

# The Aryl Hydrocarbon Receptor Affects Distinct Tissue Compartments during Ontogeny of the Immune System

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There is growing evidence that prenatal and early postnatal environmental factors influence the development and programming of the immune system, causing long-lasting negative health consequences. The aryl hydrocarbon receptor (AhR) is an important modulator of the development and function of the immune system; however, the mechanism is poorly understood. Exposure to the AhR agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin throughout gestation and during lactation yields adult offspring with persistent defects in their immune response to influenza virus. These functional alterations include suppressed lymphocyte responses and increased inflammation in the infected lung despite normal cellularity and anatomical development of lymphoid organs. The studies presented here were conducted to determine the critical period during immune ontogeny that is particularly sensitive to inappropriate AhR activation. We also investigated the contribution of AhR-mediated events within and extrinsic to hematopoietic cells. Our findings show that AhR activation alters different elements of the immune system at different times during development by affecting different tissue targets. In particular, diminished T-cell responses arise due to deregulated events within bone marrow-derived cells. In contrast, increased interferon gamma levels in the infected lung result from AhR-regulated events extrinsic to bone marrow-derived cells, and require AhR agonist exposure during early gestation. The persistence of AhR activation induced immune modulation was also compared, revealing that AhR activation causes long-lasting functional alterations in the developing immune system, whereas the impact on the mature immune system is transient.

**Key Words:** developmental immunotoxicity; hematopoietic cells; infection; influenza virus; dioxin.

In both mice and humans, all lineages of blood cells are derived from a small population of pluripotent hematopoietic stem cells (HSC), and lineage commitment is controlled by

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serial differentiation. This process is regulated by the expression of lineage-specific transcription factors and regulatory cytokines, which direct HSC to either undergo a process of self-renewal or differentiate into lineage specific precursors. Common lymphoid precursors give rise to T and B lymphocytes and natural killer cells, whereas other leukocytes as well as erythrocytes are derived from common myeloid precursors (Dzierzak and Medvinsky, 1995; Robin *et al.*, 2003; Zhu and Emerson, 2002). Although understanding of HSC differentiation and proliferation has expanded remarkably in the past decade, less is known about how subtle changes during development affect the responses and functions of the immune system later on in life. The origins of the vertebrate immune system begins during embryonic development and does not fully mature until well after birth. In mice and humans, the regulatory processes and sites of ontogeny are similar despite the variation in gestational lengths (Zhu and Emerson, 2002). For instance, the primary sites of hematopoiesis migrate throughout development to different anatomical locations that are the same for both mice and humans, and homologous cytokines and transcription factors regulate this process. In mice, the earliest detection of HSC is in the yolk sac, at about gestational day (gd) 7 (Dzierzak and Medvinsky, 1995). HSC are detected in the aorta-gonads-mesonephros (AGM) region at about gd 9 (Dzierzak and Medvinsky, 1995; Robin *et al.*, 2003). On gd 9–12, HSC migrate from the yolk sac and AGM to the fetal liver and spleen, which then take over as the primary sites of hematopoiesis in the fetus. At or near parturition, HSC migrate to the bone marrow, which serves as the primary site of hematopoiesis from then on.

*Per-Arnt-Sim* proteins are a family of basic helix–loop–helix transcription factors that act as environmental sensors to regulate toxin metabolism, circadian rhythm and responses to oxygen levels (Furness *et al.*, 2007). One particular member of this family, the aryl hydrocarbon receptor (AhR), is an important modulator of the development and function of the immune system. This was initially appreciated because the AhR binds many common environmental contaminants, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), coplanar polychlorinated biphenyls (PCBs), and polyaromatic

hydrocarbons such as benzo[*a*]pyrene, and exposure to these chemicals has profound effects on the immune system (Lawrence and Kerkvliet, 2006). In fact, the sensitivity of the developing immune system to AhR agonists was recognized over 30 years ago (Vos and Moore, 1974).

Specific effects of developmental exposure to TCDD on the immune system include thymic atrophy and alterations in the proportion of thymocyte subpopulations (Camacho *et al.*, 2004; Fine *et al.*, 1990; Gehrs *et al.*, 1997; Holladay *et al.*, 1991). Interestingly, this has not been observed in all studies (Nohara *et al.*, 2000; Vorderstrasse *et al.*, 2004). Moreover, alterations in thymocytes appear transient, as adult mice exposed developmentally to TCDD did not exhibit thymic atrophy or alterations in the proportion of thymocyte subpopulations, and skewing of T-cell subpopulations is not observed in secondary lymphoid organs (Fine *et al.*, 1989; Holladay *et al.*, 1991; Nohara *et al.*, 2000; Vorderstrasse *et al.*, 2004). In addition to effects on the thymus, deficiencies in T cell-dependent functions have been reported following developmental exposure to TCDD. For example, developmental exposure to TCDD between gd 6 and gd 14 results in offspring with diminished cytotoxic T lymphocytes (CTL) activity against allogeneic tumor cells (Holladay *et al.*, 1991). Suppression of delayed type hypersensitivity and contact hypersensitivity responses were observed in rats exposed *in utero* on gd 14 (Gehrs and Samialowicz, 1999; Walker *et al.*, 2004). Functional defects in the immune response to influenza virus infection have also been reported, and include suppressed expansion and differentiation of T cells and decreased antibody responses (Vorderstrasse *et al.*, 2004, 2006). In addition to these diminished responses, infected offspring from TCDD-treated dams exhibited an increased number of neutrophils and higher interferon gamma (IFN- $\gamma$ ) levels in their lungs. This indicates that the consequences of developmental exposure to TCDD are not exclusively immunosuppressive, as some functions are aberrantly upregulated. In support of this idea, offspring exposed to TCDD via lactation showed decreased host resistance and higher serum IFN- $\gamma$  levels after infection with *Listeria monocytogenes* (Sugita-Konishi *et al.*, 2003). Thus, developmental exposure to TCDD suppresses lymphocyte responses and enhances aspects of inflammation, and in contrast to thymic atrophy, these alterations in leukocyte function appear to persist into adulthood (Gehrs *et al.*, 1997; Genhsa and 1999; Luster *et al.*, 1980; Walker *et al.*, 2004).

Epidemiological evidence further supports the idea that exposure to AhR agonists causes alterations in the development of the immune system. For example, increased dioxin/PCB levels in breast milk and cord blood correlate with increased incidence of otitis media, respiratory tract infections in infants, and reduced antibody responses to childhood vaccinations (Dallaire *et al.*, 2004; Dewailly *et al.*, 2000; Heilmann *et al.*, 2006), which suggests a possible impairment in host resistance to infection. Correlations between prenatal exposure to dioxins

and PCBs and altered T-cell subpopulations and leukocyte recruitment have been reported (Weisglas-Kuperus, 1998; Weisglas-Kuperus *et al.*, 1995). Decreased lung function and increased chest congestion have also been associated with high perinatal dioxin exposure (Nagayama *et al.*, 1998; ten Tusscher *et al.*, 2001, 2003).

Despite information documenting that exposure to AhR ligands during development disrupts the function of the mature immune system at exposure levels that are not overtly hematotoxic, very little information is available regarding the mechanisms by which inappropriate AhR activation during development causes these changes. In particular, we do not know if there are periods during the development of the immune system that are more or less sensitive to perturbation by AhR activation. Differences between species, endpoints examined, dose of AhR agonist used, and time period of exposure (e.g., continuous, late gestation, perinatal/lactational) have made it difficult to compare findings among published studies. Another issue addressed by this study is whether the developing immune system is more sensitive to perturbation by sustained AhR activation than the mature immune system. Finally, it is not clear whether deregulated immune function in developmentally exposed offspring stems from AhR-mediated events within or extrinsic to the hemopoietic system. The present study assesses whether there is a critical period during immune development that is particularly sensitive to perturbation by AhR activation, directly compares immune function in exposed dams and their adult offspring and, through the creation of bone marrow chimeras evaluates the contribution of AhR-mediated events within and extrinsic to hematopoietic cells.

## MATERIALS AND METHODS

**Mice, TCDD treatment, viruses and infection.** C57BL/6 (B6, CD45.2) mice (age 5 weeks) were obtained from the Jackson Laboratory (Bar Harbor, ME) or National Cancer Institute (Frederick, MD). Female B6.SJL<sup>*ptprcaep3b*</sup>/BoyJ (CD45.1) mice were purchased from the Jackson Laboratory. Nulliparous B6 females were housed with B6 males, and checked daily for presence of a vaginal plug. The day a vaginal plug was found was designated as day 0 of gestation. Pregnant mice were then individually housed for the remainder of the study. Offspring from vehicle- and TCDD-treated dams were weaned at age 20–21 days. All mice were housed in microisolator cages in a specified pathogen-free facility at either Washington State University or the University of Rochester Medical Center, and were provided food and water *ad libitum*. All animal treatments were conducted with approval of Institutional Animal Care and Use Committees at Washington State University and the University of Rochester.

TCDD ( $\geq 99\%$  pure, Cambridge Isotope Laboratories, Inc., Woburn, MA) was dissolved in anisole and diluted in peanut oil to 0.1  $\mu\text{g}/\text{ml}$ . Except where indicated, impregnated mice were given 1  $\mu\text{g}$  TCDD/kg body weight by gavage on days 0, 7, and 14 of pregnancy, and 2 days after parturition. In some studies, only a subset of these four doses was administered (see Fig. 2). Control mice received the peanut oil-anisole vehicle in the same manner as described above. The cumulative dose of TCDD to the dams was  $\leq 4$   $\mu\text{g}/\text{kg}$ , which is well below the LD<sub>50</sub> for adult mice of this strain (160  $\mu\text{g}/\text{kg}$ ), and is not toxic to the fetus or

fetal lymphoid organs (Vorderstrasse *et al.*, 2004, 2006). This is an important point, because higher doses of TCDD administered in very late gestation or perinatally result in transient hypocellularity of primary immune organs (Fine *et al.*, 1990). Due to the relatively short half-life of TCDD in mice (7–11 days), dams were treated every 7 days in order to maintain roughly constant TCDD levels throughout pregnancy. As previously described (Vorderstrasse *et al.*, 2004), this dosing regimen does not affect litter size or the overall health status and viability of the offspring. Moreover, this dosing paradigm is not overtly hematotoxic, as there are no defects in cell number or cellular composition of the bone marrow, thymus, spleen, or lymph nodes.

Influenza virus strain A/HK-x31 (x31; H3N2) was prepared, titered and stored as previously described (Warren *et al.*, 2000). Adult female mice (6–12 weeks of age) were anesthetized by ip injection of 2,2,2-tribromoethanol and intranasally (i.n.) infected with 120 hemagglutinating units (HAU) of x31, which is a sublethal dose of virus. Mock-infected mice received 25  $\mu$ l of sterile endotoxin-tested phosphate buffered saline (PBS), and were included in some experiments as nonimmune controls for the infection. In other studies, dams and their adult offspring were sensitized with two separate 200- $\mu$ l injections (ip) of ovalbumin (OVA, 100  $\mu$ g) adsorbed with 1 mg Imject Alum in PBS (Pierce, Biotechnology, Rockford, IL) on days 1 and 5. On day 12, mice were challenged by inhalation of OVA aerosol in a plastic exposure chamber for 60 min. Aerosolized OVA (1% in PBS) was generated using a Shuco 3000 nebulizer.

**Collection and preparation of cells.** Leukocytes were obtained from lung airways by bronchoalveolar lavage (BAL) into cold RPMI 1640 containing 1% bovine serum albumin or 2.5% fetal bovine serum, and 10mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Teske *et al.*, 2005). Airway-derived immune cells were separated from BAL fluid by centrifugation. Lung-derived immune cells were obtained by digesting lungs with collagenase as previously described (Neff-LaFord *et al.*, 2007; Teske *et al.*, 2005). Immune cells were separated from erythrocytes and parenchymal lung cells using Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada). Single cell suspensions of mediastinal lymph nodes (MLN) were prepared by disrupting tissues using frosted microscope slides. Erythrocytes were removed by treatment with ammonium chloride. Cells were enumerated using either a hemacytometer or a Coulter Counter (Beckman Coulter Corp., Miami, FL).

**Immunophenotypic analyses.** Cells were stained with allophycocyanin (APC)- or phycoerythrin-labeled major histocompatibility complex (MHC) class I tetramers containing a peptide corresponding an immunodominant epitope of x31 (nucleoprotein, NP<sub>366–374</sub>/D<sup>b</sup>) and then incubated with previously determined optimal concentrations of fluorochrome-conjugated antibodies against the following cell surface antigens: CD8 $\alpha$ , CD44, CD45.1, CD45.2, CD62L. Other cells were stained with antibodies against Gr-1, CD11b, F4/80, NK1.1, TCR $\gamma\delta$ , or CD19. All antibodies were purchased from BD/Pharmingen (San Diego, CA), e-Biosciences (San Diego, CA), or Caltag Laboratories (Burlingame, CA). Nonspecific staining was blocked by incubating cells with a combination of rat IgG and an anti-mouse CD16/CD32 MAb. Appropriately labeled, isotype-matched immunoglobulins were used as controls for non-specific fluorescence. Data were collected from 50,000 to 200,000 cells by listmode acquisition using FACSCalibur and FACSsort flow cytometers (Becton Dickinson, San Jose, CA). Data were analyzed using FlowJo (TreeStar, Ashland, OR) software program. To identify IFN- $\gamma$ -producing cells, cells were incubated in 24-well plates at 37°C, 5% CO<sub>2</sub> for 5 h in the presence of 12.5 U/ml recombinant mouse interleukin-2, 1 $\mu$ M x31 nucleoprotein peptide (NP<sub>366–374</sub>. ASNENMETM), and 10  $\mu$ g/ml brefeldin A (Neff-LaFord *et al.*, 2007). Cells were stained with mAbs specific for cell surface molecules, fixed with 2% formalin, permeabilized with 1% saponin, and incubated with an APC-labeled anti-IFN- $\gamma$  mAb (BD Biosciences or eBiosciences).

**Bone marrow chimeric mice.** Bone marrow chimeric mice were generated as outlined in Figure 4. Age-matched female offspring of dams that were treated with either vehicle control or 1  $\mu$ g/kg TCDD on gd 0, 7, 14 and post-natal day 2 were used as either recipients or bone marrow cell donors. To

provide a means to validate chimerization, untreated congenic B6.SJL<sup>ptprc<sup>cap</sup>ep3b</sup>/BoyJ (B6.CD45.1<sup>+</sup>) mice were used as irradiated recipients of bone marrow cells from developmentally exposed mice or as bone marrow cell donors for irradiated offspring. Female B6.CD45.1 mice were age matched, antigenically naïve, and had not been previously treated with TCDD. Irradiation and reconstitution were performed as described previously (Lawrence *et al.*, 2006; Neff-LaFord *et al.*, 2007). Briefly, recipient mice were lethally irradiated with two doses of 600 rad, spaced 3.5-h apart. One hour after the second irradiation, mice were given  $1.5 \times 10^6$  bone marrow cells (iv) from either B6 (CD45.2<sup>+</sup>) or B6.SJL<sup>ptprc<sup>cap</sup>ep3b</sup>/BoyJ (B6.CD45.1<sup>+</sup>) mice. Six to 8 weeks after transplantation, bone marrow chimeric mice were infected, as described above. Irradiated mice that did not receive bone marrow cells did not survive. Survival rates for recipients of bone marrow cells were > 95%, regardless of exposure history of the recipient or donated cells. Chimerization was further validated using flow cytometry, which showed that for recipients of either CD45.1<sup>+</sup> or CD45.2<sup>+</sup> cells, chimerization success is not affected by the TCDD exposure history of donor cells and irradiated recipients (see Fig. 4B).

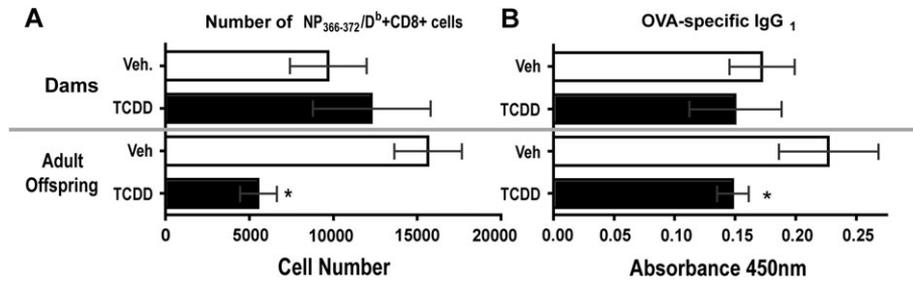
**Enzyme-linked immunosorbent assay.** Levels of IFN- $\gamma$  in BAL fluid were determined by sandwich enzyme-linked immunosorbent assay (ELISA) using matched antibody pairs and recombinant murine IFN- $\gamma$  from BD Biosciences. The assay was performed following the manufacturer's instructions. The lower limit of detection was 125 pg/ml. OVA-specific IgG1 and IgE were measured in serially diluted plasma using biotinylated anti-isotype-specific antibodies (Southern Biotech, Birmingham, AL).

**Statistical analyses.** Statistical analyses were performed using Statview (SAS, Cary, NC). Using a one-way analysis of variance, followed by *post hoc* tests (Fisher least significant difference), differences between independent variables were compared between each treatment group. Differences between two groups at a single point in time were evaluated using a Student's *t*-test. Differences were considered significant when *p* values were less than 0.05.

## RESULTS

### *The Developing and Mature Immune Systems are Differentially Sensitive to AhR Activation*

Maternal exposure to AhR agonists causes deregulated immune function later in the offspring, and there is a general sense that the developing immune system is more sensitive to immunomodulatory agents and environmental toxins than the mature immune system (Dietert, 2005; Holladay and Smialowicz, 2000; Luebke *et al.*, 2006). However, with regard to AhR agonists, this concept has not been directly tested. To determine whether the developing immune system is more sensitive than the mature immune system to perturbation by sustained and inappropriate AhR activation, we compared the expansion of influenza virus-specific CD8<sup>+</sup> T cells in exposed dams and their adult offspring. When considering these data, it is important to bear in mind that the exposed dams were challenged with influenza virus ~8 weeks after their last dose of TCDD (i.e., they were infected at the same time as their adult offspring). Virus-specific CD8<sup>+</sup> T cells were detected using fluorescently labeled MHC class I molecules complexed with an immunodominant viral peptide (NP<sub>366–372</sub>/D<sup>b</sup>). This population expands upon infection, with peak levels detected between days 8–11, after which time the virus is cleared from the lung and this population retracts (Flynn *et al.*, 1998;



**FIG. 1.** AhR activation during the ontogeny of the immune system causes long-lasting functional deregulation. Responses to influenza virus and OVA were compared in vehicle- and TCDD-treated dams and their developmentally exposed adult offspring. Antigen was administered to dams 8 weeks after their last dose of TCDD (i.e., when their offspring had reached maturity). Treated dams were of the same age, and data from TCDD-exposed offspring are compared with age-matched vehicle-exposed offspring. (A) Graphs depict the average number of virus-specific CD8<sup>+</sup> cells from vehicle- and TCDD-treated dams and their adult offspring 9 days after infection (i.n.) with 120 HAU influenza A virus (x31). MLN cells were stained with fluorescently labeled MHC class I molecules complexed with NP<sub>366-372</sub> and antibodies against CD8 and analyzed by flow cytometry (B) The average level of OVA-specific IgG<sub>1</sub> in the plasma was determined in a separate group of treated dams and their offspring, which had been sensitized and challenged with OVA as described in the “Materials and Methods.” OVA-specific IgG<sub>1</sub> was measured by isotype-specific ELISA. Absorbance (optical density) readings represent those from a 1:2.6 × 10<sup>6</sup> dilution of plasma, which was within the linear range of the dilution series. Findings were similar when plasma OVA-specific IgE levels were examined (data not shown). For these studies, there were three to four dams or six to eight pups per treatment group. Error bars indicate SEM. An \* indicates a significant difference from vehicle-exposed controls ( $p \leq 0.05$ ).

Lawrence *et al.*, 2006b). Nine days after infection, the number of NP<sub>366-372</sub>/D<sup>b</sup>-specific CD8<sup>+</sup> cells was equivalent in MLN from vehicle- and TCDD-treated dams (Fig. 1A). In contrast, mature offspring that were exposed to TCDD during gestation and lactation show a 50% reduction in the number of NP<sub>366-372</sub>/D<sup>b</sup>-specific CD8<sup>+</sup> cells compared with offspring from control dams.

To further test this using a different model antigen, we compared the antibody response to immunization with OVA in dams and their adult offspring. Compared with offspring of vehicle-exposed dams, plasma levels of OVA-specific antibodies were significantly decreased in mature animals that were exposed developmentally to TCDD. In contrast, levels of OVA-specific antibodies were not different among vehicle and TCDD-treated dams (Fig. 1B). Thus, based upon the endpoints examined, it appears that AhR activation in adults does not cause long-lasting deregulation of the mature immune system, but when inappropriate AhR activation occurs during the ontogeny of the hematopoietic system, it results in long-lasting functional deregulation.

#### *Sustained AhR Activation throughout Gestation and Lactation Leads to the Most Marked Deregulation of Immune Function Later in Life*

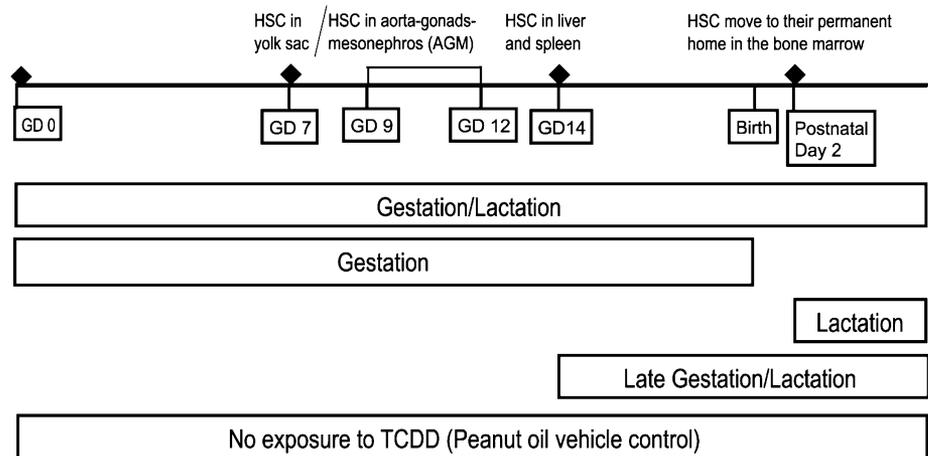
We next determined the developmental time period in which inappropriate AhR activation leads to long-lasting alterations in immune function. To accomplish this, we compared the expansion of virus-specific CD8<sup>+</sup> T cells in the MLN, the recruitment of neutrophils to the lung, and pulmonary IFN- $\gamma$  levels in animals exposed to TCDD during windows of time that span critical periods of immune ontogeny (Fig. 2). Specifically, exposure to TCDD during all of gestation and then via lactation, gestation only, lactation only, or during late

gestation and lactation was compared with offspring for which there was no developmental exposure to TCDD (vehicle control-exposed group). Compared with infected adult offspring of control dams, there was substantially reduced expansion of NP<sub>366-372</sub>/D<sup>b</sup>-specific CD8<sup>+</sup> cells in mice exposed to TCDD during development (Fig. 3A). This reduction was statistically significant in adult offspring of all exposure groups, except for those exposed to TCDD only during gestation.

Although aspects of the adaptive response to infection are suppressed when the AhR is inappropriately activated during development, some innate responses are enhanced. In particular, following infection with influenza virus, pulmonary IFN- $\gamma$  levels and the number of neutrophils recruited to the lung are elevated in adult mice that were exposed to TCDD throughout gestation and via lactation (Vorderstrasse *et al.*, 2004, 2006, and Figs. 3B and 3C). Similarly, when AhR activation is not initiated until GD14 (and continues via lactational exposure), infected adult offspring exhibited increased pulmonary IFN- $\gamma$  levels and neutrophilia. However, developmental exposure to TCDD solely during gestation or only via lactation did not significantly alter infection-associated recruitment of neutrophils to the lung or IFN- $\gamma$  levels in BAL fluid.

#### *AhR-Mediated Events within Hematopoietic Cells Underlie Suppressed Response of Virus-Specific CD8<sup>+</sup> T Cells but not Enhanced Innate Responses to Infection*

Reprogramming of the developing immune system could result from AhR-mediated effects within bone marrow-derived cells, which renders responses to infection permanently altered. If this were the case, then we would expect that when moved into a new environment, functional changes would still be evident. Alternatively, inappropriate AhR activation during



**FIG. 2.** Dosing strategy of timed pregnant mice for developmental exposure to TCDD. Impregnated mice were treated with vehicle or 1  $\mu\text{g}/\text{kg}$  TCDD on gd 0, gd 7, and gd 14 and/or 2 days after parturition (filled diamond). A subset of these doses was administered to separate groups of dams, indicated by the blocks underneath the dosing strategy. Mice that received only gestational exposure to TCDD were cross-fostered to untreated dams 2 days after birth. Offspring that received only lactational exposure were born to untreated dams and cross-fostered to treated dams that had received TCDD on gd 0, gd 7, gd 14 and 2 days after parturition. This cross-fostering insured that treated dams were exposed to the same amount of TCDD. Critical events during ontogeny of the vertebrate immune system are represented above the dosing scheme.

development could act upon hematopoietic cells in an indirect manner. That is, it causes long-lasting alterations in the immune response to infection via alterations in the programming (and long-term function) of cells extrinsic to the hematopoietic system. To distinguish between these two mechanisms, we generated reciprocal bone marrow chimeras (Fig. 4A). To evaluate whether sustained AhR activation during development impinges on the intrinsic function of bone marrow-derived cells, we reconstituted lethally irradiated naïve B6.CD45.1 congenic recipient mice with bone marrow cells from C57Bl/6 mice (CD45.2<sup>+</sup>) that were exposed to TCDD during development (TCDD-CD45.2  $\rightarrow$  CD45.1). Bone marrow cells from offspring of vehicle-treated dams were injected into a separate group of irradiated B6.CD45.1 recipients to serve as a control group (VEH-CD45.2  $\rightarrow$  CD45.1). For the reverse chimeras, bone marrow cells from untreated B6.CD45.1 mice were injected into lethally irradiated adult C57Bl/6 mice that were exposed to TCDD throughout gestation and via lactation (CD45.1  $\rightarrow$  TCDD-CD45.2). As a control, lethally irradiated adult offspring from vehicle-treated dams were reconstituted with bone marrow cells from B6.CD45.1 donors (CD45.1  $\rightarrow$  VEH-CD45.2).

In response to infection, the generation of CTL effector cells (CTLe), which are defined phenotypically as CD44<sup>hi</sup>CD62L<sup>lo</sup> CD8<sup>+</sup> cells, and expansion of NP<sub>366-374</sub>/D<sup>b+</sup> CD8<sup>+</sup> cells were suppressed in recipients of bone marrow cells from offspring of TCDD-treated dams (Figs. 5A and 5B). In contrast, the response of CD8<sup>+</sup> T cells in irradiated mice that were exposed developmentally to TCDD but received bone marrow cells from untreated donors (CD45.1  $\rightarrow$  TCDD-CD45.2) was not statistically different from irradiated mice that were exposed developmentally to the peanut oil vehicle and received cells

from untreated donors. This indicates that functional defects in CD8<sup>+</sup> T cells are inherent to bone marrow-derived cells. In contrast to the response of CD8<sup>+</sup> T cells, there were no differences in the number of neutrophils in lung airways (Fig. 5C) or lung interstitium (data not shown) in either chimeric scenario. That is, regardless of the exposure background of the donor cells and recipient mice, an equivalent number of neutrophils were recruited to the lung following infection with influenza virus. This suggests that enhancement of neutrophil recruitment during infection depends upon reciprocal interactions between AhR-programmed changes in bone marrow-derived cells and AhR-regulated targets extrinsic to hematopoietic cells.

We also examined IFN- $\gamma$  levels in lung lavage fluid of infected chimeras. IFN- $\gamma$  levels were equivalent in infected recipients of cells from offspring of vehicle- and TCDD-treated dams (CD45.2  $\rightarrow$  CD45.1 mice; Fig. 5D). However, mice that were exposed developmentally to TCDD but then received bone marrow cells from untreated donors (CD45.1  $\rightarrow$  TCDD-CD45.2) had twofold more IFN- $\gamma$  than infected mice in the control group. This result suggests that AhR activation during development does not impact infection-induced IFN- $\gamma$  production in the lung by directly affecting the cells that produce IFN- $\gamma$ . Instead, these data indicate that AhR activation during development targets extra-hematopoietic cells such that signals that control IFN- $\gamma$  production by cells in the lung are cued to respond differently during infection.

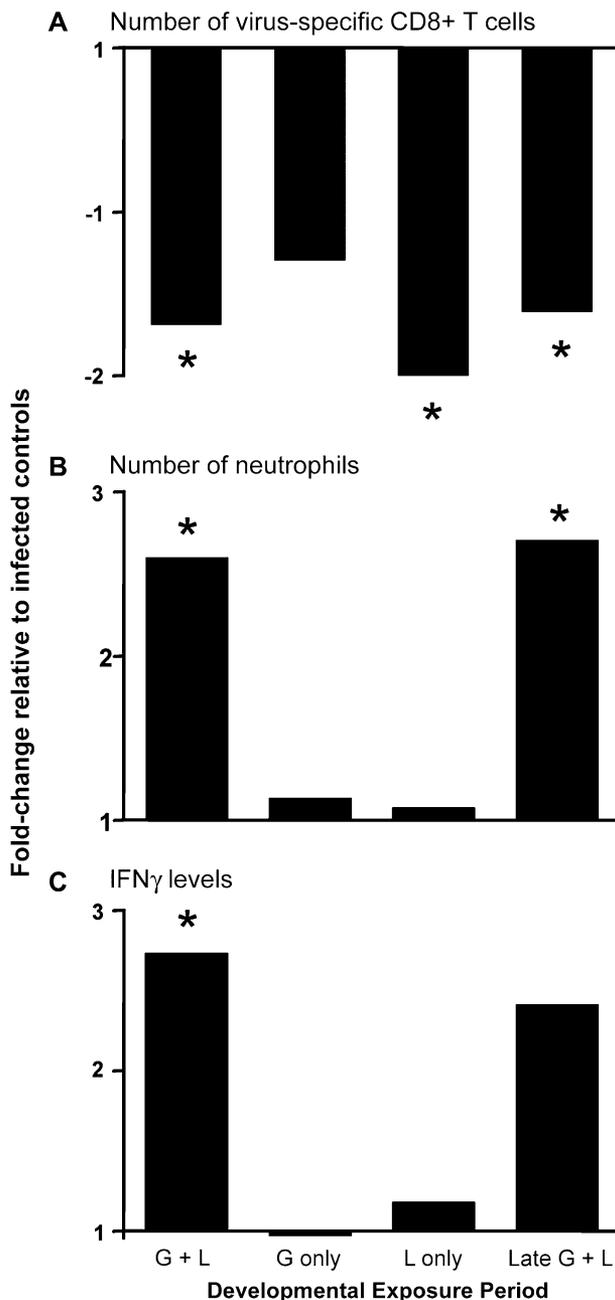
We have made similar observations in mice treated with TCDD as adults, and have found that in those mice neutrophils are the major source of excess IFN- $\gamma$  in the lung (Neff-LaFord *et al.*, 2007). However, we did not know whether increased IFN- $\gamma$  in lungs of infected offspring of TCDD-exposed dams

also comes from neutrophils. Therefore, we next determined the number and phenotype of IFN- $\gamma$ <sup>+</sup> cells in lungs of infected offspring from vehicle- and TCDD-treated dams. Compared with infected offspring of control-exposed dams, twice as many IFN- $\gamma$ <sup>+</sup> cells were present in lungs of infected mice developmentally exposed to TCDD. Regardless of maternal exposure, only a small proportion of the IFN- $\gamma$ <sup>+</sup> cells were CD8<sup>+</sup>, CD4<sup>+</sup>, NK1.1<sup>+</sup>, or CD19<sup>+</sup>, whereas the majority of IFN- $\gamma$ <sup>+</sup> cells were Gr1<sup>+</sup> and CD11b<sup>+</sup> (Fig. 6B). This suggests that the increased IFN- $\gamma$  levels in the lung stems primarily from myeloid cells recruited to the lung.

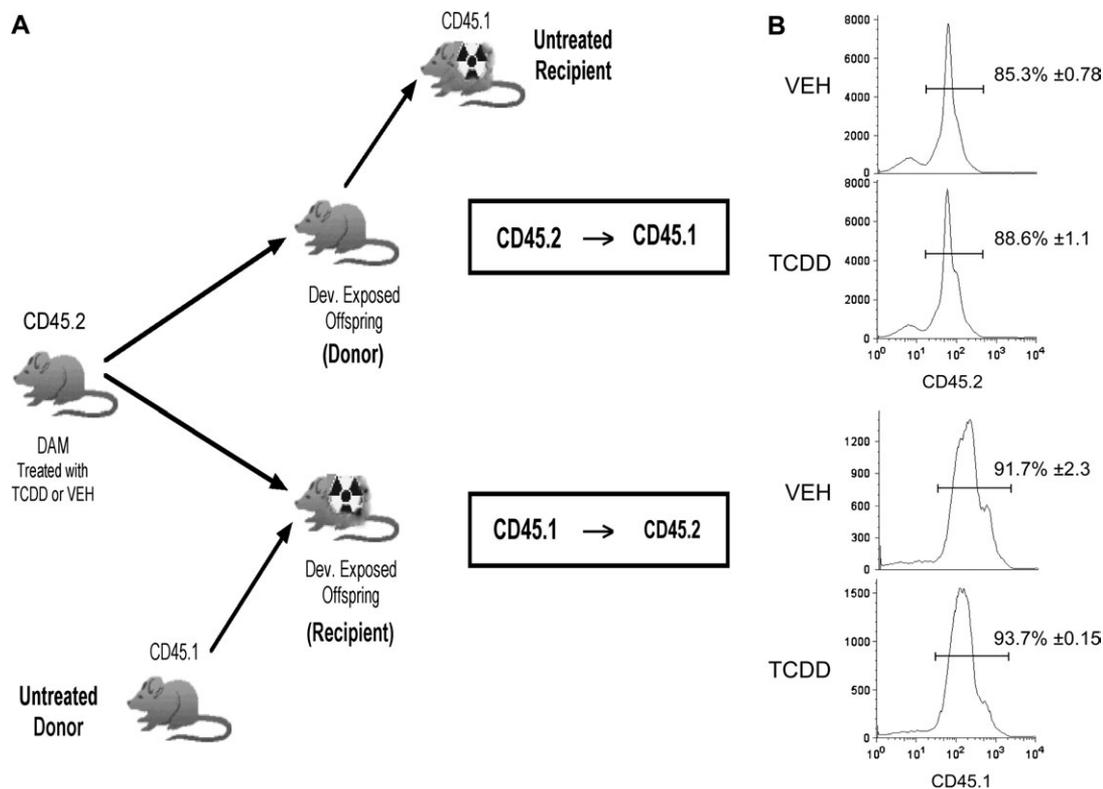
## DISCUSSION

Epidemiological studies provide very compelling evidence that prenatal and early postnatal environmental factors influence the risk of developing chronic diseases, such as diabetes, cardiovascular disease, and maybe even obesity (Gluckman and Hanson, 2004; Jirtle and Skinner, 2007; Rhind *et al.*, 2003). Although the evidence is not as extensive, environmental factors appear to influence the development and programming of the immune system as well (Dietert, 2005; Holladay and Smialowicz, 2000; Luebke *et al.*, 2006; Merlot *et al.*, 2007; Pinkerton and Joad, 2006; Prescott, 2006; Zhang *et al.*, 2005). Even subtle changes in immune function could result in poor vaccine efficacy and decreased resistance to infectious disease. Growing concern over emerging and re-emerging infectious diseases necessitates a better understanding of how environmental factors influence immunity and contribute to differential susceptibility to infectious diseases. Furthermore, imbalances in immune function can also enhance responsiveness to nonpathogenic antigens, as is the case in autoimmune disease and hypersensitivity reactions.

Among the many factors that contribute to the establishment of proper immunoregulatory balance, the AhR clearly plays an important role. Inappropriate or sustained AhR activation during fetal and neonatal development results in changes in the immune system that persist into adulthood. At higher doses of TCDD, thymic atrophy and bone marrow hypocellularity have been reported. However, at lower doses of TCDD, functional alterations are present in adult offspring in the absence of detectable alterations in the cellular composition of primary and secondary immune organs (Vorderstrasse *et al.*, 2004, 2006). This suggests AhR activation during development may reprogram the immune system such that certain responses are inappropriately elevated and other responses are dampened. Numerous epidemiological and animal studies have suggested



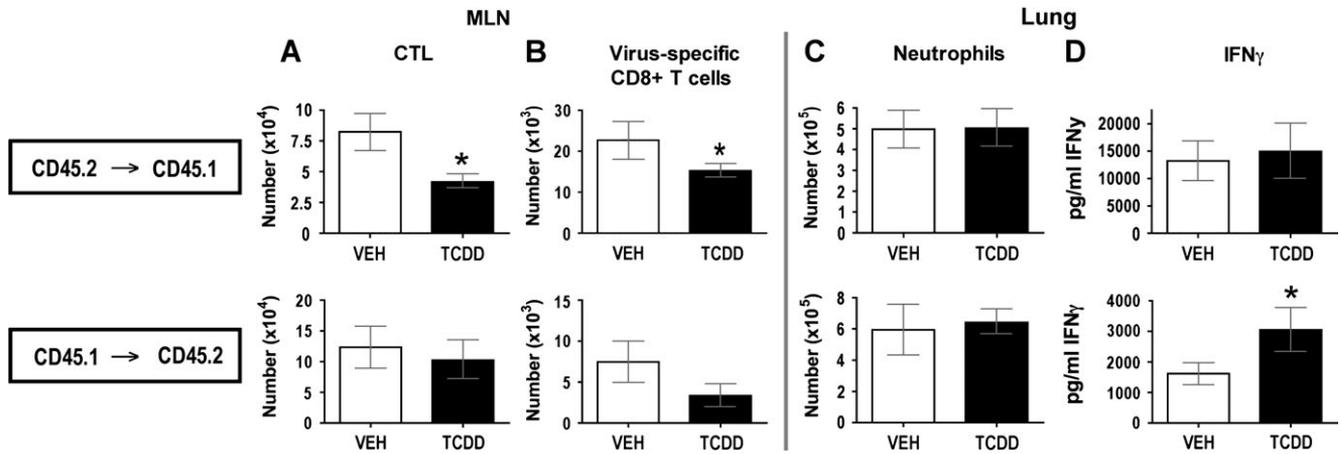
**FIG. 3.** Developmental exposure to TCDD throughout all of gestation and then via lactation results in impaired immune function. Developmentally exposed adult mice (6–8 weeks of age) were treated as described in Figure 2, and the following abbreviations are used: exposure during all of gestation and via lactation (G + L), gestation only (G only), lactation only (L only), or late gestation and lactation (late G + L). Data from TCDD-exposed offspring are compared with age-matched vehicle-exposed offspring that were infected at the same time. BAL cells and BAL fluid were collected 7 days after infection with influenza virus, whereas MLN cells were collected on day 9. All graphs depict the average fold change relative to infected adult offspring of control dams (TCDD/Veh), in which a value of 1 means that there was no difference between the groups. (A) The number of NP<sub>366–372</sub>/D<sup>b</sup>CD8<sup>+</sup> cells was determined by flow cytometry. (B) The average number of neutrophils in the lung was determined by differential cell counting. (C) The average amount of IFN- $\gamma$  in lung lavage fluid was determined by ELISA. There were five to eight female offspring per treatment group at each point in time, and each infected mouse was from a different exposed dam. Error bars indicate SEM. An \* indicates a significant difference from infected offspring of vehicle-exposed dams ( $p \leq 0.05$ ).



**FIG. 4.** Generation of bone marrow chimeras is not affected by maternal TCDD exposure. (A) Experimental design. CD45.2 → CD45.1 chimeras were generated by transferring bone marrow cells (BMC) from age-matched female C57Bl/6 CD45.2 mice that were developmentally exposed to TCDD or vehicle (as described in Fig. 2, G + L) into lethally irradiated (but otherwise untreated) adult B6.CD45.1 congenic mice. CD45.1 → CD45.2 chimeras were created by transferring BMC from untreated B6.CD45.1 congenic female mice into lethally irradiated, age-matched adult C57Bl/6 CD45.2 mice that had been developmentally exposed to TCDD or vehicle (as described in Fig. 2, G + L). (B) Representative histograms show that 6–8 weeks after reconstitution, bone marrow cells in the recipient mice are derived from the donor. The number beside each histogram shows the average percentage of cells in bone marrow from each treatment group ( $\pm$  SEM). There were no significant differences in chimerization among the groups.

that prenatal and early life environmental factors, including exposure to exogenous chemicals and maternal stress, influence states of health and disease later in life (Jirtle and Skinner, 2007; Merlot *et al.*, 2007; Rhind *et al.*, 2003; Zhang *et al.*, 2005). Delineating the epigenetic mechanisms by which developmental programming occurs is an active area of study. In addition to determining how AhR modulates the programming of the immune system, it will be important to delineate whether exposure to TCDD, and other AhR ligands, during development skews programming due to interference with the function of endogenous AhR ligands, or via inappropriate alterations of AhR-responsive genes and signaling pathways. Interestingly, phenotypic characterization of transgenic mice with a constitutively active AhR suggests that alterations in immune parameters occur in the absence of TCDD (Andersson *et al.*, 2003; Tauchi *et al.*, 2005). Although this supposition awaits validation in the context of assessing specific immune responses *in vivo*, it provides further evidence that the consequences of sustained AhR activation during the development of the immune system extend beyond toxicity caused by dioxin exposure.

The observation that the dose of TCDD administered to pregnant mice did not cause long-lasting changes their responses to influenza virus and OVA challenge strongly suggests that the persistence of functional changes following developmental exposure stems from reprogramming rather than residual TCDD. In support of this idea, it is important to recall that only a very small amount ( $\leq 0.5\%$ ) of the maternal dose is transferred to the fetus (Birnbaum, 1986; Weber and Birnbaum, 1985), and that hepatic levels of TCDD in 5-week-old offspring exposed gestationally and via lactation were 75% lower than levels in the fetus (Nau *et al.*, 1986). Thus, even when exposure continues via lactation, TCDD levels in adult offspring are substantially lower than levels detected in the fetus. In our exposure paradigm, where the maximum cumulative dose to the dam was 4  $\mu\text{g}/\text{kg}$ , the amount of TCDD remaining in adult offspring is likely well below levels that are immunomodulatory. In fact, we have previously shown that adverse effects of TCDD on the immune response to influenza virus are dose-dependent (Vorderstrasse *et al.*, 2003). Administration of a single dose of 5  $\mu\text{g}$  TCDD/kg modulated IFN- $\gamma$  levels, T cell expansion in the lymph node, and the

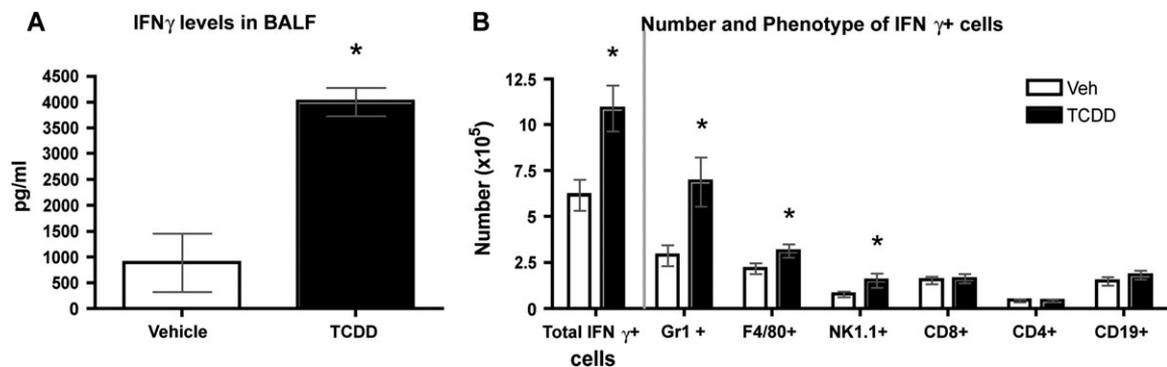


**FIG. 5.** AhR-mediated events within hematopoietic cells underlie suppressed response of virus-specific CD8<sup>+</sup> T cells, but not enhanced innate responses to infection. Reciprocal chimeras, generated as described in Figure 4, were challenged with influenza virus 6–8 weeks later. (A, B) MLN cells were obtained 9 days after x31 infection. Graphs depict the average number of (A) CTLs (CD44<sup>hi</sup>CD62L<sup>lo</sup>CD8<sup>+</sup> cells) and (B) NP<sub>366–372</sub>/D<sup>b+</sup>CD8<sup>+</sup> cells in each group of chimeras. (C, D) Airway cells and BAL fluid were collected on day 7 following infection with x31, and graphs show (C) the average number of neutrophils and (D) level of IFN- $\gamma$  in BAL fluid. Fluorescently labeled antibodies against CD45.1 and CD45.2 were used to validate chimerization, and to distinguish chimeric cells from residual endogenous cells (see Fig. 4B). Results are representative of at least two separate experiments. In each experiment, there were five to six female mice in each treatment group at each point in time. Error bars indicate SEM. An \* indicates a significant difference from the vehicle-treatment group ( $p \leq 0.05$ ).

number of CD8<sup>+</sup> T cells and neutrophils in the lung. However, at the lowest dose administered (1  $\mu\text{g}/\text{kg}$ ) these endpoints were unaffected. Given that we show here that TCDD causes long-lasting changes in developmentally exposed animals versus transient immune modulation in pregnant adult animals that were exposed acutely to the same chemical, it seems very likely that the developing immune system is more sensitive to modulation by AhR activation. This conclusion is consistent with the general thinking that the developing immune system is more sensitive to perturbation by environmental factors, and that adverse effects of a chemical are more persistent when exposure occurs during development rather than during adulthood. What remains to be determined is whether AhR

activation modulates the mature and developing immune systems through the same or different molecular mechanisms.

A step-wise understanding of when different leukocyte subpopulations and their direct progenitors arise is an active area of study. Current information indicates that different leukocyte lineages begin to form at different times. Cells of the myeloid lineage are first detected during mid-gestation, whereas T cells and a thymus are not detected until late gestation, and lymphocyte maturation continues into early postnatal life (Dzierzak and Medvinsky, 1995; Zhu and Emerson, 2002). This suggests that factors that influence neutrophils may be sensitive to AhR-mediated programming during an earlier window of time than T cells. Our data support



**FIG. 6.** Excess IFN- $\gamma$  in lungs of infected mice that were developmentally exposed to TCDD comes primarily from phagocytic cells. Lung-derived immune cells and lung lavage fluid from vehicle and TCDD developmentally exposed offspring (8 weeks of age; G + L, Fig. 2) were collected 7 days after infection with influenza virus (x31). (A) Average level of IFN- $\gamma$  in the BAL fluid, as measured by ELISA. (B) Bar graphs depict the number and phenotype of IFN- $\gamma$ <sup>+</sup> cells in the lung. Lung-derived leukocytes were restimulated *in vitro*, stained with fluorescently labeled antibodies and analyzed by flow cytometry as described in the ‘‘Materials and Methods.’’ There were 7–10 female mice in each treatment group, and each infected mouse was from a different exposed dam. Error bars indicate SEM. An \* indicates a significant difference from infected offspring of vehicle-exposed dams ( $p \leq 0.05$ ). Data are representative of two separate experiments.

this idea. The representative functional responses examined herein suggest subtle but distinct differences in the critical periods during development that are the most sensitive to long-lasting perturbation by AhR activation. Impairment in the expansion of virus-specific CD8<sup>+</sup> T cells was apparent when AhR was activated in late gestation and lactation and solely during lactation, suggesting that events that occur after parturition may be key regulatory targets of CD8<sup>+</sup> T cell function. These events may include the generation of lymphoid lineage precursors and the selection of T cells in the thymus. In contrast, neutrophil recruitment and increased IFN- $\gamma$  levels in the lung only showed significant treatment effects when AhR activation was initiated during gestation and continued via lactation. Thus, perturbation of these responses requires sustained AhR activation during an earlier period of time and lasting for a longer amount of time, and suggests that events in the AGM or fetal liver and spleen may be sensitive targets of AhR. One variable not fully accounted for in the present study is the slight variation in the total dose administered to the dam. For offspring exposed during gestation and lactation, gestation only and lactation only, the total cumulative dose to the dam was the same (4  $\mu\text{g}/\text{kg}$ ). However, for offspring exposed only during late gestation/lactation, the total dose administered to the dam was 2  $\mu\text{g}/\text{kg}$ . Regardless of these subtle differences, the most striking effect of developmental exposure was observed in offspring of dams treated throughout gestation and 2 days after parturition.

Although AhR activation during development causes long-lasting changes in innate and adaptive responses to infection, these effects likely result from distinct AhR-mediated events within and extrinsic to bone marrow-derived cells. We show here that AhR activation during development modulates programming of bone marrow-derived cells directly, such that CD8<sup>+</sup> T cells demonstrate decreased responsiveness to antigen even when transferred to recipients that have not been given exogenous AhR agonists. This does not prove TCDD acts directly within T cells, but indicates that this altered functional capacity is inherent in bone marrow-derived cells. In contrast, increased IFN- $\gamma$  levels in the lung are probably not inherent to hematopoietic cells. This is a surprising result and suggests that AhR-regulated pathways extrinsic to bone marrow-derived cells play a pivotal role in enhancing IFN- $\gamma$  levels in the infected lung. This is consistent with findings in adult mice acutely exposed to TCDD, in which AhR-mediated events extrinsic to hematopoietic cells direct enhanced IFN- $\gamma$  production by phagocytic cells in the lung (Neff-LaFord *et al.*, 2007). Because the majority of IFN- $\gamma$ + cells in the lung are myeloid cells (primarily neutrophils), it is tempting to speculate that increased IFN- $\gamma$  is simply due to the increased recruitment of neutrophils to the lung. However, the answer may not be that simple. Although the amount of IFN- $\gamma$  in BAL fluid was enhanced in TCDD-exposed chimeras reconstituted with naïve CD45.1<sup>+</sup> bone marrow cells, there was not a corresponding increase in the number of neutrophils. Thus, increased IFN- $\gamma$

levels likely reflect changes in extrahematopoietic pathways that regulate IFN- $\gamma$  production.

This dichotomy between AhR activation affecting functions of cells in the innate and adaptive arms of the immune system via distinct pathways is consistent with recent reports in adult mice exposed acutely to TCDD. Using a combination of adoptive transfer and bone marrow chimeras, it is clear that AhR-mediated modulation of T cell responses is dependent upon the presence of AhR within bone marrow-derived cells, whereas AhR-mediated increases in IFN- $\gamma$  production by neutrophils and macrophages are not (Funatake *et al.*, 2005; Kerkvliet *et al.*, 2002; Lawrence *et al.*, 2006b; Neff-LaFord *et al.*, 2007). Similarly, the idea that the AhR modulates the immune system through a combination of direct effects in bone marrow-derived cells and indirect action in extrahematopoietic tissues is supported by mechanistic studies of TCDD-induced thymic atrophy and bone marrow hypocellularity. Thymic atrophy caused by TCDD has been shown to require AhR in hematopoietic cells, but not in thymic stromal tissues (Staples *et al.*, 1998). Direct effects on thymocytes are further supported by studies using a T-cell specific *lck*-conditional knockout of ARNT, which showed that mice that do not express ARNT in their T cells do not exhibit thymic involution following TCDD exposure (Laiosia *et al.*, 2003; Tomita *et al.*, 2003). However, there is also evidence that thymic stromal cells contribute to TCDD-induced thymic atrophy (Camacho *et al.*, 2005; Greenlee *et al.*, 1985; Kremer *et al.*, 1994; Riecke *et al.*, 2003). Similarly, effects of AhR on hematopoietic cells in the bone marrow likely reflect direct and indirect mechanisms. HSC from mice that were acutely treated with TCDD (40  $\mu\text{g}/\text{kg}$ ) were less able to reconstitute bone marrow of irradiated recipients (Sakai *et al.*, 2003). These effects were not observed at lower doses of TCDD, and were not observed in AhR-deficient mice, suggesting a direct, AhR-mediated effect on hematopoietic cells, at least at high doses of TCDD. However, AhR-dependent events in bone marrow stromal cells contribute to suppressed differentiation of B cell precursors, suggesting that, at least for the B cell compartment, AhR-mediated events in stromal cells also play a role (Allan *et al.*, 2003; Wyman *et al.*, 2002; Yamaguchi *et al.*, 1997). When taken together, these findings suggest that AhR-mediated alterations in primary immune organs (thymus and bone marrow) reflect a composite of changes in regulatory pathways within and extrinsic to bone marrow-derived cells.

The new findings reported here have important implications with regard to the design and interpretation of studies aimed at delineating how AhR contributes to immunoregulatory balance. For instance, in order to comprehend the mechanism at a more detailed level, we need to better understand how extrahematopoietic cells modulate the magnitude and nature of the response of leukocytes to antigens. More broadly, this study reiterates the point made by others (Dietert, 2005, 2006; Holsapple *et al.*, 2004) that a full understanding of how environmental factors in-play during development cause

long-lasting and often subtle changes in immune function, we will need to conduct studies in which functional endpoints form a critical component.

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