninfected) or on day 1 following infection with *S. pneumoniae* (n = 6 per treatment group for each time point). The number of iNKT cells recovered from (A) collagenase-digested lungs and (B) spleen were identified by flow cytometry using anti-TCR $\alpha\beta$ antibody and PE-CD1d-PBS57 tetramers. (C) iNKT cells were also enumerated in blood samples that ranged from 300–500 μ l, and are expressed as a percentage of total blood leukocytes. Bars represent the mean $\pm$ SEM.

Fig. 6. Adherence molecule expression and *in vitro* assays of bacterial adherence

(A) Proteins were isolated from lungs of vehicle- and TCDD-treated mice 1 day after infection with *S. pneumoniae*. 40 μ g of protein from each sample was analyzed by western blotting for E-cadherin (120 kDa), platelet-activating factor receptor (PAFR; 48 kDa), and polymeric Ig receptor (pIgR; 140 kDa). Actin was used to confirm equal sample loading for each gel (not shown). (B) A549 cells were treated with 1 nM TCDD or vehicle (0.1% DMSO) for 24 hours prior to co-culturing with *S. pneumoniae* (strain DS 2341-94). Adherent bacteria were visualized by overlaying washed cultures with agar containing TTC, and incubating overnight at 37°C. Bars represent the average number of colonies ($\pm$ SEM) from triplicate wells. (C) *S. pneumoniae* were pre-incubated in plasma collected from vehicle- and TCDD-treated mice 1 day after infection. The bacteria were then co-cultured with untreated A549 cells, and the number of adherent bacteria was determined as described for (B). Bars represent the average numbers of colonies ($\pm$ SEM) from plasma samples from 7–8 mice per treatment group. Similar results (not shown) were obtained using plasma from vehicle- and TCDD-treated uninfected mice (for panel C), and for E-cadherin and PAFR expression in lungs of uninfected mice (for panel A).

Fig. 7. *S. pneumoniae* growth in lung homogenate and lavage fluid from TCDD-treated mice is equivalent to vehicle control

(A) Lung lavage fluid was collected from vehicle- and TCDD-treated mice ($n = 6$ per group) 1 day after infection with *S. pneumoniae*, and filtered to remove any bacteria washed from the lung airways. (B) Lungs were aseptically removed from mice one day after treatment with vehicle or TCDD and homogenized in PBS. For both A and B, a fresh culture of *S. pneumoniae* was mixed with the lavage fluid or homogenate, sampled at times 0, 3 and 6 hours of culture, then serially diluted and plated on blood agar. Bars represent the mean ($\pm$ SEM) of the number of colonies in the indicated dilution for each time point. Similar results were obtained when bacteria were cultured in lung lavage fluid samples collected from vehicle- and TCDD-treated uninfected mice (not shown).

Table 1

Number of macrophages and neutrophils recovered from lung parenchyma

	10 hours		Day 1	
	Vehicle	TCDD	Vehicle	TCDD
Macrophages ^I	25.9 (3.2)	29.3 (4.2)	32.0 (3.4)	38.1 (6.1)
Neutrophils	21.9 (4.5)	29.9 (5.5)	29.6 (6.1)	29.4 (6.5)

^IThe number of cells $\times 10^4$ ($\pm$ SEM) was determined by flow cytometry. Immune cells were recovered from collagenase-digested lung tissue at 10 hours or day 1 following infection with *S. pneumoniae*. (n = 6–7 per treatment group per time point)