

Effects of Developmental Activation of the AhR
on CD4⁺ T-Cell Responses to Influenza Virus Infection
in Adult Mice

Lisbeth A. Boule, Bethany Winans, and B. Paige Lawrence

<http://dx.doi.org/10.1289/ehp.1408110>

Received: 10 January 2014

Accepted: 21 July 2014

Advance Publication: 22 July 2014

Effects of Developmental Activation of the AhR on CD4⁺ T-Cell Responses to Influenza Virus Infection in Adult Mice

Lisbeth A. Boule,¹ Bethany Winans,² and B. Paige Lawrence^{1,2}

¹Department of Microbiology and Immunology and ²Department of Environmental Medicine, University of Rochester, Rochester, New York, USA

Address correspondence to B. Paige Lawrence, Department of Environmental Medicine, University of Rochester School of Medicine and Dentistry, 601 Elmwood Ave, MRBX 3-11108, Rochester, NY, 14642 USA. Telephone: (585) 275-1974. E-mail:

paige_lawrence@urmc.rochester.edu.

Running title: Developmental AhR activation alters CD4⁺ T cells

Acknowledgments: We thank Dr. Guangbi Jin and Mr. Kyle Martin for technical support, Dr. Deborah Fowell and Mr. Zachery Knowlden for advice on CD4⁺ T cell subset analyses, and Dr. Jennifer Wheeler for critique of this manuscript. We are grateful to Dr. Timothy Bushnell and the URM C Flow Cytometry Core, and the staff at the NIAID Tetramer Core Facility. This work was supported by grants from the National Institutes of Health (R01-ES017250, R01-HL097141, T32-ES07026, T32-AI007285, T32-HL066988 and P30-ES01247) and funds from the University of Rochester.

Competing financial interests: The authors declare they have no actual or potential competing financial interests.

Abstract

Background: Epidemiological and animal studies indicate that maternal exposure to pollutants that bind the aryl hydrocarbon receptor (AhR) correlates with poorer ability to combat respiratory infection and lower antibody levels in the offspring. These observations point to an impact on CD4⁺ T cells. Yet, the consequence of developmental exposure to AhR ligands on the activation and differentiation of CD4⁺ T cells has not been directly examined.

Objectives: Our goal was to determine whether maternal exposure to an AhR ligand directly alters CD4⁺ T cell differentiation and function later in life.

Methods: C57Bl/6 mice were exposed to a prototypical AhR ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) *in utero* and via suckling. CD4⁺ T cell activation and differentiation into distinct effector populations was measured in adult offspring that were infected with influenza A virus (IAV). Reciprocal adoptive transfers were used to define whether modifications in CD4⁺ T cell responses resulted from direct effects of developmental exposure on CD4⁺ T cells.

Results: Developmental exposure skewed CD4⁺ T cell responses to IAV infection. There were fewer virus-specific, activated CD4⁺ T cells, and a reduced frequency of conventional CD4⁺ effector cell subsets. However, there was an increase in regulatory CD4⁺ T cells. Impaired differentiation into conventional effector subsets was due to direct effects of AhR activation on CD4⁺ T cells, as this defect can be transferred to mice that had not been developmentally exposed to TCDD.

Conclusions: Maternal exposure to TCDD causes durable changes in the responsive capacity and differentiation of CD4⁺ T cells in adult C57Bl/6 mice.

Introduction

Prenatal and early life environmental factors, including exposure to exogenous chemicals, have been linked to increased risk of cancer, diabetes, cardiovascular disease, and obesity (Boekelheide et al. 2012). Although it has been the focus of fewer studies, maternal exposures also influence the immune system (Winans et al. 2011). The consequences of affecting the immune system are potentially serious because even subtle changes can diminish resistance to infections and reduce responses to vaccines. In fact, several recent reports suggest that these are real-world consequences of developmental exposures. For example, maternal and cord blood levels of polychlorinated biphenyls and dioxins correlate with decreased responses to routine vaccinations, and increased respiratory infections in children (Dallaire et al. 2006; Glynn et al. 2008; Heilmann et al. 2010; Hochstenbach et al. 2012; Stolevik et al. 2013). Exposure to these chemicals occurs regularly through the diet, and it is estimated that fetuses and infants are exposed to higher levels due to bioaccumulation (Institute of Medicine 2003; Schechter et al. 2001). However, the cellular targets and mechanisms by which developmental exposures cause persistent changes in the function of the immune system are unknown.

CD4⁺ T cells are critical immune effector cells, and alteration in their function can have grave consequences on responses to primary infection and the acquisition of immunity. Infection initiates naïve CD4⁺ T cells to differentiate into phenotypically and functionally distinct subsets, although the precise subset depends upon particular pathogen-derived and tissue-specific cues (Yamane and Paul 2013). Th1 and T follicular helper (Tfh) cells are two major conventional CD4⁺ effector subsets elicited by respiratory infection (Boyden et al. 2012; Chapman et al. 2005). Th1 cells produce the cytokine interferon gamma (IFN γ), and Tfh are critical for T cell-

dependent B cell responses. While their precise role during infection is not fully understood, Th17 cells correlate with reduced mortality in mice and humans (Almansa et al. 2011; McKinstry et al. 2009). While Th2 cells contribute to responses to parasites and many allergic diseases, they represent a small fraction of CD4⁺ effectors during respiratory viral infections. The aforementioned are considered conventional CD4⁺ T cells, whereas regulatory CD4⁺ T cells (Tregs) maintain peripheral tolerance and down-regulate responses in the context of numerous infections (Fontenot and Rudensky 2005). Changing the capacity of CD4⁺ T cells to differentiate into distinct effector subsets has major implications on the progression and resolution of infection.

Exposure to aryl hydrocarbon receptor (AhR) ligands alters CD4⁺ T cell differentiation and function in developmentally mature organisms. For example, AhR ligands modulate conventional CD4⁺ T cell responses, altering the proportion of Th1, Th2, and Th17 cells (Quintana and Sherr 2013). Direct treatment with AhR ligands also alters the frequency of Tregs, but often in the opposite direction compared to conventional CD4⁺ T cells, resulting in a greater frequency of Tregs (Quintana and Sherr 2013). Collectively, these studies indicate that exposure of the fully mature immune system to AhR ligands changes the proportion of functionally distinct effector sub-populations of CD4⁺ T cells and influences disease outcome. In contrast, the consequences of AhR activation during development on CD4⁺ T cells later in life have not been empirically studied. Yet, several pieces of evidence suggest developmental exposure impacts CD4⁺ T cells. First, developmental exposure to AhR ligands decreases antibody responses to sheep erythrocytes and influenza A virus (IAV), and reduces delayed-type hypersensitivity responses in adult offspring (Gehrs and Smialowicz 1999; Thomas and Hinsdill 1979;

Vorderstrasse et al. 2006). Developmental exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) increases mortality after infection with *Listeria monocytogenes* (Sugita-Konishi et al. 2003), and enhances susceptibility to tumor challenge (Luster et al. 1980). These processes all depend upon the function of CD4⁺ T cells; however whether CD4⁺ T cell function is actually altered by AhR activation during development has not been directly examined.

In this study, we report an assessment of whether AhR activation during development changes the response of CD4⁺ T cells to infection with IAV later in life. Specifically, following maternal exposure to an oral dose of TCDD that does not cause thymic or bone marrow hypocellularity or other signs of toxicity (Vorderstrasse et al. 2004), we determined the frequency of conventional, activated, and virus-specific CD4⁺ T cells. Further, we examined regulatory CD4⁺ T cells and the ratio of conventional:regulatory CD4⁺ T cells. Then, using reciprocal adoptive transfers, we evaluated whether CD4⁺ T cell responses to infection are modulated via intrinsic or extrinsic effects of AhR activation during development on CD4⁺ T cells. Delineating which aspects of CD4⁺ T cell function are intrinsically altered by developmental exposure furthers our understanding of how AhR ligands can cause durable changes to CD4⁺ T cell responses, and expands current understanding of how developmental exposures to AhR ligands affect the immune responses later in life.

Materials and methods

Animal treatment and cell preparation

C57BL/6 (B6, CD90.2⁺CD45.2⁺), B6-LY5.2/Cr (CD90.2⁺CD45.1⁺), and B6.PL-*Thy1^a*/CyJ (CD90.1⁺CD45.2⁺) mice (age 5 weeks) were obtained from the NCI Mouse Repository (Frederick, MD) or the Jackson Laboratory (Bar Harbor, ME) and are all *Ahr*^{+/+}. A colony of

B6.AhR^{tm1Bra} (*Ahr*^{-/-}) mice is maintained at the University of Rochester Medical Center (URMC), as previously described (Teske et al. 2005). Nulliparous females (age 8-10 weeks) were housed with males after spending at least one week in the vivarium at the URMC, and checked daily for the presence of a vaginal plug (day 0 of gestation). All offspring (regardless of experimental endpoint measured) remained with their mother until they were weaned (age 20-21 days), and then housed in with same sex littermates. Mice were housed in washed polysulfone microisolator cages in a specific-pathogen free facility, with controlled light (12 hr light/dark), temperature and humidity, and were provided standard mouse chow (LabDiet, 5010) and water *ad libitum*. C57BL/6 (B6, CD90.2⁺CD45.2⁺) mice were used for all time course studies and examination of CD4⁺ T cell subsets. *Ahr*^{-/-} mice crossed with C57BL/6 mice (*Ahr*^{+/+}) for experiments to determine whether offspring need to express AhR to experience changes due to TCDD exposure during development. C57BL/6 (B6, CD90.2⁺CD45.2⁺), B6-LY5.2/Cr (CD90.2⁺CD45.1⁺), and B6.PL-*Thy1^a*/CyJ (CD90.1⁺CD45.2⁺) mice were used for adoptive transfer studies.

Impregnated female mice were treated with 1 µg/kg body weight of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; ≥99% purity; Cambridge Isotope Laboratories, Woburn, MA) or peanut oil (vehicle) by gavage in the afternoon on days 0, 7, and 14 of gestation, and 2 days post parturition (Vorderstrasse et al. 2004). The concentration of TCDD stock was maintained at 1 µg/ml in peanut oil so that dosing required 10 µl TCDD (or peanut oil vehicle) per gram of body weight. The time to parturition, litter size, and sex distribution of the offspring were not changed by exposure to this dose of TCDD (data not shown and Vorderstrasse et al. 2004). IAV strain HKx31 (H3N2) was prepared, titered, and stored as previously described (Warren et al. 2000).

Adult offspring (6-8 weeks of age) were anesthetized by intraperitoneal (i.p.) injection of avertin (2,2,2-tribromoethanol; Sigma Aldrich, Milwaukee, WI) and infected intranasally (i.n.) with 120 hemagglutinating units (HAU) of IAV (Warren et al. 2000). For the time course experiments, all mice were infected on the same day and groups of mice were sacrificed at each time point (the number of mice used on each day post infection is specified in the appropriate figure legend). Naïve (also denoted as day 0 post infection) adult offspring were included as controls in experiments quantifying the number of CD4⁺ T cells and examining the IAV-specific antibody response. Spleens and lymph nodes were removed in the morning of each day post infection (e.g., day 3, 6, 9 or 12 post infection, as specified in the figure legends) and single cell suspensions were made, and blood was collected by cardiac puncture into heparin-loaded syringes. Erythrocytes were removed by hypotonic lysis. Unless otherwise specified, four to six female offspring from separate dams were used at each point in time for each and for each treatment group. All animal treatments and work with infectious agents were conducted with prior approval of Institutional Animal Care and Use Committee and Institutional Biosafety Committee of the University of Rochester. All animals used were treated humanely and with regard for alleviation of suffering.

Flow cytometry

Isolated cells were co-labeled with fluorochrome-conjugated antibodies to specific cell surface molecules, to define specific populations cells, including CD4⁺ T cells, effector (CD44^{hi}CD62L^{lo}) CD4⁺ T cells, T follicular helper cells (CD4⁺CD44^{hi}CXCR5⁺PD-1⁺), germinal center B cells (GL-7⁺CD95⁺B220⁺), and plasma cells (B220^{int}CD138⁺) (Jin et al. 2014; Vorderstrasse et al. 2004; Wheeler et al. 2013). Where indicated, cells were incubated with major

histocompatibility (MHC) class II tetramers containing an immunodominant peptide epitope of HKx31 (nucleoprotein, I-A^b/NP₃₁₁₋₃₂₅, NIAID Tetramer Core Facility, <http://tetramer.yerkes.emory.edu/>). For intracellular molecules, cells were fixed, permeabilized, and co-incubated with antibodies against Foxp3, GATA3, ROR γ t, and TBet. Non-specific staining was blocked using anti-mouse CD16/32 mAb. All antibodies were from BD Biosciences (San Diego, CA) or eBiosciences (San Diego, CA). Data were collected using an LSRII flow cytometer (BD Bioscience), and analyzed using FlowJo software (TreeStar, Ashland, OR). Fluorescence minus one (FMO) was used to define gating parameters. Details regarding gating strategies and cell number calculations are provided in Supplemental Materials (see Supplemental Material, Figure S1). Data from individual animals are used in all experiments; however, for adoptive transfer experiments, data were concatenated due to the low number of gated events for T cell subsets. Concatenation was performed after analysis to better visualize our findings. It did not affect the distribution of the data or conclusions.

ELISA and ELISPOT assays

IAV-specific antibodies were detected in serum collected on day 9-post infection, using isotype-specific enzyme linked immunosorbent assay (ELISA) (Vorderstrasse et al. 2006). IFN γ -producing CD4⁺ T cells were enumerated by enzyme-linked immunosorbent spot (ELISPOT) assay. Briefly, CD4⁺ T cells were negatively enriched (R&D, Minneapolis, MN) and serially diluted, starting with 1 x 10⁵ cells/well, and added to 96-well plates (Millipore, Bedford, MA) coated with anti-IFN γ antibody (Mabtech, Nacka, Sweden). Virus-pulsed, irradiated DC2.4 cells (provided by Dr. Ken Rock, Dana Farber Cancer Institute, Boston, MA) were used as antigen presenting cells (5 x 10⁴ cells/well). Biotinylated anti-IFN γ antibody (Mabtech) and avidin-

alkaline phosphatase (Southern Biotech, Birmingham, AL) were added, and spots visualized with Vector Blue Alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA). Spots were counted using a CTL plate reader and Immunospot software (Cellular Technologies, Shaker Heights, OH).

Adoptive transfers

CD4⁺ T cells from peripheral lymph nodes were negatively enriched (R&D, Minneapolis, MN) and sorted (FACS Aria, BD Bioscience) to obtain CD44^{lo} (naïve) CD4⁺ cells ($\geq 95\%$ purity). Five to seven offspring from TCDD or vehicle treated dams were pooled to obtain CD44^{lo}CD4⁺ T cells to transfer. CD44^{lo}CD4⁺ cells (5×10^5) were diluted in phosphate buffered saline and transferred intravenously (200 μ l) into recipients (five to ten recipients per experiment). For dual transfers, CD44^{lo}CD4⁺ cells from each group were combined and co-transferred (i.e., recipients received 2.5×10^5 from each donor; 200 μ l total volume). At 36 hr post transfer, cells from blood, peripheral lymph nodes, and spleen were harvested from a subset of recipients, while the remainder was infected with IAV.

Statistical analysis

The dam is defined as the statistical unit for all experiments; thus, offspring in each treatment group and at each point in time relative to infection are from a different treated dam. Data were analyzed using JMP software (SAS Software, Cary, NC). Differences between two groups at a single point in time were evaluated using a Student's *t*-test. A one-way or two-way analysis of variance, followed by a Tukey multiple comparisons *post hoc* test, was used to compare differences between offspring of treated dams over time or at various dilutions of cells or serum.

Differences were considered significant when p values were less than 0.05, and all data presented \pm SEM. All experiments have been independently repeated at least once with similar results.

Results

CD4⁺ T cell responses in developmentally exposed offspring

Respiratory infections trigger clonal expansion and differentiation of pathogen-specific T lymphocytes; a process that largely takes place in the secondary lymphoid organs that drain the respiratory tract, such as the mediastinal lymph nodes (MLN). Prior to infection, the number of CD4⁺ T cells in the MLN was not different in naïve mice (day 0 post infection) developmentally exposed to vehicle or TCDD (Figure 1A). However, after infection there was a significant reduction in the number, but not the percentage, of CD4⁺ T cells in MLN of mice that were developmentally exposed to TCDD (Figure 1A,B). This reduction persisted until nine days after infection. There was also a decrease in number, not percentage, of CD44^{hi}CD62L^{lo}CD4⁺ cells, indicating fewer activated effector CD4⁺ T cells (Figure 1C,D). This decrease in effector CD4⁺ T cells is due to the reduction in the total number of CD4⁺ T cells (Figure 1B). Additionally, the frequency of CD4⁺ T cells specific for a dominant IAV epitope (a viral nucleoprotein-derived peptide, NP₃₁₁₋₃₂₅) was reduced by 50% in infected offspring of TCDD-treated dams (Figure 1E, F). Using *AhR*^{+/-} dams, we confirmed that the presence of the AhR in the offspring is required in order for maternal treatment with TCDD to alter the CD4⁺ T cell response to infection (Figure 1G). Only infected *AhR*^{+/+} offspring from TCDD-treated dams have a reduced number of effector CD4⁺ T cells, whereas *AhR*^{-/-} littermates from TCDD-treated dams do not. Male and female adult offspring didn't differ in their response to IAV infection, as both exhibit the same changes after TCDD exposure during development (Vorderstrasse et al. 2004).

An important role of CD4⁺ T cells during infection is aiding in the formation of a robust virus-specific antibody response (Alam et al. 2014). Thus, we compared the impact of developmental exposure on CD4⁺ T cell-dependent and independent influenza-specific antibody levels, and CD4⁺ T cell-dependent B cell differentiation. Consistent with prior reports (Vorderstrasse et al. 2006), offspring of dams exposed to TCDD have significantly reduced virus-specific IgG2a levels compared to offspring of vehicle treated dams (CD4⁺ T cell-dependent; Figure 2A). In contrast, virus-specific IgM (CD4⁺ T cell-independent) levels were not different (Figure 2B). To further examine CD4⁺ T cell-dependent B cell responses, we determined the frequency of germinal center B cells and plasma cells in the MLN. The overall frequency of germinal center B cells (Figure 2C,D) and plasma cells (Figure 2E,F) was reduced by two- to three-fold in infected adult offspring of TCDD-treated dams compared to those of control-treated dams.

Upstream of T cell-dependent antibody production, infection initiates CD4⁺ T cell differentiation into several subpopulations of conventional effector cell subsets. Compared to infected offspring of control dams, there was a reduction in the frequency of all four conventional CD4⁺ T cell subsets. Specifically, there was a statistically significant decrease in the percentage of Th1 and Tfh cells (Figure 3A and Supplemental Material, Figure S1A, B), and the number of Th1, Tfh, Th17, and Th2 cells in the MLN of infected mice that were developmentally exposed to TCDD was reduced by approximately 50% (Figure 3B and Supplemental Material, Figure S1A-D). We further examined the functional capacity of Th1 cells by determining the frequency of IFN γ ⁺CD4⁺ T cells. Developmental exposure significantly reduced the number of IFN γ ⁺CD4⁺ T cells (Figure 3C,D), indicating that there were fewer Th1 cells, as defined using both phenotypic and functional makers.

Exposure of adult mice to AhR ligands increases the frequency of Tregs (Quintana and Sherr 2013); hence, we examined whether triggering the AhR during development has a similar effect. In the absence of infection, there was no discernable difference in the percentage or number of Tregs in adult offspring of vehicle and TCDD-treated dams (Figure 3E,F and Supplemental Material, Figure S1E). However, after infection there was an increase in the percentage of Tregs in developmentally exposed offspring (Figure 3G). Unlike conventional CD4⁺ T cell subsets, the number of Tregs did not decrease after developmental exposure. When all CD4 subsets are quantified from the same experiment, mice developmentally exposed to TCDD have a decrease in conventional CD4⁺ T cells, yet an increase in Tregs. Therefore, the ratio of Tregs:Th1, Tregs:Th17, and Tregs:Tfh cells was increased by developmental activation of the AhR (Figure 3H-J). Thus, a greater proportion of the total CD4⁺ T cell population is comprised of Tregs rather than conventional effector CD4⁺ T cells in infected offspring of TCDD-treated dams.

Developmental AhR activation directly alters CD4⁺ T cells

To determine whether AhR activation during development changes CD4⁺ T cells in an intrinsic or extrinsic fashion, we performed reciprocal adoptive transfer experiments. Naïve (CD44^{lo}) CD4⁺ T cells from offspring developmentally exposed to TCDD (CD90.2⁺CD45.2⁺) or vehicle (CD90.2⁺CD45.1⁺) were combined in a 1:1 ratio and transferred into unexposed congenic recipients (CD90.1⁺CD45.2⁺) to ascertain whether triggering AhR during development causes intrinsic changes in CD4⁺ T cells (Figure 4A). Congenic markers permit distinction of the two sets of donor-derived cells from each other and from recipient cells (see Supplemental Material, Figure S2A). Importantly, the distribution of transferred CD4⁺ T cells from the two donor pools was not different (see Supplemental Material, Figure S2B). In separate studies, we determined

that developmental AhR activation caused the same alterations in CD4⁺ T cell responsiveness to infection in all three congenic strains of mice (data not shown). On day nine post IAV infection (the height of the CD4⁺ T cell response), there was a significant reduction in the percentage and number of CD4⁺ T cells in the MLN that were derived from donors developmentally exposed to TCDD (Figure 4B, C). In addition, the percentage of transferred CD4⁺ T cells from donors developmentally exposed to TCDD that differentiated into Th1, Th17, or Th2 cells was reduced by approximately two-fold; yet there was no change in the percentage of Tregs (Figure 4D).

To examine potential extrinsic influences, we transferred naïve CD4⁺ T cells from unexposed CD45.1⁺ mice into congenic (CD45.2⁺) adult offspring of dams that were treated with vehicle or TCDD (Figure 4E). There were no differences in the transfer success rate or distribution of transferred cells into naïve recipients regardless of dam treatment (data not shown). Nine days after infection, there were fewer transferred CD4⁺ T cells in recipients that were developmentally exposed to TCDD compared to recipient offspring of vehicle-treated dams (Figure 4F,G). Yet, the percentage of conventional or regulatory effector CD4⁺ T cell subsets was the same regardless of the developmental exposure history of the recipients (Figure 4H). This is in direct contrast to observations when the donor cells were developmentally exposed and transferred to unexposed recipients (Figure 4D).

Discussion

The developmental basis of adult disease suggests that early life exposures alter health and contribute to disease later in life. The mechanism by which developmental exposures lead to persistent changes in immune function is unknown, yet modifications to immunologically based processes are known to occur (Winans et al. 2011). Circumstantial evidence suggests that

maternal exposure to AhR-binding chemicals impacts CD4⁺ T cell-dependent processes and pathologies (Gehrs and Smialowicz 1999; Luster et al. 1980; Mustafa et al. 2011; Thomas and Hinsdill 1979; Vorderstrasse et al. 2006). We specifically examined CD4⁺ T cells, and showed that early life AhR activation changed the proportion of functionally distinct CD4⁺ T cell subsets responding to infection. These durable changes resulted from intrinsic and extrinsic effects on CD4⁺ T cells, indicating that AhR-mediated events in multiple cell types likely contributed to the immunomodulatory effects of developmental exposure. Specifically, changes in the ability of CD4⁺ T cells to differentiate into conventional effector subsets were due to effects that are intrinsic to CD4⁺ T cells. In contrast, an increase in the proportion of Tregs was likely due to a combination of intrinsic and extrinsic influences, because in both adoptive transfer scenarios the skewed frequency of Tregs was lost. The requirement of both intrinsic and extrinsic consequences of developmental AhR activation on the increase in Tregs suggests that changes are required in both the CD4⁺ T cells and other cells of the organism. The overall expansion in CD4⁺ T cell number upon infection is likely also influenced by intrinsic and extrinsic factors, as this effect was retained in reciprocal transfers. These data suggest that while AhR activation during development influenced extrinsic signals that can diminish the total number of CD4⁺ T cells responding to infection, these CD4⁺ T cell-extrinsic events did not change the ability of the CD4⁺ T cells to differentiate.

A potential CD4⁺ T cell lineage intrinsic effect of developmental AhR activation is a modification in epigenetic regulatory machinery. Major epigenetic programming events occur during development, and variations in the epigenetic marks laid down can impinge on cellular function (Cantone and Fisher 2013). While epigenetic regulation in hematopoietic cells remains

poorly understood, epigenetic mechanisms influence CD4⁺ T cell development and function. For example, CD4⁺ T cell function is altered by changes in DNA methylation, histone modifications, and other manipulators of chromatin structure (Brand et al. 2012; Carson et al. 2010). Also, although examined in only a few studies, AhR activation alters the pattern of epigenetic marks in other model systems (Manikkam et al. 2012; Papoutsis et al. 2013; Singh et al. 2011). Thus, it is plausible that AhR activation during development changes epigenetic regulatory machinery in CD4⁺ T cells, leading to intrinsic differences when these cells respond to viral infection.

Potential extrinsic factors include other immune cells, such as antigen presenting cells (APCs) and B cells, which interact bi-directionally with CD4⁺ T cells to shape the response to infection (Alam et al. 2014; Smith et al. 2004). B cells and APCs express the AhR, and their function is modulated by AhR activation in adult animals (Jin et al. 2014; Quintana and Sherr 2013; Sulentic and Kaminski 2011). Non-hematopoietic cells are also important for CD4⁺ T cell development and function (Mebius 2003). While not examined in the context of developmental exposure, AhR ligands modulate immune function via direct effects on non-hematopoietic cells (Camacho et al. 2005; Jensen et al. 2003; Wheeler et al. 2013). Also, in the context of developmental exposure, TCDD alters pulmonary inflammation after infection via effects on extrinsic to immune cells (Hogaboam et al. 2008). Therefore, AhR-mediated changes in other leukocytes and non-hematopoietic cells may contribute to alterations in CD4⁺ T cell responses in developmentally exposed offspring.

It has long been known that developmental exposure to AhR ligands suppresses antibody responses in the offspring. However, much of the prior data was generated using higher maternal or perinatal doses that caused transient thymic atrophy or other signs of hematotoxicity (Faith

and Moore 1977; Thomas and Hinsdill 1979; Vos and Moore 1974). Other prior studies used maternal doses of TCDD only slightly higher than the present study, which decreased CD4⁺ T cell-dependent and independent antibody responses (Vorderstrasse et al. 2006). The present study used a lower, more environmentally relevant dose of TCDD, which reduced CD4⁺ T cell-dependent, but not independent, antibody responses to IAV. Also, we demonstrated that the frequency of Tfh and Th1 cells, key contributors to T cell-dependent antibody and antiviral responses, were correspondingly diminished in the present study. This suggests that CD4⁺ T cell functions may be more sensitive to perturbation by developmental exposure to AhR ligands than other immune cell types, such as B cells. Thus, this work has implications for thinking about how early life exposures affect immune function in the human population, where antibody responses are often the sole measurement. Although it is challenging to equate doses in animal models to human exposures, the maternal dose we administered did not cause overt toxicity, which is consistent with reports that exposed human populations present changes in immune function without obvious changes in immune organ cellularity. For instance, epidemiological data show that early life exposures to AhR ligands correlate with decreases in vaccine specific IgG levels in children, which require a robust CD4⁺ T cell response (Heilmann et al. 2010; Hochstenbach et al. 2012; Stolevik et al. 2013). Therefore, it may be important to isolate and more closely examine CD4⁺ T cell responses in future studies with human cohorts.

The work reported herein focuses on the response of CD4⁺ T cells to primary acute infection with a common human pathogen. Yet, the implications extend beyond IAV. For instance, *Toxoplasma gondii*, *Streptococcus pneumoniae*, and *Mycobacterium tuberculosis* require CD4⁺ T cells for pathogen clearance (Cohen et al. 2013; Leveton et al. 1989; Malley et al. 2005). Also,

pathogens that have not been major burdens due to successful immunization strategies may reemerge if vaccine efficacy is reduced by exposures experienced during development. Furthermore, many autoimmune and allergic diseases are CD4⁺ T cell dependent, suggesting that these diseases may be altered by developmental exposures to AhR ligands. Support for this idea comes from studies showing that developmental exposure to TCDD enhances autoimmune symptoms later in life (Mustafa et al. 2011). Therefore, in addition to revealing that developmental activation of the AhR directly impinged upon the function of CD4⁺ T cells in the context of infection, the present study suggests that AhR-mediated events in CD4⁺ T cells may be an important underlying factor in other infectious and immune-mediated diseases.

Conclusions

Developmental exposure to AhR ligands caused lasting changes in CD4⁺ T cell responses to infection due to direct effects on the CD4⁺ T cell lineage. These results have global implications, as CD4⁺ T cells are critical in appropriate immune responses to many pathogens and vaccines.

References

- Alam S, Knowlden ZA, Sangster MY, Sant AJ. 2014. CD4 T cell help is limiting and selective during the primary B cell response to influenza virus infection. *J Virol* 88(1):314-324.
- Almansa R, Socias L, Ramirez P, Martin-Loeches I, Valles J, Loza A, et al. 2011. Imbalanced pro- and anti-Th17 responses (IL-17/granulocyte colony-stimulating factor) predict fatal outcome in 2009 pandemic influenza. *Crit Care* 15(5):448.
- Boekelheide K, Blumberg B, Chapin RE, Cote I, Graziano JH, Janesick A, et al. 2012. Predicting later-life outcomes of early-life exposures. *Environ Health Perspect* 120(10):1353-1361.
- Boyden AW, Legge KL, Waldschmidt TJ. 2012. Pulmonary infection with influenza A virus induces site-specific germinal center and T follicular helper cell responses. *PLoS One* 7(7):e40733.
- Brand S, Kesper DA, Teich R, Kilic-Niebergall E, Pinkenburg O, Bothur E, et al. 2012. DNA methylation of TH1/TH2 cytokine genes affects sensitization and progress of experimental asthma. *J Allergy Clin Immunol* 129(6):1602-1610 e1606.
- Camacho IA, Singh N, Hegde VL, Nagarkatti M, Nagarkatti PS. 2005. Treatment of mice with 2,3,7,8-tetrachlorodibenzo-p-dioxin leads to aryl hydrocarbon receptor-dependent nuclear translocation of NF-kappaB and expression of Fas ligand in thymic stromal cells and consequent apoptosis in T cells. *J Immunol* 175(1):90-103.
- Cantone I, Fisher AG. 2013. Epigenetic programming and reprogramming during development. *Nat Struct Mol Biol* 20(3):282-289.
- Carson WF, Cavassani KA, Ito T, Schaller M, Ishii M, Dou Y, et al. 2010. Impaired CD4+ T-cell proliferation and effector function correlates with repressive histone methylation events in a mouse model of severe sepsis. *Eur J Immunol* 40(4):998-1010.
- Chapman TJ, Castrucci MR, Padrick RC, Bradley LM, Topham DJ. 2005. Antigen-specific and non-specific CD4+ T cell recruitment and proliferation during influenza infection. *Virology* 340(2):296-306.
- Cohen SB, Maurer KJ, Egan CE, Oghumu S, Satoskar AR, Denkers EY. 2013. CXCR3-Dependent CD4(+) T Cells Are Required to Activate Inflammatory Monocytes for Defense against Intestinal Infection. *PLoS Pathog* 9(10):e1003706.

- Dallaire F, Dewailly E, Vezina C, Muckle G, Weber JP, Bruneau S, et al. 2006. Effect of prenatal exposure to polychlorinated biphenyls on incidence of acute respiratory infections in preschool Inuit children. *Environ Health Perspect* 114(8):1301-1305.
- Faith RE, Moore JA. 1977. Impairment of thymus-dependent immune functions by exposure of the developing immune system to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *J Toxicol Environ Health* 3(3):451-464.
- Fontenot JD, Rudensky AY. 2005. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol* 6(4):331-337.
- Gehrs BC, Smialowicz RJ. 1999. Persistent suppression of delayed-type hypersensitivity in adult F344 rats after perinatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicology* 134(1):79-88.
- Glynn A, Thuvander A, Aune M, Johannisson A, Darnerud PO, Ronquist G, et al. 2008. Immune cell counts and risks of respiratory infections among infants exposed pre- and postnatally to organochlorine compounds: a prospective study. *Environ Health* 7:62. DOI: 10.1186/1476-1069X-1187-1162.
- Heilmann C, Budtz-Jorgensen E, Nielsen F, Heinzow B, Weihe P, Grandjean P. 2010. Serum concentrations of antibodies against vaccine toxoids in children exposed perinatally to immunotoxicants. *Environ Health Perspect* 118(10):1434-1438.
- Hochstenbach K, van Leeuwen DM, Gmuender H, Gottschalk RW, Stolevik SB, Nygaard UC, et al. 2012. Toxicogenomic profiles in relation to maternal immunotoxic exposure and immune functionality in newborns. *Toxicol Sci* 129(2):315-324.
- Hogaboam JP, Moore AJ, Lawrence BP. 2008. The aryl hydrocarbon receptor affects distinct tissue compartments during ontogeny of the immune system. *Toxicol Sci* 102(1):160-170.
- Institute of Medicine. 2003. *Dioxins and Dioxin-like Compounds in the Food Supply*. Washington, D.C: National Academy of Sciences.
- Jensen BA, Leeman RJ, Schlezinger JJ, Sherr DH. 2003. Aryl hydrocarbon receptor (AhR) agonists suppress interleukin-6 expression by bone marrow stromal cells: an immunotoxicology study. *Environ Health* 2(1):16. DOI: 10.1186/1476-1069X-1182-1116.

- Jin GB, Winans B, Martin KC, Lawrence BP. 2014. New insights into the role of the aryl hydrocarbon receptor in the function of CD11c cells during respiratory viral infection. *Eur J Immunol*:DOI: 10.1002/eji.201343980.
- Leveton C, Barnass S, Champion B, Lucas S, De Souza B, Nicol M, et al. 1989. T-cell-mediated protection of mice against virulent *Mycobacterium tuberculosis*. *Infect Immun* 57(2):390-395.
- Luster MI, Boorman GA, Dean JH, Harris MW, Luebke RW, Padarathsingh ML, et al. 1980. Examination of bone marrow, immunologic parameters and host susceptibility following pre- and postnatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Int J Immunopharmacol* 2(4):301-310.
- Malley R, Trzcinski K, Srivastava A, Thompson CM, Anderson PW, Lipsitch M. 2005. CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. *Proc Natl Acad Sci U S A* 102(13):4848-4853.
- Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK. 2012. Dioxin (TCDD) induces epigenetic transgenerational inheritance of adult onset disease and sperm epimutations. *PLoS One* 7(9):e46249.
- McKinstry KK, Strutt TM, Buck A, Curtis JD, Dibble JP, Huston G, et al. 2009. IL-10 deficiency unleashes an influenza-specific Th17 response and enhances survival against high-dose challenge. *J Immunol* 182(12):7353-7363.
- Mebius RE. 2003. Organogenesis of lymphoid tissues. *Nat Rev Immunol* 3(4):292-303.
- Mustafa A, Holladay SD, Witonsky S, Sponenberg DP, Karpuzoglu E, Gogal RM, Jr. 2011. A single mid-gestation exposure to TCDD yields a postnatal autoimmune signature, differing by sex, in early geriatric C57BL/6 mice. *Toxicology* 290(2-3):156-168.
- Papoutsis AJ, Selmin OI, Borg JL, Romagnolo DF. 2013. Gestational exposure to the AhR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin induces BRCA-1 promoter hypermethylation and reduces BRCA-1 expression in mammary tissue of rat offspring: Preventive effects of resveratrol. *Mol Carcinog*:10.1002/mc.22095.
- Quintana FJ, Sherr DH. 2013. Aryl hydrocarbon receptor control of adaptive immunity. *Pharmacol Rev* 65(4):1148-1161.

- Schechter A, Cramer P, Boggess K, Stanley J, Papke O, Olson J, et al. 2001. Intake of dioxins and related compounds from food in the U.S. population. *J Toxicol Environ Health A* 63(1):1-18.
- Singh NP, Singh UP, Singh B, Price RL, Nagarkatti M, Nagarkatti PS. 2011. Activation of aryl hydrocarbon receptor (AhR) leads to reciprocal epigenetic regulation of FoxP3 and IL-17 expression and amelioration of experimental colitis. *PLoS One* 6(8):e23522.
- Smith CM, Wilson NS, Waithman J, Villadangos JA, Carbone FR, Heath WR, et al. 2004. Cognate CD4(+) T cell licensing of dendritic cells in CD8(+) T cell immunity. *Nat Immunol* 5(11):1143-1148.
- Stolevik SB, Nygaard UC, Namork E, Haugen M, Meltzer HM, Alexander J, et al. 2013. Prenatal exposure to polychlorinated biphenyls and dioxins from the maternal diet may be associated with immunosuppressive effects that persist into early childhood. *Food Chem Toxicol* 51:165-172.
- Sugita-Konishi Y, Kobayashi K, Naito H, Miura K, Suzuki Y. 2003. Effect of lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin on the susceptibility to *Listeria* infection. *Biosci Biotechnol Biochem* 67(1):89-93.
- Sulentic CE, Kaminski NE. 2011. The long winding road toward understanding the molecular mechanisms for B-cell suppression by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Sci* 120 Suppl 1:S171-191.
- Teske S, Bohn AA, Regal JF, Neumiller JJ, Lawrence BP. 2005. Activation of the aryl hydrocarbon receptor increases pulmonary neutrophilia and diminishes host resistance to influenza A virus. *Am J Physiol Lung Cell Mol Physiol* 289(1):L111-124.
- Thomas PT, Hinsdill RD. 1979. The effect of perinatal exposure to tetrachlorodibenzo-p-dioxin on the immune response of young mice. *Drug Chem Toxicol* 2(1-2):77-98.
- Vorderstrasse BA, Cundiff JA, Lawrence BP. 2006. A dose-response study of the effects of prenatal and lactational exposure to TCDD on the immune response to influenza a virus. *J Toxicol Environ Health A* 69(6):445-463.

- Vorderstrasse BA, Cundiff JA, Lawrence BP. 2004. Developmental exposure to the potent aryl hydrocarbon receptor agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin Impairs the cell-mediated immune response to infection with influenza A virus, but enhances elements of innate immunity. *J Immunotoxicol* 1(2):103-112.
- Vos JG, Moore JA. 1974. Suppression of cellular immunity in rats and mice by maternal treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Int Arch Allergy Appl Immunol* 47(5):777-794.
- Warren TK, Mitchell KA, Lawrence BP. 2000. 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* 56(1):114-123.
- Wheeler JL, Martin KC, Lawrence BP. 2013. Novel cellular targets of AhR underlie alterations in neutrophilic inflammation and inducible nitric oxide synthase expression during influenza virus infection. *J Immunol* 190(2):659-668.
- Winans B, Humble MC, Lawrence BP. 2011. Environmental toxicants and the developing immune system: a missing link in the global battle against infectious disease? *Reprod Toxicol* 31(3):327-336.
- Yamane H, Paul WE. 2013. Early signaling events that underlie fate decisions of naive CD4(+) T cells toward distinct T-helper cell subsets. *Immunol Rev* 252(1):12-23.

Figure legends

Figure 1. Developmental activation of the AhR diminishes the virus-specific CD4⁺ T cell response to infection. Adult offspring developmentally exposed to vehicle (V) or TCDD (T) were infected (i.n.) with IAV, and MLN cells were stained for flow cytometry. The average (A) percentage and (B) number of CD4⁺ T cells over time. The average (C) percentage and (D) number of effector (CD44^{hi}CD62L^{lo}) CD4⁺ T cells over time. Representative plots (A, C) are from day 3 and 9 post-infection, respectively. The average (E) percentage and (F) number of I-A^bNP₃₁₁₋₃₂₅⁺CD4⁺ T cells (day 9). (G) The number of CD44^{hi}CD62L^{lo}CD4⁺ T cells in AhR^{+/+} and AhR^{-/-} offspring. Five to six female offspring from separate dams were used at each point in time. * p ≤ 0.05.

Figure 2. The virus-specific, class-switched antibody response to IAV is reduced by activation of the AhR during development. Mice were developmentally exposed and infected as adults as in Figure 1. IAV-specific (A) IgG2a and (B) IgM levels in serially diluted serum were determined on day 9 of infection. Serum from naïve mice served as the control. The percentage and number of germinal center B cells (C, D, B220⁺CD95⁺GL-7⁺) and plasma cells (D, E, CD138⁺B220^{int}). Four to five female mice from separate dams were used for each group. * p ≤ 0.05.

Figure 3. AhR activation during development alters CD4⁺ T cell subsets after IAV infection. Mice were developmentally exposed to vehicle (V) or TCDD (T) and infected as adults. Nine days post infection, the (A) percentage and (B) number of conventional CD4⁺ T cell subsets were enumerated as follows: Th1 cells, TBet⁺CD4⁺; Th2 cells, GATA3⁺CD4⁺; Th17 cells, RORγt⁺CD4⁺; and Tfh cells, CD44^{hi}PD-1⁺CXCR5⁺CD4⁺ cells (see Supplemental Material, Figure S1). IFNγ⁺CD4⁺T cells enumerated by ELISPOST assay: (C) representative wells and (D)

average number of IFN γ ⁺CD4⁺ T cells/MLN. The (E) percentage and (F) number of Tregs prior to infection. The (G) percentage of Tregs, and the ratio of (H) Treg:Th1, (I) Treg:Th17, and (J) Treg:Tfh cells in the MLN 9 days after infection. Five to six female offspring from separate dams were used for each experiment; * $p \leq 0.05$.

Figure 4. AhR activation during development leads to intrinsic changes in CD4⁺ T cell responsiveness. (A) Dual adoptive transfer: Naïve (CD44^{lo}) CD4⁺ T cells (5×10^5) from offspring of dams treated with vehicle (V) or TCDD (T) were transferred (1:1 ratio; 2.5×10^5 cells/treatment) into unexposed congenic recipients. Recipients were infected. Nine days later the average (B) percentage and (C) number of transferred CD90.2⁺CD4⁺ T cells from each donor was determined. (D) The percentage of CD4⁺ T cell subsets derived from each donor. (E) Single adoptive transfer: Naïve CD4⁺ T cells (5×10^5) from unexposed donors were transferred into congenic adult recipients that were developmentally exposed to vehicle (V) or TCDD (T). Recipients were infected with IAV. The average (F) percentage and (G) number of transferred CD4⁺ T cells, and (H) percentage of effector CD4⁺ T cell subsets derived from each donor on day 9 post-infection. Five to ten recipients were used. *, $p \leq 0.05$; ns denotes no significant difference ($p > 0.05$).

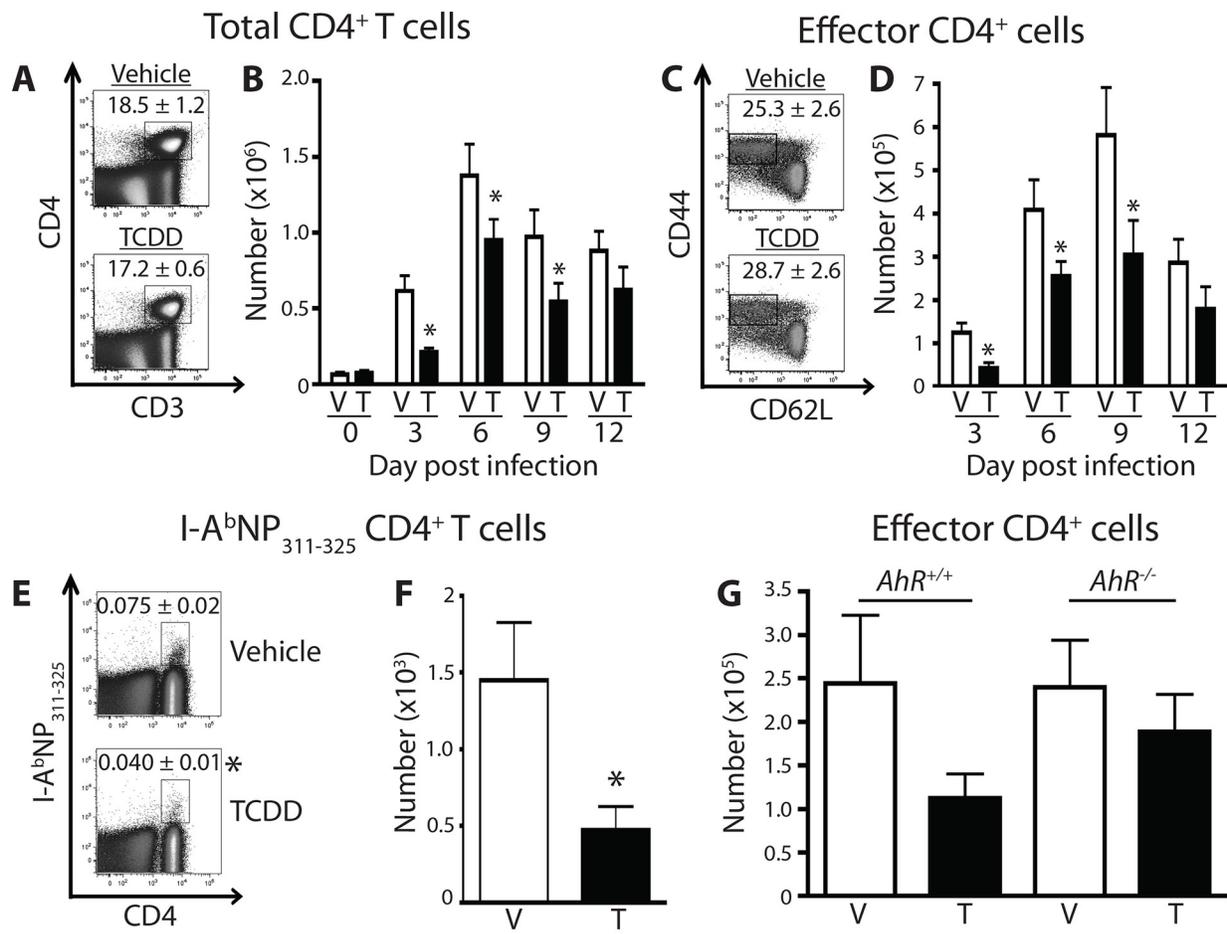


Figure 1

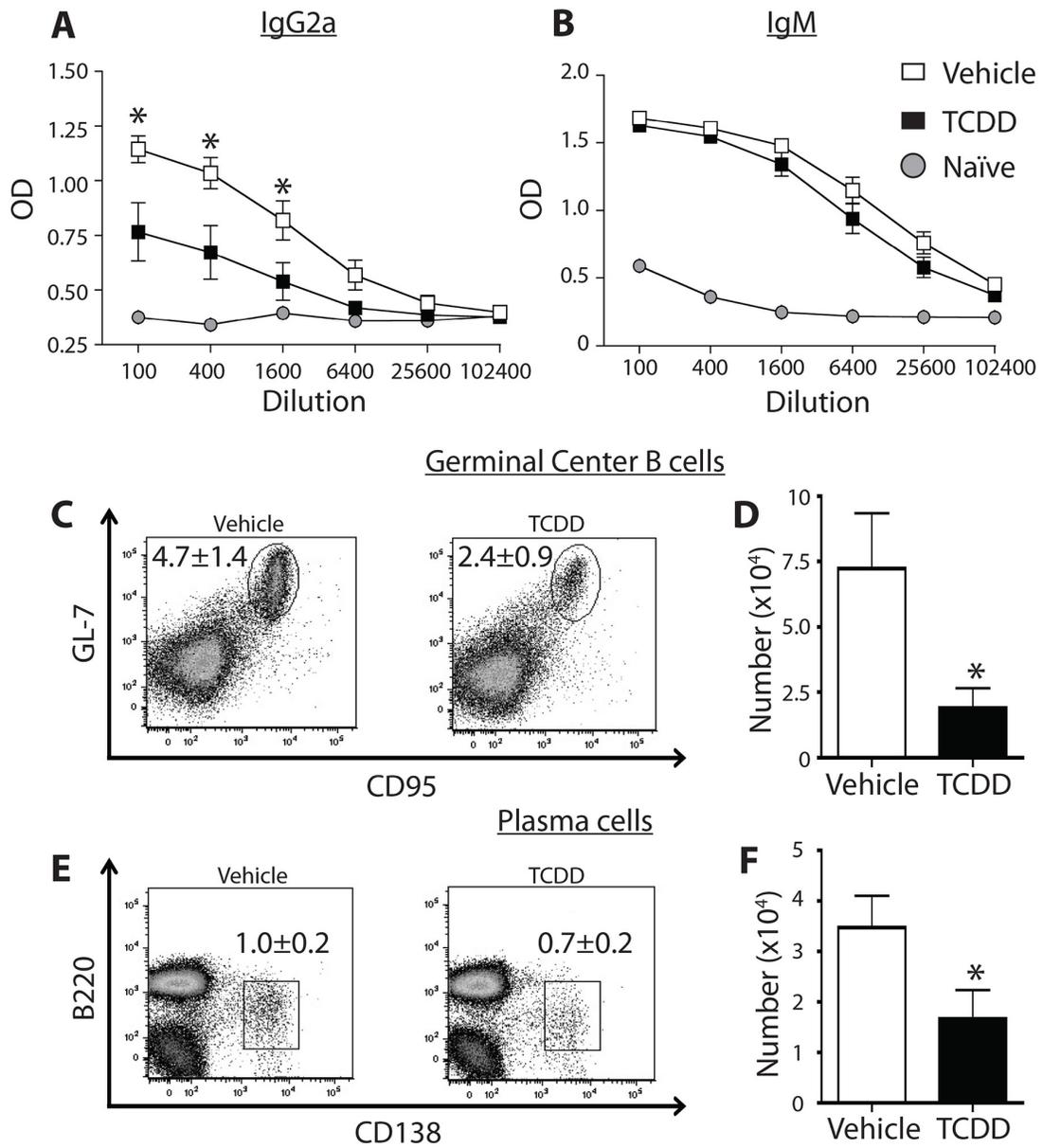
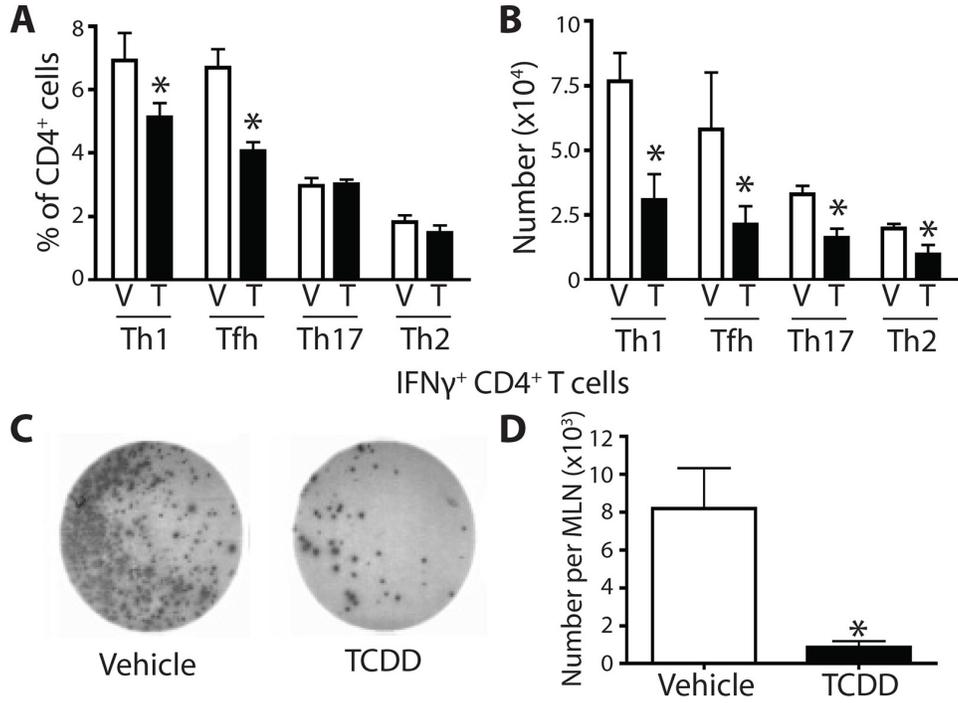


Figure 2

Conventional CD4⁺ T cell subsets



Regulatory CD4⁺ T cells

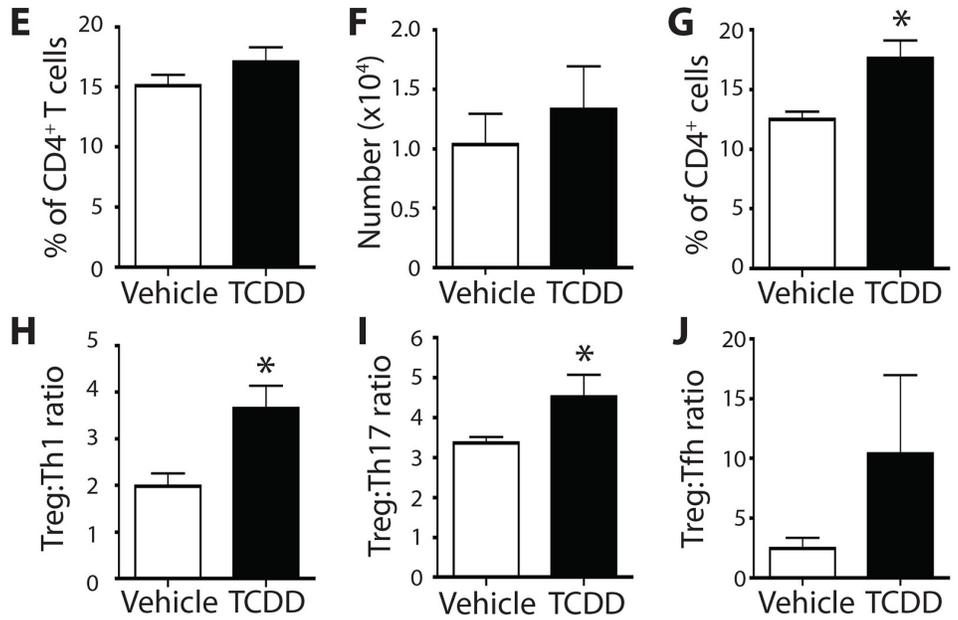


Figure 3

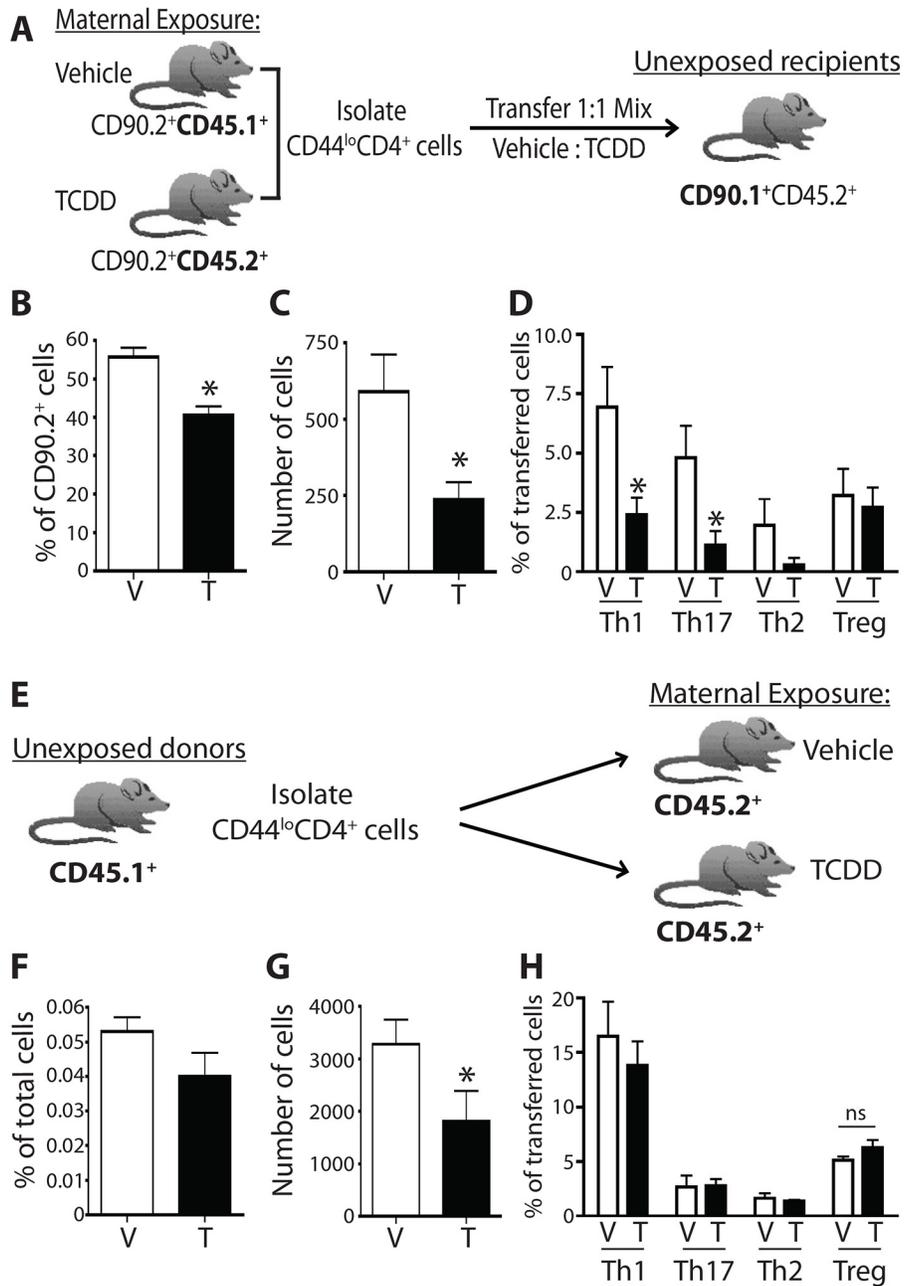


Figure 4