5-1-2020

Effects of TCDD on B Cell Populations in Various Anatomic Locations in EAE

Amye McDonald
Mississippi State University

Effect of TCDD on B Cell Populations in Various Anatomic Locations in EAE

By

Amye McDonald

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for an Honors Thesis in the Shackouls Honors College
Mississippi State, Mississippi
May 2020

Name: Amye McDonald

Date of Degree: May 1, 2020

Institution: Mississippi State University

Major Field: Microbiology

Title of Study: Effect of TCDD on B Cell Populations in Various Anatomic Locations in EAE

Pages in Study: 27

Undergraduate Honors Thesis

TCDD is an environmental toxin that has been well characterized to attenuate experimental autoimmune encephalomyelitis (EAE), a mouse model used to study multiple sclerosis. To evaluate the effect of TCDD on B cells in various anatomic locations in EAE, the expression of B cell markers, including CD19, B220, and CD5; the expression of IgG; and the expression of CD24 and CD38, which together signify a regulatory B cell, were evaluated in each location. In mice with EAE, TCDD increased CD19 and B220 expression and suppressed IgG expression in the spleen and increased the CD5+CD24+CD38+ population in the spinal cord, suggesting that TCDD's target organs at end-stage disease are spleen and spinal cord. These findings provide insight into the mechanism of immunotoxicity of TCDD and contribute to the understanding of how AhR ligands affect EAE, which could lead to development of a less toxic AhR compound for treatment of autoimmune disease.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Immunotoxicology. This seemingly big word, when broken down, essentially means the study of toxic substances and their effects on the immune system. Toxic substances are prevalent throughout the world and in a variety of settings, and the immune system is crucial for human survival. Often, toxins target the immune system in order to exert their harmful effects on the human body (Dean, 1994). Therefore, studying the ways in which immunology and toxicology overlap is crucial to not only preventing harmful health effects from toxins but also developing less toxic treatments for certain diseases. The studies described in the following pages fall into the category of immunotoxicology because they seek to evaluate the effects of a ubiquitous environmental toxin, 2,3,7,8-terachlorodibenzo-p-dioxin (TCDD) on the immune system using the mouse model of multiple sclerosis (MS), an autoimmune disease.

Although it is well-known for being a contaminant in the herbicide Agent Orange, TCDD is also an unintentional byproduct of many chemical and industrial processes, including the combustion of fossil fuels, the burning of waste, the production of pulp and paper, and the manufacture of some organic chemicals. Low levels of TCDD may be found in the air, soil, and food, including meat, fish, and dairy products. Therefore, it is likely that all humans have been exposed to TCDD at some point in their life. Because TCDD is the most toxic of all known dioxins, a specific group of environmental pollutants, studying its effects on human health is very important. These effects are vast and diverse; TCDD has been linked to cardiovascular

disease, liver abnormalities, skin deformities, and cancer. However, for the purpose of this paper, it is important to note that TCDD has been well characterized to be immunosuppressive (White and Birnbaum, 2009).

TCDD is also a prototypical aryl hydrocarbon receptor (AhR) ligand. AhR is a transcription factor located in the cytosol. When activated by the binding of a ligand, AhR moves to the nucleus where it regulates the expression of many genes. Studies have shown that activated AhR can integrate signals from the environment and other cell processes to modulate the immune response (Wheeler et al., 2017). Because TCDD is an AhR ligand, it is thought that TCDD exerts its immunosuppressive effects by binding and activating AhR, leading to a cascade of immune processes that reduce inflammation (Neavin et al., 2018).

The suppression of the immune system can be deleterious and lessen a person's ability to fight disease, but suppression may also be beneficial, specifically in the presence of autoimmune disease. One such disease is MS, an autoimmune-mediated disorder of the central nervous system. Although the exact cause of MS can vary, the disorder leads to the death of neurons and demyelination of nerves. This destruction of nerve tissue results in an inflammatory response in which lesions form in the CNS. These lesions then interfere with normal neuronal functions and can result in visual disturbances, fatigue, loss of muscle control and coordination, and paralysis, as well as emotional difficulties and thinking problems (Ghasemi et al., 2017).

To study MS, an animal model called experimental autoimmune encephalomyelitis (EAE) may be used (Constantinescu et al., 2011). In previous studies using the EAE model, TCDD has been shown to slow the onset of disease and lessen disease severity. Although TCDD attenuates EAE, it is still important to understand how it is exerting its immunosuppressive effects. Our previous studies have mainly examined TCDD's effect on T cell function, a

component of the specific immune response, in the spleens of diseased mice (Yang et al., 2016). The purpose of these studies was to assess TCDD's effect on B cells, which make up the humoral immune response. Comparing the effect of TCDD on specific B cell subsets within different anatomic locations, including the spleen, bone marrow, lymph nodes, and spinal cord provides a more holistic look at the ways in which TCDD attenuates EAE disease via immunosuppressive mechanisms in the nervous system.

Through these studies, we aimed to gain novel information about TCDD's effect in multiple tissues at end-stage disease. The effects on the spleen and spinal cord have been studied more in depth, so we sought to understand if TCDD was also having an effect in the bone marrow and lymph nodes. To evaluate the effects on the different tissues, we analyzed the expression of multiple B cell markers, including two that have not been studied extensively. Additionally, we evaluated the production of IgG, an antibody crucial to mounting an immune response, and the expression of a B cell regulatory population at end stage disease. Lastly, by conducting independent studies with both male and female mice, we sought to determine if TCDD had any sex-specific immunosuppressive mechanisms.

Overall, these studies aid in understanding the toxicity of TCDD and identifying its mechanism of immune suppression. These findings can then be used to help prevent the deleterious effects of environmental toxins and to potentially identify less toxic, AhR-binding compounds as treatments for autoimmune disease. Altogether, results from immunotoxicology studies like this one help to make progress in the realm of public and individual human health.

CHAPTER II

BACKGROUND

As mentioned above, EAE is an animal model that closely mimics MS in humans. EAE is similar to MS in that it leads to inflammation and demyelination. However, EAE is different in that it must be induced via active immunization with an emulsion of complete Freud's adjuvant (CFA) and myelin oligodendrocyte glycoprotein (MOG). CFA contains heat-killed *Mycobacterium tuberculosis*, which activates the innate immune response, and MOG is a component of the myelin sheath surrounding neurons. Together, CFA and MOG induce an inflammatory CNS immune response similar to that of MS (Constantinescu et al., 2011).

TCDD has previously been shown to attenuate disease and affect the immune response in the EAE model by suppressing T cell effector function and increasing CD4+CD25+FOXP3+ T regulatory cells (Yang et al., 2016). Our preliminary results also show that TCDD inhibited IgG production in EAE; TCDD suppressed MOG-specific IgG, as well as intracellular IgG production in the spleen and spinal cord in CD19- cells (Kummari et al.). The inhibition of IgG production by TCDD demonstrates that the AhR ligand affects B cells. To further evaluate TCDD's effect, we analyzed IgG expression in other B cells, specifically CD19, B220, and CD5 negative populations, in the spleen, lymph nodes, and bone marrow. CD19, a transmembrane glycoprotein, and B220, a CD45 isoform specific to murine B cells, are B cell markers expressed in all stages of B cell development but lost upon plasma cell differentiation (Wang et al., 2012, Cascalho et al., 2000). CD5 is a surface glycoprotein expressed in one subset of innate

B cells, known as B1a. Its expression is highest in B cells with regulatory functions (Fenutría et al., 2014).

Since our previous studies showed that TCDD suppressed EAE by inhibiting T effector function and inducing T regulatory function (Yang et al., 2016), we sought to determine if TCDD would affect a regulatory B cell population. Regulatory B cells help modulate the immune response by either suppressing other immune cells or inducing regulatory cells (Mauri and Menon, 2017). Our preliminary results showed that TCDD modestly upregulated FasL expression in CD19+ B cells in EAE, and FasL is just one cell surface marker on B cells that defines the B cells as regulatory (Kummari et al.). Therefore the focus of these studies was to determine if TCDD would affect another regulatory B cell population, one defined by co-expression of CD24 and CD38 (Mauri and Menon, 2017), which play a role in controlling T effector function and inducing T regulatory function. We hypothesized that TCDD would suppress IgG and induce CD24 and CD38 in B cells isolated from the spleens, lymph nodes, bone marrow, and spinal cords of male and female mice with EAE.

CHAPTER III

MATERIALS AND METHODS

Mice

Male and female C57BL/6 wild type (WT) mice were obtained from Envigo (Indianapolis, IN) at 6-8 weeks of age. The mice were kept 3 to a cage in a sterile, temperature (22 +/- °C) and light controlled (12-h light: 12-h dark) room. Each experimental group contained 3 mice. Food and water were provided *ad libitum*. Mississippi State University Institutional Animal Care and Use Committee approved the experimental protocols.

EAE Induction and Clinical Evaluation

Mice were immunized with 100 μg of myelin oligodendrocyte glycoprotein (MOG) peptide (Anaspec, Fremont, CA; MEVGWYRSPFSRVVHLYRNGK) in Complete Freund's Adjuvant (CFA) supplemented to 5 μg/ml heat-killed *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). Mice were anesthetized with isoflurane and injected with the MOG/CFA emulsion in four flanks (25 μl each; 2 at the hips, 2 at the shoulders) subcutaneously. EAE was induced in mice on day 0 and TCDD or CO was administered by oral gavage on days 1-12. Mice were weighed every other day until day 10 then daily thereafter. Mice were assigned clinical scores based on the following scoring system: 0, healthy; 0.5, flaccid tail; 1, awkward gait; 2; able to be flipped over; 3, single hind limb paralysis; 4, dual hind limb paralysis. Mice were not permitted to progress beyond a clinical score of 4.

TCDD Administration

TCDD (Accustandard, New Haven, CT) was dissolved in corn oil (CO) to make a stock solution. Prior to administration, the stock solution was further diluted in CO. Mice were given 2.5 μg/kg/day of TCDD or CO via oral gavage each day for twelve days, starting the day after disease induction. Dosage was chosen based on previous experiments in which 2.5 μg/kg/day of TCDD attenuated EAE. Using this dosing paradigm allowed for the evaluation of subchronic, low dose TCDD exposure prior to the development of clinical signs of disease in the mice.

Splenocyte, Lymph Node, Bone Marrow, and Spinal Cord Cell Isolation

Mice were necropsied on day 18. The spleen was harvested and placed in a plate and mechanically disrupted in 1ml of serum-free 1 x Roswell Park Memorial Institute (RPMI) 1640 media (Gibco, Grand Island, New York). The crude single cell preparation was collected and centrifuged at 400 x g for 5 minutes at RT. The SPLC pellet was resuspended in 1ml culture media (RPMI with 5% bovine calf serum, 1% penicillin-streptomycin and 50 μM 2-mercaptoethanol) and counted using an ACEA Novocyte flow cytometer. Lymph nodes (pooled from axillary and inguinal lymph nodes) were collected and mechanically disrupted. Bone marrow was isolated from the femur. The spinal column was harvested, and the spinal cord was excised and placed in 1X phosphate-buffered saline (PBS). Spinal cords were cut into pieces and digested with collagenase and DNase for 45 min at 37°C after which lymphocytes were separated using a 30%-70% percoll gradient. Lymphocytes were isolated from the buffy coat in the middle of the gradient after centrifugation.

Cell Staining

Cells were stained on the day of necropsy. Isolated cells were resuspended in 1X PBS. Cells were washed once with 1X PBS, centrifuged at 500 x g for 5 min at RT, and the supernatant was discarded. Cells were incubated with Near-IR Fixable Viability Dye (FVD, BioLegend, San Diego, California) for 30 min at room temperature (RT) to collect live cells via negative fluorescence intensity of FVD. Cells were washed again with PBS and then with flow cytometry (FCM) buffer. Then cells were treated with mouse Fc block for 15 minutes at RT. Cells for IgG staining were also blocked with extracellular IgG. Next, cells were labeled with the fluorescently conjugated antibodies for surface markers in the dark for 30 min at RT. The antibodies used for staining of cell surface markers are as follows (all antibodies were purchased from BioLegend): panel 1: APC IgG, PE-Cy7 CD19, BV785 B220, BV421 CD5; panel 2: PE-Cy7 CD19, BV785 B220, BV421 CD5, PE CD24, APC CD38. The antibodies were delivered in 50 µl of FCM buffer. Cells were washed with FCM buffer and fixed using Cytofix (BD Biosciences). Cells for IgG staining were permeabilized with Perm Wash Buffer (BD Pharmingen, San Jose, California). IgG antibody was delivered in Perm Wash Buffer for intracellular staining. All cells were analyzed using an ACEA Novocyte.

Flow Cytometry Analysis

Data analysis was performed using NovoExpress software. High fluorescent intensity of FVD was used to exclude dead cells. Forward scatter vs. side scatter properties of the live cells were used to gate the lymphocyte population, and the lymphocyte population was further gated on side scatter area versus height to exclude doublets. Bead controls were prepared for compensation, and fluorescence minus one cell controls were used to establish the gating for each fluorochrome in the staining mixture.

Statistical Analysis

GraphPad Prism 7 was used to perform statistical analysis. Comparisons between the experimental groups were calculated by two-way ANOVA tests. The level of significance was defined as $p < 0.05$.

CHAPTER IV

RESULTS

Clinical Evaluation

There were no significant changes in body weight (data not shown). EAE mice that received CO started showing clinical signs around day 17. In the first experiment, disease at day 18 was still very mild in all mice. In experiments 2 and 3, most EAE/CO mice reached a clinical score of 0.5-1.5 by day 18, meaning they exhibited loss of tail tone and had an awkward gait. TCDD modestly attenuated clinical disease in all three experiments, and TCDD-treated EAE mice had clinical scores very similar to mice without EAE (Figure 4.1).

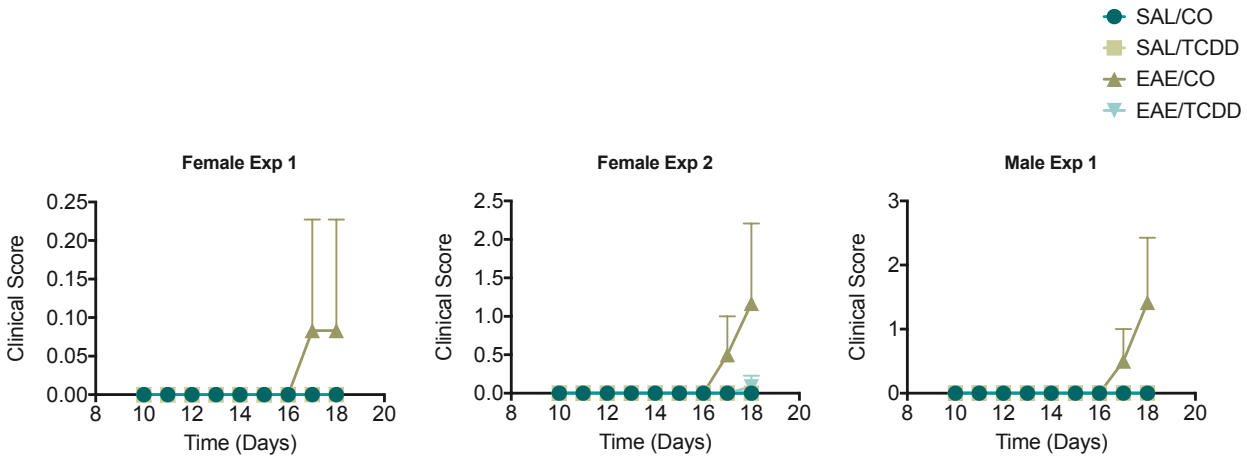

Figure 4.1 TCDD modestly reduced clinical scores

EAE was induced in C57BL/6 mice on day 0 (n=3). Mice received or 2.5 µg/kg/day TCDD by oral gavage on days 1-12. Clinical scores were assessed every other day for the first 10 days then daily until necropsy on day 18. Examples of clinical scores are: 0.5, flaccid tail; 1, awkward gait;

2, susceptible to flipping over without ability to right self; 3, single hind limb paralysis; 4, dual hind limb paralysis.

CD19, B220, and CD5 Expression in Various Tissues

Overall, CD19 was most highly expressed in the spleens of both male and female mice. TCDD modestly increased CD19 expression in the spleens of mice with and without EAE. In the bone marrow and lymph nodes, CD19 expression was variable and was not consistently affected by TCDD (Table 4.1).

In general, B220 was also most highly expressed in the spleen, and TCDD tended to increase B220 expression in the spleens of male and female mice. TCDD slightly increased B220 in the bone marrow of male and female mice with disease. B220 expression in the lymph nodes was the most variable. In the lymph nodes of females, TCDD increased B220 expression. In the lymph nodes of males, B220 expression was lower than in females, and TCDD did not have a consistent effect on B220 expression (Table 4.2).

CD5 was most highly expressed in the lymph nodes of both male and female mice. The effect of TCDD on CD5 expression in the lymph nodes was variable in male and female mice with and without disease. TCDD did not affect CD5 expression in the spleen and had inconsistent effects on CD5 expression in the bone marrow (Table 4.3).

Interestingly, CD19 and B220 expression was correlated, especially in the bone marrow and lymph nodes, regardless of disease or treatment (Figure 4.2).

Table 4.1 CD19 expression in splenocytes (SPLC), bone marrow (BM), and lymph nodes (LN) in two independent experiments.

%CD19						
	Female SPLC	Male SPLC	Female BM	Male BM	Female LN	Male LN
SAL/CO	41.89 +/- 7.91	41.17 +/- 9.69	32.79 +/- 2.61	42.12 +/- 2.45	30.51 +/- 2.777	16.72 +/- 7.17
SAL/TCDD	54.87 +/- 10.76	67.91 +/- 0.39	28.83 +/- 2.22	37.93 +/- 3.45	32.18 +/- 3.08	10.46 +/- 2.62
EAE/CO	30.24 +/- 1.86	23.57 +/- 4.93	13.92 +/- 1.83	3.29 +/- 0.38	59.99 +/- 5.88	28.68 +/- 7.43
EAE/TCDD	44.73 +/- 3.00	25.60 +/- 1.78	20.23 +/- 3.10	4.21 +/- 0.28	50.94 +/- 1.82	34.93 +/- 4.85

One experiment used female C57BL/6 mice and the other used male (n=3). CD19 (% live single lymphocytes) was detected using extracellular staining and flow cytometry.

Table 4.2 B220 expression in splenocytes (SPLC), bone marrow (BM), and lymph nodes (LN) in two independent experiments.

%B220						
	Female SPLC	Male SPLC	Female BM	Male BM	Female LN	Male LN
SAL/CO	35.30 +/- 11.86	10.46 +/- 1.73	26.56 +/- 2.34	36.44 +/- 3.55	30.40 +/- 2.72	11.23 +/- 3.87
SAL/TCDD	43.90 +/- 14.49	60.06 +/- 3,71	26.37 +/- 3.46	34.93 +/- 2.62	31.56 +/- 3.06	7.04 +/- 1.39
EAE/CO	26.75 +/- 2.92	18.91 +/- 4.14	10.40 +/- 3.09	3.02 +/- 0.28	58.97 +/- 5.92	16.99 +/- 4.87
EAE/TCDD	39.57 +/- 3.83	22.04 +/- 1.31	16.66 +/- 3.00	3.47 +/- 0.41	50.13 +/- 2.07	22.90 +/- 3.19

One experiment used female C57BL/6 mice and the other used male (n=3). B220 (% live single lymphocytes) was detected using extracellular staining and flow cytometry.

Table 4.3 CD5 expression in splenocytes (SPLC), bone marrow (BM), and lymph nodes (LN) in two independent experiments.

%CD5						
	Female SPLC	Male SPLC	Female BM	Male BM	Female LN	Male LN
SAL/CO	18.94 +/- 10.53	13.22 +/- 2.86	4.41 +/- 0.56	7.96 +/- 1.11	64.79 +/- 2.90	32.23 +/- 16.65
SAL/TCDD	14.22 +/- 9.05	28.05 +/- 0.33	5.27 +/- 0.84	3.90 +/- 0.42	60.08 +/- 2.65	14.55 +/- 3.71
EAE/CO	21.87 +/- 2.51	11.52 +/- 11.43	5.69 +/- 0.95	1.08 +/- 0.24	35.26 +/- 5.18	36.11 +/- 13.51
EAE/TCDD	13.84 +/- 2.56	12.89 +/- 0.20	5.13 +/- 1.21	0.79 +/- 0.16	43. 16 +/- 1.47	44.53 +/- 9.26

One experiment used female C57BL/6 mice and the other used male (n=3). CD5 (% live single lymphocytes) was detected using extracellular staining and flow cytometry.

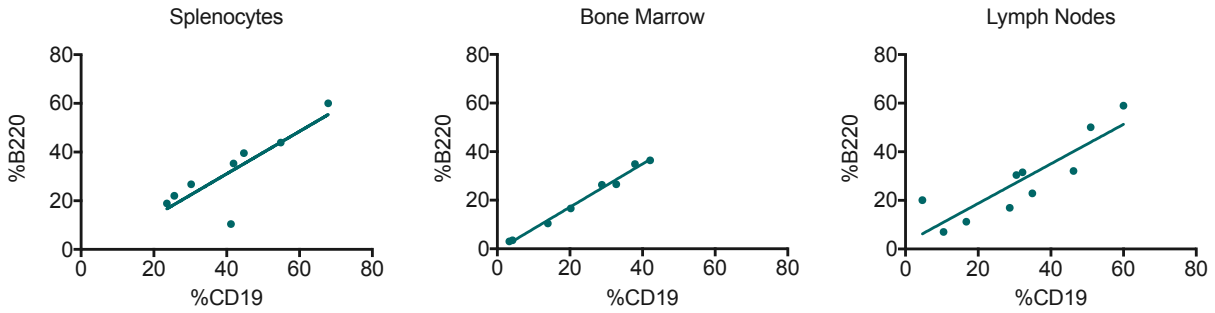

Figure 4.2 Expression of B220 and CD19 is positively correlated.

CD19 and B220 were detected in splenocytes, lymphocytes, and bone marrow using flow cytometry. Data from all four experimental groups from two independent experiments, one with female and the other with male C57BL/6 mice, was pooled (n=24). R square values: 0.6873 (A), 0.9902 (B), and 0.7713 (C).

TCDD Effect on IgG Production in B cells in Various Tissues

Preliminary results showed that TCDD most affected IgG production in CD19- populations of B cells (Figure 4.3). Therefore, these studies focused on IgG production in CD19-, B220-, and CD5- populations in the spleen, lymph nodes, and bone marrow.

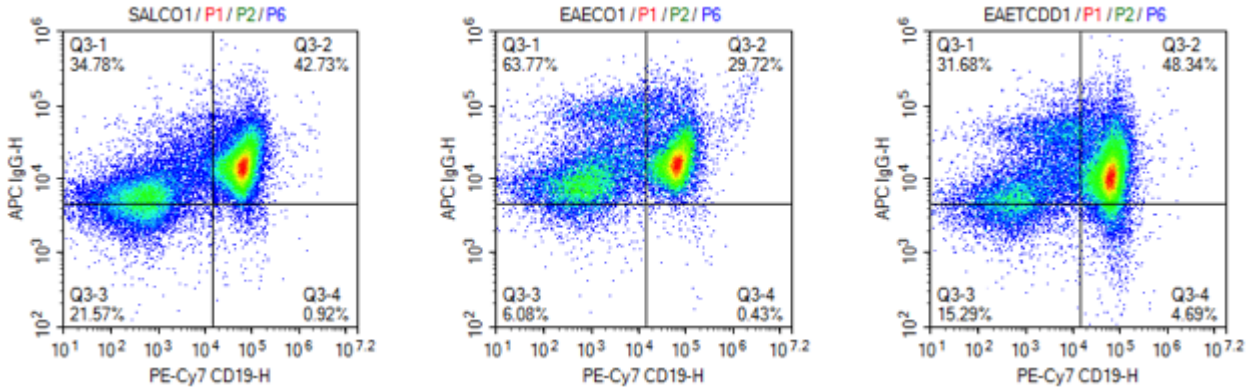

Figure 4.3 Intracellular IgG increased was most notably increased in CD19- B cells.

This upregulation was sensitive to inhibition by TCDD. This effect is demonstrated in Q3-1. Cells were stained with viability dye followed by Fc receptor blockade. Extracellular IgG was blocked using an unstained IgG antibody prior to permeabilization. Cells were stained with extracellular CD19-PeCy7 and intracellular IgG-APC. Cells are gated on live lymphocytes.

TCDD modestly reduced IgG expression in CD19-, B220-, and CD5- B cells in the splenocytes of females but increased or did not affect expression in the splenocytes of males. TCDD modestly suppressed IgG expression in CD19- and B220- populations in the lymph nodes of female mice. In males, TCDD did not affect IgG expression in CD19-, B220-, or CD5- populations in the lymph nodes. In both female and male mice, TCDD did not have much of an effect on IgG expression in any of the measured B cell subsets in the bone marrow (Figure 4.4).

TCDD Effect on CD24+CD38+ Expression in B cells in Various Tissues

TCDD did not significantly affect CD24 and CD38 expression in CD19+ or B220+ populations in the spleens, lymph nodes, bone marrow, or spinal cords of female mice. In males, where CD24 and CD38 expression was overall lower than in females, TCDD significantly suppressed CD24 and CD38 expression in CD19+, B220+, and CD5+ populations in the spleens of mice without disease and in the lymph nodes of mice with disease. Interestingly, TCDD slightly increased the CD5+CD24+CD38+ population in the spinal cords of both male and female mice (Figure 4.5).

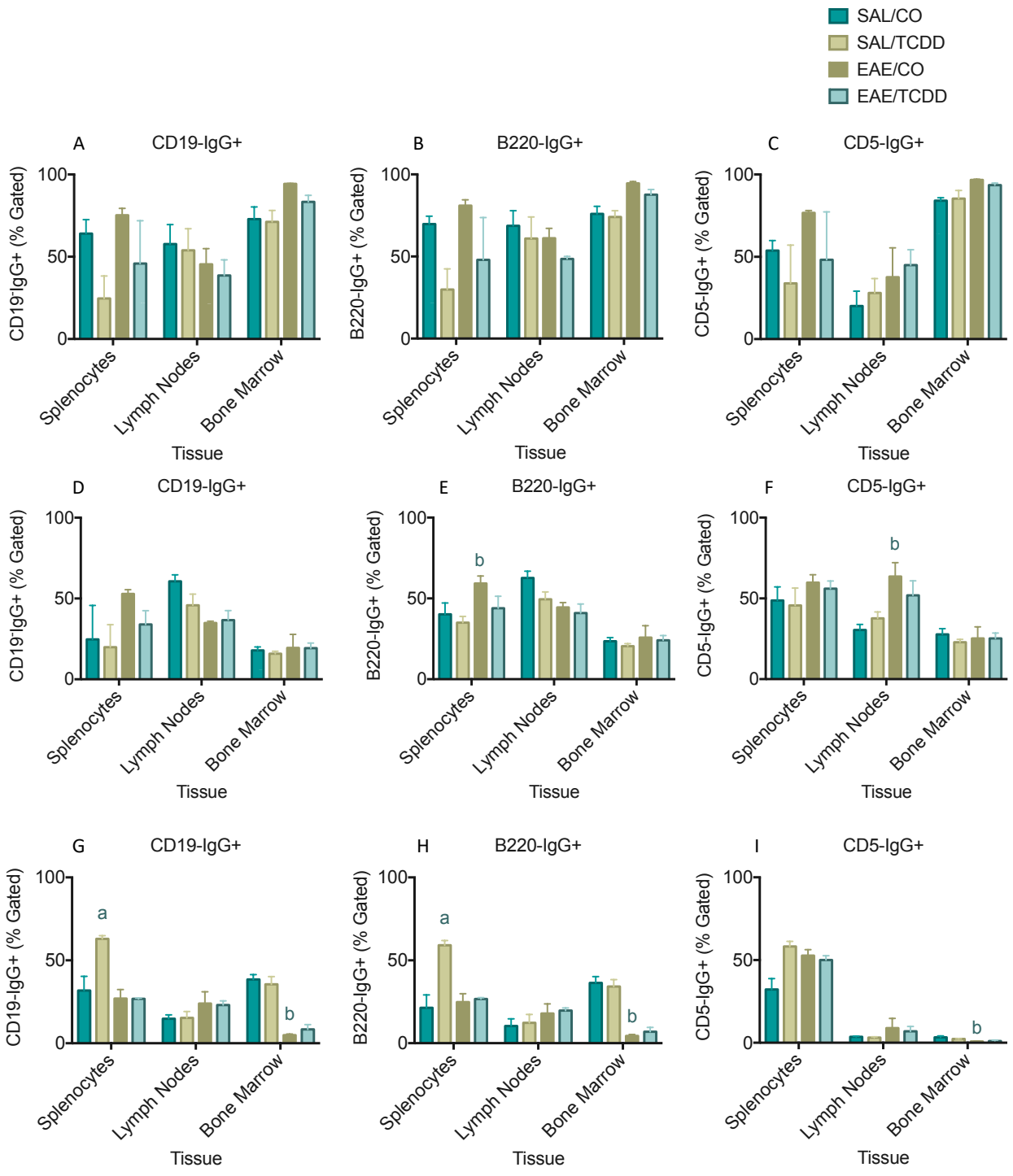

Figure 4.4 Expression of IgG in CD19, B220, and CD5 negative populations varies

Mice were euthanized on Day 18 and intracellular IgG was detected in splenocytes, lymph nodes, and bone marrow using flow cytometry (n=3). Female mice were used in experiments 1

(A, B, C) and 2 (D, E, F). Male mice were evaluated in experiment 3 (G, H, I). a p <0.05 difference between SAL/TCDD and SAL/CO. b p< 0.05 difference between EAE/CO and SAL/CO. c p<0.05 difference between EAE/TCDD and EAE/CO.

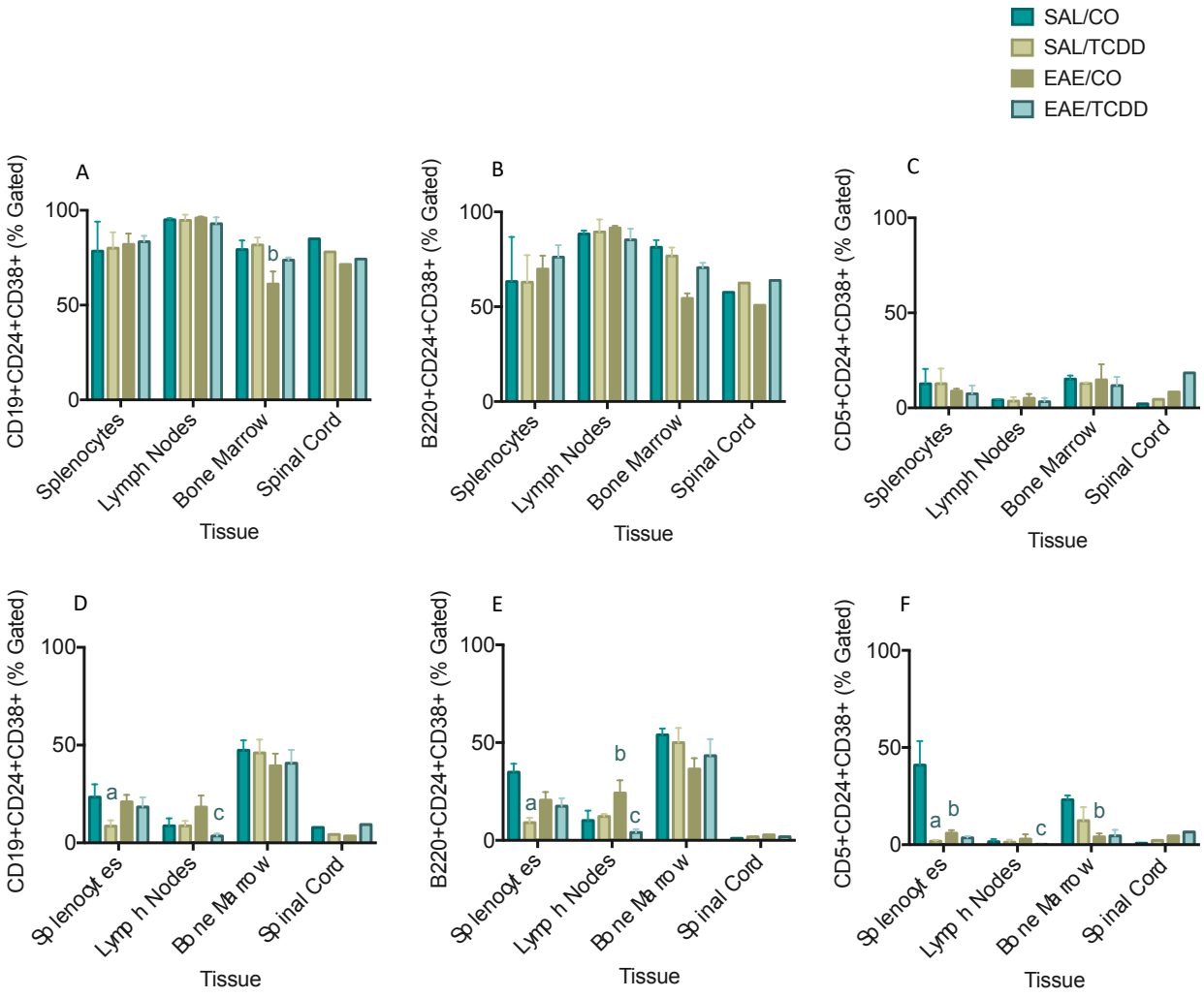

Figure 4.5 Expression of CD24 and CD38 in CD19, B220, and CD5 positive populations varies

Mice were euthanized on Day 18, and extracellular CD24 and CD38 was detected in splenocytes, lymph nodes, bone marrow, and spinal cord using flow cytometry (n=3). Female mice were used in experiments 1 (A, B, C) and 2 (D, E, F). Male mice were evaluated in experiment 3 (G, H, I). a p <0.05 difference between SAL/TCDD and SAL/CO. b p< 0.05 difference between EAE/CO and SAL/CO. c p<0.05 difference between EAE/TCDD and EAE/CO.

CHAPTER V

DISCUSSION

Development of MS has been linked to multiple causes, from viral infections to exposure to environmental contaminants. In studies evaluating EAE after AhR ligand exposure, the results have varied. Both TCDD and FICZ, another AhR ligand, have been shown to attenuate disease in some instances and exacerbate disease in others, depending on the dosing paradigm (Yang et al., 2016). These studies focused on B cell responses following subchronic, low dose TCDD treatment in EAE without pertussis toxin (PTX). EAE disease was induced, although it was quite modest, especially in experiment 1. This could be due to the fact that we don't use the additional immune adjuvant, pertussis toxin, in our EAE model. The absence of PTX, which is often used to exacerbate EAE, more closely mimics a milder form of MS that exists at disease onset in humans but does lead to lower clinical scores overall in EAE mice (Hofstetter et al., 2002). However, TCDD still attenuated disease, but whether or not B cell marker expression or IgG production aided attenuation remains the question.

When evaluating the results of this study, it is important to note the typical time course of the development of the immune response against EAE. When EAE is initiated via active immunization, the MOG emulsified in CFA triggers an inflammatory immune response against the central nervous symptom. In about the first five days after disease induction, local dendritic cells bind the MOG/CFA antigen and then infiltrate the lymph nodes. In the lymph nodes, dendritic cells present the antigen to T cells and B cells, which then become activated. From

about day 5 to day 10, these activated T cells and B cells circulate throughout the body, and many move to the spleen, where differentiation takes place. Around day 10 to day 18, the T cells and B cells have begun to move to the CNS where they release inflammatory cytokines and recruit macrophages that result in demyelination and CNS injury (Kuerten and Lehmann, 2011).

Overall, CD19, B220, and CD5 expression in the spleen, bone marrow, and lymph nodes of both male and female mice varied. However, there were some consistent patterns that should be noted. For example, in both male and female mice, the presence of disease correlated to a decrease in CD19 and B220 expression in the bone marrow and spleen but in increase in CD19 and B220 expression in the lymph nodes. In mice with disease, TCDD modestly increased CD19 and B220 expression in the spleen and bone marrow and did not consistently affect expression of the markers in the lymph nodes. Interestingly, treatment with TCDD countered EAE's effect by increasing expression, although it did not increase CD19/B220 expression back to the levels demonstrated in the control mice. Nevertheless, since CD19 plays a role in early B cell differentiation in the bone marrow and late maturation in the spleen (Wang et al., 2012), the increase of CD19/B220 expression may signify that the presence of TCDD leads to a downregulation of B cell differentiation in the spleen and bone marrow. Again, it is important to note that the data analyzed here comes from a single time point, day 18. Therefore, it is possible that CD19 and B220 expression was more significantly affected by TCDD, but the effect may have occurred earlier in the immune response.

As noted in the patterns above, expression of CD19 and B220 was correlated, especially in the bone marrow and lymph nodes. This co-expression supports the idea that both CD19 and B220 are pan-B cell markers in mice (Wang et al., 2012 , Rodig et al., 2005) Since CD5 expression was not strongly correlated with CD19 and B220 expression, the cells expressing CD19 and B220 are

most likely B2 B cells (Sindhava and Bondada, 2012). B2 B cells make up the largest subpopulation of B cells and differentiate into a variety of B cells, depending on chemokines, BCR signaling, and presence of antigen. Because they can use the BCR to engulf an antigen, process it, and present it to T cells, B2 cells play a role in T-dependent immune responses (Cano and Lopera, 2013), such as EAE (Fletcher et al., 2010). Although it seems clear as to why B2 cells may be needed to mount an immune response in EAE, it is not as clear as to why both CD19 and B220 are expressed and why their expression is correlated, regardless of the presence of disease or TCDD treatment. It is known that CD19 is involved in B cell signaling by modulating the B cell receptor (BCR)-dependent and BCR-independent signaling pathways, as well as MHC Class II-mediated signaling. CD19 also plays an important role in B cell activation (Wang et al., 2012). B220 also plays a role in the positive regulation of antigen receptor signaling, but its function is more critical for T cell signaling and development than B cell signaling (Coughlin et al., 2015). Therefore, it is possible that CD19 and B220 are expressed together because the former plays a more significant role in the B cell signaling while the latter is needed for T cell signaling.

The third marker that was evaluated was CD5. Expression of CD5 did not correlate with expression of CD19 and B220 and did not show consistent patterns of expression in the presence of disease with or without treatment. Although the CD5 in our studies was variable, one EAE study demonstrated that disease onset was delayed and disease severity was attenuated in mice lacking CD5. Researchers found that T cells in CD5- mice had a higher rate of apoptosis and suggested that CD5 helps to regulate T cell survival in EAE (Axtell et al., 2004). Therefore, CD5 may be playing a bigger role in the progression of EAE than we initially thought.

As a whole, the expression of the three different B cell markers, CD19, B220, and CD5, was variable in all three tissues. It is important to note that these data reflect expression of B cell

markers at end-stage disease, day 18. Based on the timeline of disease progression described above, it is known that the CNS is the main target organ at end-stage disease. On the other hand, it is not likely that the lymph nodes and bone marrow are a target organ at end-stage disease, and we are not still sure about the spleen. It is possible that TCDD does affect the B cell populations spleen, bone marrow, and lymph nodes, but it may happen at an earlier stage in the immune response, so future studies could evaluate expression at an earlier day. However, the focus of these studies was end-stage disease, and the inconsistent data points to the need to evaluate more than just expression of the markers in order to fully understand how B cell populations in EAE mice are affected by TCDD. A more in depth look at the function of these markers could provide this information. It is known that T cells from mice that were actively induced with EAE can be transferred to healthy mice and initiate disease, a process known as passive induction (Stromnes and Goverman, 2006). In order to study the function of B cells, B cells from the spleens of diseased mice, both untreated and treated with TCDD, could be injected into new mice. These mice would then be evaluated for physical changes to see if disease is induced as well as changes to expression of cytokines and lymphocytes. Thus, we could gain greater insight into the actual function of B cells in EAE and TCDD's effect on that function.

In addition to evaluating expression of CD19, B220, and CD5, the production of IgG was evaluated in populations lacking these markers. In the last few decades, there has been increasing evidence of the role of immunoglobins in the pathogenesis of MS. It is thought that immunoglobulins may bind to cells expressing MOG, causing tissue destruction (Wootla et al., 2011). More notably, IgG in particular is able to fix and activate the classical complement cascade, which leads to cell death. Therefore, IgG directed against MOG can activate the complement cascade and destroy the cells making up the myelin sheath, leading to demyelination

(Magliozzi et al., 2006). Another study demonstrated a direct correlation between antibody-producing B cells and demyelination in the spinal cord. In sections of the spinal cord containing many B cells, there was also significant demyelination whereas sections without B cell infiltration maintained the myelin sheath. This study also found that IgG expression in the spinal cord increased upon EAE induction but was mainly produced in B cells lacking CD19 (Kummari et al., 2019). Our preliminary study also demonstrated that that IgG expression was increased in CD19- populations upon EAE disease induction and then decreased upon treatment with TCDD (Kummari et al.). This pattern suggests that B cells that have differentiated and lost CD19, and most likely B220, expression are more susceptible to changes in IgG expression. Therefore, the studies presented here focused on IgG expression in B cell populations lacking either CD19, B220, or CD5 and since we already know that TCDD affects IgG expression in the spinal cord, we evaluated IgG expression in the B cell populations of the spleen, bone marrow, and lymph nodes.

We found that the effect of TCDD on IgG was most prominent in the spleen. In females, TCDD modestly suppressed IgG expression in CD19-, B220-, and CD5- B cell populations in the spleen. In males, TCDD significantly increased IgG expression in CD19- and B220- splenic B cells and modestly increased IgG in CD5- splenic B cells in saline mice but had little effect on IgG expression in EAE mice. These differences between female and male mice are not clear and point to the need for repeated IgG studies, especially in male mice since they presented the most inconsistent data. However, the data as a whole does show that IgG expression in the spleen was more affected by TCDD than in the bone marrow and lymph nodes, suggesting that the spleen may play a more important role as a target organ in end-stage disease.

Although TCDD had modest effects on B cell marker expression and IgG expression, its effect on a regulatory B cell population, identified by the co-expression CD24 and CD38, was inconsistent in almost all B cell populations and tissues in male and female mice. However, TCDD did increase the CD5+CD24+CD38+ population in the spinal cords of both male and female mice with EAE. This observation is not consistent with the other study that supported the idea that CD5 exacerbates disease (Axtell et al., 2004), but it does show that CD5 is an important marker in the progression of EAE and should be more thoroughly evaluated in future studies involving EAE. Additionally, the variable and inconsistent data regarding CD24 and CD38 expression in each of the populations may have also been affected by the fact that the data came from day 18 of disease. This regulatory population could still be playing an important role in disease development or in the TCDD mechanism of attenuation, but the effects may be occurring at an earlier timepoint.

CHAPTER VI

CONCLUSION

In summary, most of the data regarding the expression of different B cell markers in the different tissues was variable. However, we did find that CD19 and B220 expression was decreased in the spleen and bone marrow in EAE mice but then increased upon treatment with TCDD, suggesting a possible mechanism of disease attenuation. Another interesting finding was that CD19 and B220 expression was correlated in all experimental groups in all three tissues. Although we are not sure why both markers are upregulated or suppressed at the same time, one hypothesis is that both are needed, or not needed, in certain B cells because CD19 plays a more significant role in B cell signaling while B220 plays a more significant role in T cell signaling.

Because previous studies demonstrated that IgG was most affected in CD19- B cell populations in the spinal cord, we decided to evaluate the effect of TCDD on IgG expression in CD19-, B220-, and CD5- populations in the spleen, lymph node, and bone marrow. Our results showed that IgG expression was most affected in the spleen and also may be a possible sex difference because TCDD decreased IgG expression in the B cell populations of females but increased it in the B cells of males.

In order to study TCDD's effect on a regulatory B cell subset, we analyzed the expression of CD24 and CD38, which together can represent a regulatory cell. For this evaluation, we evaluated CD19+CD24+CD38+, B220+CD24+CD38+, and CD5+CD24+CD38+ in the spleen, bone marrow, lymph nodes, and spinal cord since this regulatory population has not been well

characterized in any of these tissues. We found that TCDD had no significant or consistent effects on the expression of CD24 and CD38 in almost all of the experimental groups in any of the tissues, except that TCDD modestly increased the CD5+CD24+CD38+ population in the spinal cords of both male and female mice with EAE, which could be important since the spinal cord is known to be the primary target organ for end stage disease.

It is important to again point out that in these studies we looked at cells harvested 18 days following disease initiation, which is considered end-stage disease in our model of EAE. Although there were not many significant effects in the B cells of the bone marrow and lymph nodes, the data we gathered still provides important information because it confirms that the CNS, and possibly the spleen, are still the most likely target organs at end-stage disease. Before these studies, we did not know what was happening in the bone marrow and lymph nodes, but based on the results, we believe that if TCDD is affecting B cells in these tissues, it is most likely exerting its effects earlier in the disease progression. Future studies could evaluate this possibility.

Overall, the results of this study provide novel information about the effect of TCDD in B cells in EAE, especially in the bone marrow and lymph nodes, at end-stage disease. Future studies are needed to verify the results, particularly in males, which we looked at for the first time, and to further access the changes in the function of the B cells, since expression of different markers alone is not telling us enough. In light of these findings, we hope to contribute to the understanding of TCDD's mechanism of toxicity and immunosuppression and to further our knowledge of AhR ligands as a potential treatment for autoimmune diseases.

REFERENCES

Axtell, R.C., Webb, M.S., Barnum, S.R., and Raman, C. (2004). Cutting Edge: Critical Role for CD5 in Experimental Autoimmune Encephalomyelitis: Inhibition of Engagement Reverses Disease in Mice. J Immunol *173*, 2928–2932.

Cano, R.L.E., and Lopera, H.D.E. (2013). Introduction to T and B lymphocytes (El Rosario University Press).

Cascalho, M., Wong, J., Brown, J., Jäck, H.-M., Steinberg, C., and Wabl, M. (2000). A B220–, CD19– population of B cells in the peripheral blood of quasimonoclonal mice. International Immunology *12*, 29–35.

Constantinescu, C.S., Farooqi, N., O'Brien, K., and Gran, B. (2011). Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). Br J Pharmacol *164*, 1079–1106.

Coughlin, S., Noviski, M., Mueller, J.L., Chuwonpad, A., Raschke, W.C., Weiss, A., and Zikherman, J. (2015). An extracatalytic function of CD45 in B cells is mediated by CD22. Proc Natl Acad Sci USA *112*, E6515–E6524.

Dean, J.H. (1994). IMMUNOTOXICOLOGY: AN OVERVIEW. Toxicology in Vitro *8*, 933–937.

Fenutría, R., Martinez, V.G., Simões, I., Postigo, J., Gil, V., Martínez-Florensa, M., Sintes, J., Naves, R., Cashman, K.S., Alberola-Ila, J., et al. (2014). Transgenic Expression of Soluble Human CD5 Enhances Experimentally-Induced Autoimmune and Anti-Tumoral Immune Responses. PLoS One *9*.

Fletcher, J.M., Lalor, S.J., Sweeney, C.M., Tubridy, N., and Mills, K.H.G. (2010). T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. Clin Exp Immunol *162*, 1–11.

Ghasemi, N., Razavi, S., and Nikzad, E. (2017). Multiple Sclerosis: Pathogenesis, Symptoms, Diagnoses and Cell-Based Therapy. Cell J *19*, 1–10.

Hofstetter, H.H., Shive, C.L., and Forsthuber, T.G. (2002). Pertussis Toxin Modulates the Immune Response to Neuroantigens Injected in Incomplete Freund's Adjuvant: Induction of Th1 Cells and Experimental Autoimmune Encephalomyelitis in the Presence of High Frequencies of Th2 Cells. J Immunol *169*, 117–125.

Kuerten, S., and Lehmann, P.V. (2011). The Immune Pathogenesis of Experimental Autoimmune Encephalomyelitis: Lessons Learned for Multiple Sclerosis? Journal of Interferon & Cytokine Research *31*, 907–916.

Kummari, E., Nichols, J.M., Yang, E.-J., and Kaplan, B.L.F. (2019). Neuroinflammation and B-Cell Phenotypes in Cervical and Lumbosacral Regions of the Spinal Cord in Experimental Autoimmune Encephalomyelitis in the Absence of Pertussis Toxin. Neuroimmunomodulation *26*, 198–207.

Kummari, E., Rushing, E., Nicaise, A., McDonald, A., and Kaplan, B.L.F. TCDD Attenuates EAE Through Inhibition of IgG Production and FasL Induction on B Cells. In Production.

Magliozzi, R., Howell, O., Vora, A., Serafini, B., Nicholas, R., Puopolo, M., Reynolds, R., and Aloisi, F. (2006). Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. Brain *130*, 1089–1104.

Mauri, C., and Menon, M. (2017). Human regulatory B cells in health and disease: therapeutic potential. Journal of Clinical Investigation *127*, 772–779.

Neavin, D.R., Liu, D., Ray, B., and Weinshilboum, R.M. (2018). The Role of the Aryl Hydrocarbon Receptor (AHR) in Immune and Inflammatory Diseases. Int J Mol Sci *19*.

Rodig, S.J., Shahsafaei, A., Li, B., and Dorfman, D.M. (2005). The CD45 isoform B220 identifies select subsets of human B cells and B-cell lymphoproliferative disorders. Human Pathology *36*, 51–57.

Sindhava, V., and Bondada, S. (2012). Multiple Regulatory Mechanisms Control B-1 B Cell Activation. Front. Immunol. *3*.

Stromnes, I.M., and Goverman, J.M. (2006). Passive induction of experimental allergic encephalomyelitis. Nature Protocols *1*, 1952–1960.

Wang, K., Wei, G., and Liu, D. (2012). CD19: a biomarker for B cell development, lymphoma diagnosis and therapy. Exp Hematol Oncol *1*, 36.

Wheeler, M.A., Rothhammer, V., and Quintana, F.J. (2017). Control of immune-mediated pathology via the aryl hydrocarbon receptor. J Biol Chem *292*, 12383–12389.

White, S.S., and Birnbaum, L.S. (2009). An Overview of the Effects of Dioxins and Dioxin-like Compounds on Vertebrates, as Documented in Human and Ecological Epidemiology. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev *27*, 197–211.

Wootla, B., Denic, A., Keegan, B.M., Winters, J.L., Astapenko, D., Warrington, A.E., Bieber, A.J., and Rodriguez, M. (2011). Evidence for the Role of B Cells and Immunoglobulins in the Pathogenesis of Multiple Sclerosis. Neurol Res Int *2011*.

Yang, E.-J., Stokes, J.V., Kummari, E., Eells, J., and Kaplan, B.L.F. (2016). Immunomodulation By Subchronic Low Dose 2,3,7,8-Tetrachlorodibenzo- *p* -Dioxin in Experimental Autoimmune Encephalomyelitis in the Absence of Pertussis Toxin. Toxicol. Sci. *151*, 35–43.