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Sebastian Ramos
University of Northern Colorado, ramo7503@bears.unco.edu

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Sebastian Ramos
Mentor: Gregory DeKrey, Ph.D., Biological Sciences

Abstract: Activated aryl hydrocarbon receptor with ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alters immune responses, including immunoglobulin A (IgA). Manipulation of antibody production via this ligand-activated receptor may provide a novel approach to enhance vaccination in the intestines. The present study aimed to explain the mechanism responsible for increased levels of fecal IgA in TCDD-treated mice. We hypothesized that TCDD enhances migration of B cells from non-intestinal tissue to the intestines. Here we predicted that β7-integrin, an essential protein for B cell migration into the intestines, would be upregulated by TCDD exposure on B cells. Female C57BL/6 mice were treated with a single dose of peanut oil (control) or TCDD at 40 μg/kg by gavage. After 1 week, splenic leukocytes were isolated and analyzed by flow cytometry. B cells in TCDD-treated mice did not express significantly higher β7-integrin levels than controls, although a significant increase of β7 integrin expression was observed on T cells. These results do not support our hypothesis; yet suggest that elevated β7 integrin expression allowed for preferential migration of B cells out of the spleen and into the intestines before analysis. In the future, we plan to address this possibility by examining β7 expression on B cells in the intestines.

Keywords: *TCDD, IgA, aryl hydrocarbon receptor, β7-integrin*

Mucosal immunity plays an important role against pathogens and allergies. During mucosal infections, B cells go through a differentiation process that allows them to produce antibodies with high specificity and affinity for the infectious agents. IgA is one of five types of antibodies produced by B cells and it is found mainly in mucosal tissues such as the digestive tract, genital tract, and respiratory tract. IgA is linked to regulating commensal microbes in the gut, the risk of developing allergies, and defenses against pathogens and toxins (Cerutti, 2008). In the last three decades, studies on the aryl hydrocarbon receptor (AhR) have demonstrated altered B cell function in animals exposed to AhR-activating chemicals. However, limited research has focused on how AhR activation can affect IgA-expressing B cells. That is the focus of this study.

The significance of the broader work is directed in hopes to manipulate humoral responses, such as IgA secretion in mucosal membranes, via this ligand-induced AhR to potentially develop vaccines containing compounds that could maximize the isotype presence in areas of the body. Manipulation of antibody production may be the novel approach to prevent diseases that occur through vocal and nasal airways, lining of the rectus, or genital ducts, including but not limited to HIV (Su, 2014).

Significance of IgA

IgA acts as the first line of defense against pathogens at mucosal membranes in the body of mammals and is the most abundant antibody produced (Cerutti, 2008). B cells secrete IgA at mucosal membranes throughout the body to protect humans against food-, water-, and airborne pathogens because the gut and respiratory tract are the major point of contact with toxins and pathogens (Mantis, Rol, & Corthesy, 2011). In the intestines, this particular antibody is mostly produced by B cells in the lamina propria and secreted into the lumen of the intestines where it can also regulate gut microbiota populations and influence our risk of developing allergies (Wood, 2006).

Sufficient production of IgA is essential for protection against some mucosal-transmitted microbes; evidence indicates that increased specific IgA antibody production protects the body against *Helicobacter pylori* infection and suggests that lower levels of IgA allow pathogens such as *H. pylori* to colonize the gut with ease (Srivastava et al., 2013). In a study aiming to demonstrate the importance of IgA in mucosal

membranes of the gut, Uren et.al. (2005) found that pathogens such as *Vibrio cholerae* and influenza virus, localized mainly in the mucosal areas of the body, seemed to affect body weight and health recovery in IgA knockout mice more negatively than those that had IgA-secreting B cells. Data suggests that the increment of IgA levels by immunization can prevent infections in local mucosal areas. For instance, high levels of fecal anti-cholera toxin B IgA in mice due to immunization prevented weight loss and lesser symptoms than those that did not show high levels of fecal IgA from lack of immunization (Price et al., 2013).

The role that IgA plays against the human immunodeficiency virus (HIV) needs further studying, but sparse research shows the proprieties of IgA as a mucosal barrier. In a study done by Su and Moog (2014), IgA levels increased in HIV^- people who interacted with HIV^+ patients, especially in saliva and the genital tract. The implications of this can lead to the formation of protective ways to prevent infection against HIV. However, mechanisms are yet poorly understood.

AhR-Mediated Biology

The AhR is a ligand-dependent transcription factor that is known to regulate the expression of genes important for immune function, metabolism, cellular homeostasis, and others (Nebert, Puga, & Vasiliou, 1993). The AhR is found in most nucleated cells of vertebrates, including mammals (Okey, 2007). For 30 years, this receptor has been associated with mediating chemical toxicity and environmental contaminants (Stevens, Mezrich, & Bradfield, 2009). In the last decade, longstanding toxicology studies on 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) have demonstrated an additional role for this protein in immunology (Stevens, Mezrich, & Bradfield, 2009).

2,3,7,8 Tetrachlorodibenzo-p-dioxin

A number of different endogenous and exogenous ligands are known to activate the AhR, specifically polycyclic aromatic hydrocarbons (PAHs) (Denison & Nagy, 2003). However, TCDD has demonstrated to have the highest binding affinity out of all compounds related to AhR and a longer half-life (7-10 days) (Stejskalova et al., 2011). Much of our understanding regarding this receptor has been gleaned from the use of TCDD as it serves as a model to understand the effects of AhR in the immune system.

TCDD is a toxic halogenated aromatic hydrocarbon that forms as a by-product or contaminant of materials such as 2, 4, 5-trichlorophenol (TCP) which is used as an herbicide (Hay, 1979). The properties of TCDD limit its risks to direct contact because it is not volatile. The average U.S. resident has measureable levels of TCDD in their blood (up to 30 parts per trillion) that come from common foods sold in U.S. markets (Schecter et. al., 2001); no study has shown that this level of TCDD causes adverse effects in humans. However, high TCDD levels (over 1000-fold) in the skin clearly lead to adverse health conditions such as chloracne (a severe form of acne; Forrester et. al., 2014). Indication that intermediate levels of TCDD can cause disease in humans is less convincing because of its weak exposure-effect relationship. Due to the toxicity of TCDD, evidence that suggests changes in immune function in humans is scarce and poorly understood. Yet, there is ample and extensive evidence that TCDD exposure alters immune function, developmental abnormalities, metabolic disorders, and increases risk of tumor growth in mice (Baccarelli et al., 2004). In addition to altering the immune responses, lymphocytes seem to be particularly sensitive to TCDD exposure fluctuating their response against pathogens (Lawrence & Vorderstrasse, 2013). TCDD can dysregulate B cell differentiation, which causes suppression of antibody secretion in mice (Sulentic, Holsapple, & Kaminski, 1998).

AhR-Dependent Immune Function

In regards to immunology, the AhR is a ligand-dependent transcription factor that regulates the development and function of the innate and adaptive immune systems in various

ways (Zhou, 2016). Activation of the AhR has the ability to mediate immunological responses against pathogens with some responses being suppressed while others augmented. Suppressed immunity is related to the reduced ability to appropriately defend against diseases or respond to vaccination. Augmented immunity is related to enhanced inflammatory responses or dysregulation of self-tolerance mechanisms. For example, one study showed that numerous cytokines, including IL-6, IL-12, and TNF-α levels, were reduced upon activation of the AhR, though fecal IgA levels were increased (Benson & Shepherd, 2010). Low cytokine levels reduce inflammation and allows animals to recover faster. Studies like this are significant to our goal because animals treated with TCDD showed markedly reduced severity of autoimmune disease such as Cronh's disease, a chronic gastrointestinal inflammation.

This ligand-mediated activation is most potent in leukocytes comprising the adaptive immune system, meaning that a series of signaling pathways coordinate changes in the responses of several adaptive leukocytes (Sulentic, Holsapple, & Kaminski, 1998). Among the most widely reported impacts of AhR activation is suppression of IgM and IgG antibody levels (Vorderstrasse et al., 2003). However, the influence of activated AhR on IgA antibody levels is not very well understood.

Studies on humans and laboratory animals suggest that exposure to AhR-activating compounds can alter IgA levels in blood and feces. For instance, a study done on 200 adolescents in Belgium showed that lifetime exposure to TCDD increased IgA levels in serum (Van Den Heuvel et al., 2002). Oral exposure in mice with TCDD suppressed recruitment of $CD8^+$ lymphocytes to the lungs, including decreased levels of interleukin-12 and decreased antibody production in the lungs. In addition to those findings, there was a four-fold increase of IgA in plasma after TCDD exposure in C57BL/6 mice (Warren et al., 2000). Similar to those findings, TCDD exposure affected the transport of IgA to the liver, which showed an increase of serum IgA (Moran et al., 1986). Studies by Ishikawa (2009) and Kinoshita et al. (2006) found decreased fecal IgA levels in rodents within the first 3 weeks after exposure, which suggests a reduced resistance to food- or water-borne pathogens and toxins. Limitations from the previously mentioned studies indicate that circadian rhythm, TCDD dosage, time, concentration and incubation time, and type of species, may alter the results.

A handful of studies have looked at the effects of antibody levels upon treatment with TCDD. However, a clear consensus understanding regarding the mechanisms underlying AhR regulation of IgA production by B cells has yet to be achieved. Some of these previously mentioned studies suggested that the increased or decreased levels of IgA in serum or feces is not due to increased/reduced antibody production by B cells, but rather the presence/absence of B cells in the tissue.

AhR-Mediated B Cell Migration

Migration of lymphocytes, such as B cells, to the intestinal mucosa requires dimerization of β7 with α4 integrin. A study in 2007, demonstrated the proposed idea of AhR-mediated migration in macrophages by exposure of PAHs. PAHs up-regulated β7-integrin expression up to 6-fold factor at mRNA level among other α and β subunits in both human and mouse primary macrophages (Monteiro et al., 2007). AhR involvement was analyzed by using an antagonist of AhR, which prevented the upregulation of β7-integrin in mRNA levels (Monteiro et al., 2007). These data suggest that TCDD (also an AhR agonist) may have the same effects on β7-integrin in B cells, which may lead to an increase rate of migration into the intestines.

The AhR acts as one of the body's environmental detector by binding to a wide variety of artificial and natural compounds found in our food. It is important to further understand the implications that the AhR has in our bodies, specifically in how our immune system responds to disease. If we can manipulate humoral responses, such as IgA secretion in mucosal membranes, via this ligand-induced receptor, we

can potentially develop vaccines containing compounds that could maximize the isotype presence in areas of the body. Manipulation of antibody production may be the novel approach to prevent diseases that occur at mucosal barriers such as oral and nasal airways, lining of the rectus or genital ducts, including but not limited to HIV (Su, 2014).

A previous study in our laboratory has shown increased levels in fecal IgA upon activation of the AhR with TCDD exposure in mice (Metten & DeKrey, 2015). Based on existing literature and these findings, the purpose of the present study is to explain the increased levels of fecal IgA after exposure with TCDD in mice. We hypothesize that TCDD exposure enhances migration of B cells from non-intestinal tissue to the lamina propria of the intestines. If this hypothesis is supported, we will expect to find an increase in β7-integrin expression on splenic B cells. In the present study, we 1) enumerated total number of leukocytes in the spleen and 2) compared levels of β7-integrin expression on B cells and T cells with their respective control groups using flow cytometry

METHOD

Animals

Female C57BL/6 mice, 6-8 weeks of age, were obtained from a breeding colony at the University of Northern Colorado animal facility. Animals were randomly assigned to one of two treatment groups and housed on a 12-hour day/night light cycle. The University of Northern Colorado Animal Care and Use Committee approved all protocols for the use of animals in keeping with current NIH guidelines for animal usage. Across two replicates, a total of twelve mice were used, with three mice in each treatment group (vehicle-treated controls and TCDD-treated at 40µg/kg) per experiment.

Procedure

Treatment

TCDD was obtained from AccuStandard, Inc. (Haven, CT). TCDD was dissolved in a vehicle solution of peanut oil. Vehicle-treated mice (control) were only given peanut oil vehicle by gavage. The TCDD-treated group at 40µg/kg were given a single dose by gavage. After TCDD exposure, mice were observed every morning for 1 week to ensure their health.

Cell Preparation

Mice were euthanized via CO_2 asphyxiation from 10 a.m. to 11 a.m. Each spleen was removed and placed in medium containing DMEM + 1mM HEPES + 5% heat-inactivated FBS. Cells were liberated from the spleen by rubbing the tissues between the frosted ends of microscope slides. Cells were transferred from the cell suspension into centrifuge tubes and sedimented at 1300 rpm for 5 min. Supernatant was discarded and cells were re-suspended in the same medium. Erythrocytes were lysed by a 10 second exposure to water. The remaining cells (leukocytes) were stained with trypan blue and enumerated under a microscope using a hemocytometer.

Flow Cytometry

β7-integrin levels on B cells and T cells were determined using flow cytometry technique. The following antibodies were used to stain cells: Fluorescein-isothiocyanate (FITC)-conjugated anti-mouse β7-integrin (clone 11-44-2) or IgG_1 isotype control (clone KLH/G1-2-2); Alexa Fluor 647 (AF647)-conjugated anti-mouse CD19 (clone RA3-6B2), -conjugated anti-mouse CD3 (clone RA3-6B2), or IgG_1 isotype control (clone RA3-6B2). Antibodies were purchased from Southern Biotech (Birminghan, AL) or BD PharMingen (San Jose, CA). Cells were analyzed on a BD Accuri C6 flow cytometer (San Jose, CA). A minimum of 18,000 cumulative events were collected per sample. A cell was considered positive (+) for expression of a marker if its fluorescence was greater than the fluorescence of the corresponding non-stained or isotype control-stained cell population. Lymphocytes (based on 90° light scatter versus FSC gating) were considered viable if they were negative for 7-AAD fluorescence. Only single cells (based on integrated versus peak FSC) were considered for analysis.

Statistical Analysis

The data were analyzed by two-way analysis of variance (ANOVA) and SigmaPlot statistical software. Sources of variation (effects) for the statistical model included the experiment number and the dose of TCDD. All-pairwise post-hoc t-tests were used to determine differences between means. Each experiment was replicated a minimum of two times to ensure consistency of results. Values of $p \leq 0.05$ were considered significant.

RESULTS

Statistical analysis was only based on TCDD source of variation and not on experiment nor experiment x TCDD interaction because the differences in mean values were not significant. In control mice, body weight ranged between 17-21 g and was not significantly changed in comparison to the TCDD-treated group. Thymuses were observed to be smaller in TCDD-treated mice (as reported by Metten & DeKrey, 2015), and was considered a positive indicator of TCDD exposure. When compared to controls, the total number of splenic leukocytes in TCDD-treated mice was significantly reduced ($F(1,8) = 12.254$, $p = 0.008$). In contrast, the percentages of B cells and T cells in spleens were not altered by TCDD exposure with approximately 60% being B cells and 40% being T cells (see fig. 2A). The percentage of CD3+ or CD19+ cells expressing β7-integrin was not significantly different between control and TCDD-treated mice. The mean fluorescence for β7-integrin on $CD19^+$ cells was not significantly different between control and TCDD-treated groups. The mean fluorescence for β7-integrin on $CD3^+$ TCDD-treated cells, however, showed a significant increase in comparison to control ($F(1,8) = 6.998$, $p = 0.029$).

Figure 1. Total number of splenic leukocyte at both 0 and 40 ug/kg of TCDD. After isolation of splenic leukocytes, cells were counted using hemocytometers. Viability was shown by using trypan blue. Data are the means ± SD of two combined trials; they are shown comparatively to the value of β7-integrin levels found in control cells. $*p < 0.05$.

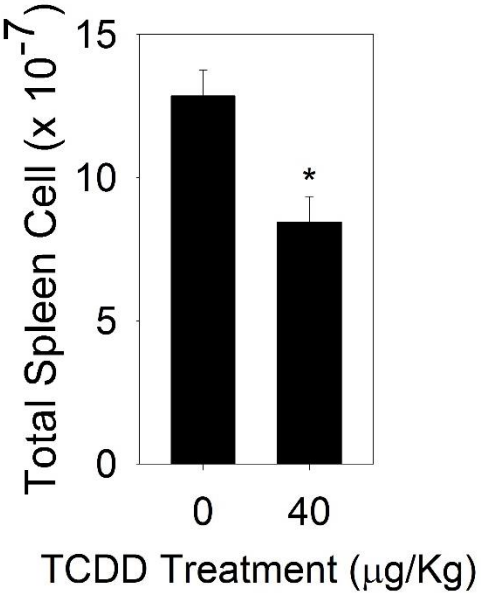
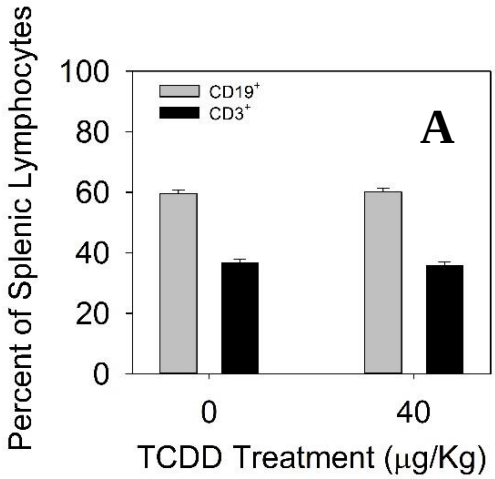
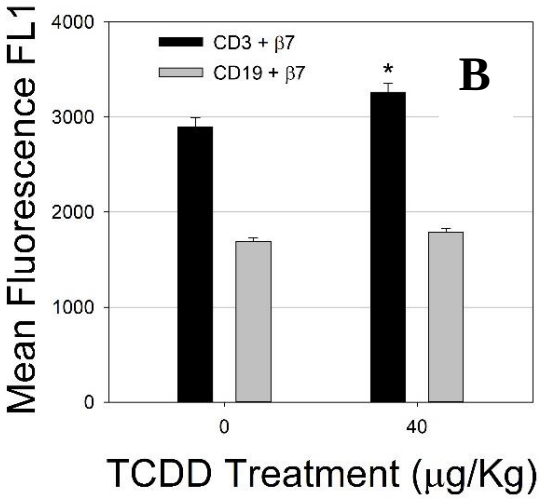


Figure 2. (A) Total percentage of $CD19^+$ and $CD3^+$ cells in the spleens at both zero, and 40 μg/Kg TCDD. (B) Mean fluorescence of FL1 depicts the mean expression of β7-integrin on both $CD19^+$ and $CD3^+$ at both 0 and 40 μg/Kg of TCDD. Splenic leukocytes were stained with AF647 (FL4) for anti-CD19 and anti-CD3 cells and FITC (FL1) for anti-β7-integrin. B7-integrin levels were then analyzed using flow cytometry. Data are the means ± SD of two combined trials; they are shown comparatively to the value of β7-integrin levels found in control cells. $*p < 0.05$.





DISCUSSION

In the present study, we were unable to demonstrate that TCDD exposure enhances B cells migration by increasing β7-integrin expression on B cells. These results suggest that elevation of fecal IgA levels in TCDD-treated mice may be caused by a different mechanism. On the other hand, data showed significantly increased average β7-integrin expression on $CD3^+$ cells from TCDD-treated mice indicating that TCDD exposure does impact β7-integrin expression on some cells. Interestingly, TCDD exposure significantly reduced total leukocyte numbers in spleens. Although a direct explanation for this change in leukocyte numbers is not provided by the results of this study, one possibility is that TCDD exposure on day 0 caused an increase of β7-integrin expression on B cells (and other cells) which allowed for B cell migration to the intestines prior to analysis on day 7. This particular information suggests that leukocytes did migrate out of the spleen into other tissues. We cannot say with certainty that these cells migrated into the intestines, but future studies may demonstrate so by directing their focus to collecting the spleens at different times or analyzing intestinal tissue instead. A high percent of interest should be placed by future researchers into understanding the effects of circadian rhythm on antibody levels if we wish to understand the implications of the AhR on IgA. The circadian rhythm has demonstrated to have an effect on antibody levels, which could aid in the understanding of B cell migration (Garrett & Gasiewicz, 2006). Our data suggest that B cells may have already migrated to the intestines or other tissues and that is why we did not obtain high levels of β7-integrin expression.

The unchanged percentage of $CD19^+$ and $CD3^+$ cells across control and treated groups (fig 2A) may suggest that there was no B cell migration at all. However, readers should see this figure as an indicator of the body's effort toward achieving homeostasis as it encounters xenobiotic compounds like TCDD. The figure does not account for migration because it is only showing percentages and not total numbers. The total number may change without necessarily yielding a different ratio.

Some limitations of the study include: (a) the time at which spleens were collected may have been a time when B cells had already migrated into the intestines; (b) the number of trials performed does not rule out the probability of random results; (c) we did not find a well-known range of β7-integrin levels in the literature for us to know how much was necessary to create a migration response; and (d) after we finished collecting our data, we received an email from BD that some of the antibodies we had purchased were being recalled for not working. In addition to that, TCDD and mice acting as models to study the AhR can be very valuable to understanding mechanisms and its effects in humans. However, it is necessary to understand the differences between mice and humans in regards to the AhR pathways, which are acknowledged by the authors but were not be investigated in this study.

The AhR acts as the body's environmental detector by binding to a wide variety of artificial and natural compounds found in places such as our own grocery stores. It is important to further understand the implications that the AhR has in our bodies, specifically in how our immune system responds to disease. More studies must be done to understand the mechanisms behind the AhR and IgA. Topics that are important to take into consideration for future studies are consolidating the period of time that takes TCDD

to cause effects in the body, the time of collection due to changes in antibody levels affected by the circadian rhythm of mice, and the usage of different ligands to exert different responses in mouse models. Our study takes the first steps towards understanding the mechanisms behind AhR-mediated migration of B cells into the intestines. AhR activation shows to be capable of mediating the migration of splenic leukocyte to other tissues of the body, which can explain altered levels of antibodies in the intestines such as IgA. However, more research must be done in order to accurately show the mechanism for increased levels of fecal IgA.

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