

MODULATION OF B CELL ACTIVATION AND EFFECTOR FUNCTION BY THE ARYL
HYDROCARBON RECEPTOR IN THE RAT

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ABSTRACT

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Suppression of the humoral immune response by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) occurs through activation of the aryl hydrocarbon receptor (AHR) and the B cell has been identified as one of the most sensitive targets to TCDD. Despite decades of studies in multiple species the exact mechanisms by which TCDD suppresses B cell activation and the primary IgM antibody response has yet to be elucidated. The magnitude of AHR regulation on B cell activation and effector function is also unknown. It was hypothesized that the targeted deletion of the AHR results in the loss of sensitivity of the B cell to suppression by TCDD and conversely in the augmentation of the primary IgM antibody response, which is mediated in part, through a greater magnitude in B cell activation in the absence of TCDD. The expression of surface markers CD80, CD86, and MHC II signify early activation events in the B cell and can be used to evaluate the magnitude of activation. Likewise, IgM secretion can be used to evaluate the magnitude in B cell effector function. Using a rat AHR knockout model, *in vitro* LPS activation of B cells resulted in higher expression of CD80, CD86 and MHC II in AHR knockout compared to wild type rats. Similarly, AHR knockout rats had an increased primary IgM antibody response with LPS activation. TCDD did not significantly alter CD80, CD86, and MHC II expression or the IgM antibody response of AHR knockout and wild type rats. This study has demonstrated that AHR is partly responsible for regulating the magnitude of B cell activation and effector function.

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LIST OF ABBREVIATIONS

ACK	Ammonium chloride potassium
AHR	Aryl hydrocarbon receptor
AP-1	Activating protein 1
APC	Antigen presenting cell
ARA9	Aryl hydrocarbon associated protein 9
ARNT	Aryl hydrocarbon receptor nuclear translocator
Bach2	BTB and CNC homology 2
BCL-6	B cell lymphoma 6
BCR	B cell receptor
bHLH	Basic helix loop helix
BLIMP-1	B lymphocyte maturation protein 1
BSA	Bovine serum albumin
CD	Cluster of differentiation
DLC	Dioxin like compound
DMSO	Dimethyl sulfoxide
DRE	Dioxin response element
ELISA	Enzyme linked immunoabsorbent assay
ERK	Extracellular signal regulated kinase
FACS	Fluorescence-activated cell sorting
Hsp90	Heat shock protein 90

Ig	Immunoglobulin
JNK	Jun amino terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MHC II	Major histocompatibility complex II
MyD88	Myeloid differentiation primary response protein 88
NF- κ B	Nuclear factor kappa B
PAMPs	Pattern-associated molecular patterns
Pax5	Paired box 5
PBS	Phosphate buffered saline
PWM	Pokeweed mitogen
ROS	Reactive oxygen species
sRBC	Sheep red blood cell
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
T _H cell	T helper cell
TLR	Toll-like receptor
XPB-1	X box paired protein 1

Introduction

A. Organization of the Immune System

The immune system plays a critical physiological role by protecting an organism against disease-causing pathogens through highly complex and regulated processes. Defense against an invading pathogen occurs through innate and adaptive divisions of the immune system. The innate immune system is the first to respond to a pathogen and does so in a non-specific manner through activation of the complement system, inflammation, mucosal barriers, phagocytosis by macrophages and direct killing of the pathogen by natural killer cells. This system is effective in the protection against an invading pathogen but it is unable to adapt to the changing environment in which the host resides. The adaptive immune system complements the innate immune system in that it is able to adapt according to the pathogens present in the host's external environment and produce specific responses to these pathogens. In addition to highly specific responses, the adaptive immune system elicits memory against the pathogen so that subsequent responses will be faster and more effective.

The adaptive immune system can be further divided into cell-mediated and humoral immunity. Generally speaking, cell-mediated immunity is mediated by T cells, macrophages, and natural killer cells and protects the organism against cancer, viral and bacterial diseases. Humoral immunity is mediated by B cells and protects the organism through the production of immunoglobulins (Ig) also called antibodies, which neutralize, destroy and activate the complement component of the innate immune system. The cell mediated and humoral immune response are antigen specific and

several cell types such as antigen presenting cells (APCs), T-helper cells (T_H cells), and B cells are responsible for coordinating their effector functions for antigen destruction. Macrophages and dendritic cells are APCs that scavenge their environment and upon encountering an antigen, present it to T cells. If the T cell recognizes the antigen as foreign, then they will either become activated into an effector T cell and secrete cytokines or differentiate into a memory T cell that will respond to the same antigen again upon subsequent exposure. B cells recognize antigens in their environment through the B cell receptor (BCR) which is a membrane bound form of antibody. Once bound to the BCR, the antigen is internalized into endosomes that fuse with an acidic lysosome that contain proteases to degrade the antigen into peptide fragments. A specialized presenting protein, major histocompatibility complex class II (MHC II) is then transferred from the endoplasmic reticulum into the peptide fragment containing endosome where the MHC II molecule is loaded with an antigen peptide fragment. This peptide bound MHC II molecule then travels to the cell surface to present the B cell recognized antigen to T_H cells initiating their activation. This T_H cell activation leads to cytokine secretion initiating B cell survival and proliferation. It is the balance of these several cell types that allows for specific and swift responses to antigens and any imbalances in the interactions of these cells can lead to autoimmune diseases.

B. The Adaptive Immune System

The cells of the adaptive immune response arise from a common lymphoid progenitor cell in the bone marrow, which have developed from hematopoietic stem cells. It is the tight contact between the stromal cells of the bone marrow and the

lymphoid progenitors that gradually commit the cells to either the myeloid or lymphoid development pathway. Cells committed to the myeloid pathway become macrophages, granulocytes, mast cells, and dendritic cells whereas cells committed to the lymphoid pathway become T or B cells. The coordinated physical interaction between lymphoid progenitors and stromal cells as well as the timed release or temporal kinetics of cytokines and chemokines leads to the development of lymphocytes from hematopoietic stem cells and gives rise to immature B cells. Next, the production of a functional BCR by an immature B cell is a critical step in the development of a mature B cell. Before migrating to secondary lymphoid tissue to mature, the BCR is tested for autoreactivity to antigens recognized as self. If the BCR is found to be autoreactive it can be amended by one of three different manners. The autoreactive B cells can undergo apoptosis by clonal deletion, the BCR can be edited so it no longer recognizes self antigens or, the BCR become anergic or non-responsive against self antigens (Hardy and Hayakawa, 2001). Once a functional BCR is in place, the immature B cells can then migrate from the bone marrow to the spleen for maturation into a naïve B cell.

As mentioned humoral immunity is specific in its response and the recognition of a specific antigen by a B cell is based on the structure of the antibody. Antibodies consist as a heterodimer of two heavy chains linked by disulfide bonds to two light chains and each of these chains contains a variable and a constant region. It is the variable region of both chains that allow for the specificity in antigen binding. Antigen specificity is made possible through a diversification processes known as VDJ recombination which genetically recombines heavy and light chain genes resulting in several isotypes of both chains. The light chain constant regions are homodimers of

either Ig kappa (Ig κ) or Ig lambda (Ig λ) isotypes where the heavy chain constant regions are homodimers of either μ , δ , γ , α , ϵ isotypes. The heavy chain isotypes designate the five classes of antibodies with μ gene producing IgM, δ producing IgD, γ producing IgG, α producing IgA, and ϵ producing IgE. The first antibody to be produced in the primary humoral immune response is IgM. This antibody has a low binding affinity but is secreted as a pentamer leading to a high avidity upon binding to antigens. It is predominately found in the bloodstream and lymph and is effective in activating the complement system. IgD is found co-expressed with IgM and signals B cells to exit the bone marrow upon maturation. IgA exists as either a monomer or dimer in epithelial surfaces where complement and phagocytes are not present to neutralize antigens. IgE only exists as a monomer and has high avidity for receptors on mast cells and functions to mediate allergic and hypersensitivity responses. IgG is the only antibody that is able to cross the placental barrier and is mostly found as a monomer. It primarily functions in secondary immune responses but can also activate the complement system and other immune cells. The multimerization of IgM and IgA is mediated through the polypeptide Ig J-chain (IgJ). As mentioned before, the BCR is a membrane bound form of antibody and each of these Ig isotypes can exist as a BCR. It is the combination of the highly diverse light and heavy chains that give such a large gamut of structurally different Igs, allowing for B cells to recognize and bind to such a wide diversity of antigens.

Before a B cell is able to differentiate into an antibody secreting plasma cell it must first become activated. B cell activation can occur in two ways, either in a T cell-dependent or T cell-independent manner. The focus of this thesis is on T_H cell-independent B cell activation and hence T_H cell-dependent B cell activation will not be

discussed in detail. T cell-dependent B cell activation requires two signals, one signal through engagement of the BCR and the second signal from CD40-CD40 ligand interactions with cognate T cells. Many antigens that activate B cells in a T_H cell independent manner do so via the activation of toll-like receptors (TLRs) and cross-linking of several BCRs. Pokeweed mitogen (PWM) is a lectin derived from the *Phytolacca americana* plant and activates B cells through cross-linking several BCRs leading to the proliferation and differentiation into IgM antibody secreting plasma cells (Bekeredjian-Ding *et al.*, 2012). TLRs recognize pathogen-associated molecular patterns (PAMPs) in the outer membrane of gram-negative and gram-positive bacteria, viruses, and fungi. Each TLR recognizes a specific PAMP molecule, for example TLR7 and 8 respond to single stranded RNA of viruses, TLR9 responds to unmethylated CpG DNA, TLR1 and TLR2 respond to lipopeptides in bacterial membranes. Lipopolysaccharide is a component in the outer cell membrane of gram negative bacteria and is recognized by TLR4. Stimulation of TLR4 in B cells leads to polyclonal activation and the production of a primary IgM antibody response (Iwasaki and Medzhitov, 2004). In both cases of LPS and PWM T_H cell-independent B cell activation, nuclear factor kappa B (NF- κ B) and activating protein-1 (AP-1) signaling cascades become activated and are integrated and converge onto mitogen-activated protein kinases (MAPK), extracellular signal regulated kinase (ERK), and Jun amino terminal kinase (JNK) through the adaptor protein myeloid differentiation primary response protein 88 (MyD88) (Iwasaki and Medzhitov, 2004; Jilling *et al.*, 2006; Shapiro-Shelef and Calame, 2005). The crosstalk between these pathways leads to an activated B cell phenotype that can be characterized by the upregulation of cell surface proteins

Cluster of Differentiation 80 (CD80, B7.1), CD86 (B7.2), and MHC II (Kaminski *et al.*, 2012; Rodo *et al.*, 2006). CD80, CD86, and MHCII surface proteins are B cell activation markers and can be used to identify B cells that have become activated in response to a stimulus.

Once activated, a B cell can differentiate into an antibody secreting plasma cell; this process is tightly controlled by a bistable regulatory network of transcription factors including B-cell lymphoma 6 (BCL-6), paired box 5 (Pax5), B lymphocyte induced maturation protein-1 (BLIMP-1), and X-box binding protein 1 (XBP-1), and BTB and CNC homology 2 (Bach2) (Bhattacharya *et al.*, 2010; Calame, 2001; Sciammas *et al.*, 2011; Shapiro-Shelef and Calame, 2005; Zhang *et al.*, 2010). Pax5 is required for the commitment of a lymphoid progenitor to the B cell lineage during development as well as positively regulating the expression of BCL-6 and Bach2 (Sciammas *et al.*, 2011). In addition, Pax5 also regulates the expression of XBP-1 which is required for MHC II transcription (Ono *et al.*, 1991; Reimold *et al.*, 2001). The maintenance of a resting naïve B cell phenotype is due to the repression of BLIMP-1 by Pax5 and BCL-6. Upon activation by LPS, AP-1 can act upon this bistable switch to induce an all-or-none response and induce the expression of BLIMP-1 (Zhang *et al.*, 2013). BLIMP-1 is considered the master regulator in B cell differentiation and its expression leads to a feed forward loop to suppress Pax5 and BCL-6 (Fairfax *et al.*, 2008; Lin *et al.*, 2002; Shaffer *et al.*, 2002). It is the rise in the transcriptional repressor BLIMP-1 and XBP-1 and fall in BCL-6 and Pax5 that ultimately lead to the terminal differentiation of B cells into plasma cells.

C. TCDD and the AHR Pathway

Halogenated aromatic hydrocarbons and halogenated polycyclic aromatic hydrocarbons are structurally related compounds comprised of dioxin and dioxin like compounds (DLCs) (Poland and Knutson, 1982). They are ubiquitous and persistent environmental contaminants produced as byproducts from the manufacturing of herbicides, combustion of chlorinated materials, chlorinated bleaching of paper pulp during paper manufacturing. Although to a lesser extent they are also found naturally as a result of natural combustion such as volcanic eruptions and forest fires. Dioxins and DLCs are highly lipophilic compounds and the greatest route of human exposure is diet due to bioaccumulation in the food chain leading to lifelong exposures (El-Shahawi *et al.*, 2010; White and Birnbaum, 2009). The most well studied and potent dioxin is 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and it has been shown to elicit toxic effects in several species. The health effects of TCDD exposure seen in several laboratory species include metabolic perturbations, thymic involution, endocrine disruption, liver and stomach hyperplasia, hepatic lesions, carcinogenesis, and respiratory irritation (Rysavy *et al.*, 2013; Safe, 1986). Many of these toxic effects of TCDD can also be seen in humans including carcinogenesis, endocrine disruption, liver damage, and immune suppression, but the most common human health effect is chloracne characterized by the hyperkeratinization of the skin with dark cysts (Marinkovic *et al.*, 2010).

Despite common species effects there is marked inter-species and inter-strain variability in the response to TCDD exposure. The LD₅₀ is an average lethal dose required to kill 50% of a tested population and is often used as a measurement of acute toxicity. It was found that the LD₅₀ of TCDD has a broad range across species and can

vary from 1 µg/kg in guinea pigs (found to be the most sensitive) to 5mg/kg in hamsters (found to be the most resistant) (McConnell, 1985). Inter-strain differences in rat species have also been found. The Long-Evans rat are one of the more sensitive strains to TCDD with an LD₅₀ of 10-20µg/kg and the Han/Wistar rat are exceedingly resistant to TCDD as the LD₅₀ is higher than 9600µg/kg (Tuomisto *et al.*, 1999). The inter-species and intra-species specific differences in the effects of TCDD has been attributed to a difference in ligand affinity resulting from allelic differences in a genetic locus identified as the Ah locus which encodes the aryl hydrocarbon receptor (AHR) (Poland and Glover, 1980; Poland and Glover, 1974; Poland *et al.*, 1994; Quintana and Sherr, 2013). The toxicity seen upon exposure to TCDD are thought to be mediated via the AHR (Rowlands and Gustafsson, 1997).

The AHR has been identified as a ligand activated transcription factor in the Per-Arnt-Sim (PAS) family containing a basic helix loop helix domain (bHLH) (Stevens *et al.*, 2009). The PAS domain in AHR contains two domains denoted as A and B and the ligand binding activity of AHR is found in the PAS B domain (Hao and Whitelaw, 2013). The PAS domain also allows for dimerization with the Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT) while the bHLH domain is responsible for DNA binding and dimerization with its associated chaperone proteins (Stevens *et al.*, 2009). In the absence of bound ligand, AHR is found in the cytosol as a complex with two heat shock protein 90 (HSP90), p23, aryl hydrocarbon associated protein 9 (ARA9), or AHR Interacting Protein (AIP). It has been suggested that the role of ARA9 and HSP90 is to maintain a proper folding configuration of AHR to enable binding as well as preventing inappropriate nuclear translocation, while the role of p23 is to maintain the association

of the AHR-HSP90 complex (Stevens *et al.*, 2009). Once ligand activated, AHR translocates to the nucleus where it dissociates from its cytosolic chaperone proteins and dimerizes with ARNT forming a heterodimer that acts as a transcription factor. The AHR/ARNT complex then binds to regulatory DNA sequences containing dioxin response elements (DRE) leading to alterations in gene expression. In addition to being a ligand activated transcription factor, the AHR has also been shown to function as an E3 ubiquitin ligase for selective protein degradation (Nguyen *et al.*, 2013). The most well characterized alteration in gene expression is increased expression of phase I and phase II xenobiotic metabolism enzymes such as the cytochrome P450s (CYP) specifically CYP1A1, CYP1A2, and CYP1B1, glutathione-S-transferase A2 (GSTA2), and UDP-glucuronosyltransferases 1A1 and 1A6 (UGT1A1, UGT1A6) (Abel and Haarmann-Stemmann, 2010). These AHR responsive genes have been characterized as the AHR gene battery and CYP1A1 and CYP1B1 are often used as an indirect biochemical endpoint of TCDD exposure (Hu *et al.*, 2007; Kerkvliet, 2002).

D. B cells as a Sensitive Target to TCDD Mediated Immunotoxicity

Most of the toxic effects of TCDD are thought to be mediated by AHR and immune suppression is one of the most sensitive end points of TCDD exposure in mouse and human (Holsapple *et al.*, 1991; Sulentic and Kaminski, 2011). One early study to demonstrate the humoral immune system as a sensitive target to TCDD-mediated immunosuppression was conducted by the Holsapple group. In this *in vivo* mouse study, TCDD elicited a concentration-dependent suppression of the primary IgM responses to sheep red blood cells (sRBC), LPS, and DNP-ficoll (Holsapple *et al.*,

1986). Additional mouse *in vitro* studies using separation and reconstitution techniques showed that only cultures containing B cells from TCDD-dosed mice had a significant suppression of the primary IgM antibody response (Dooley and Holsapple, 1988; Morris and Holsapple, 1991). Another *in vitro* study also supports evidence that IgM secretion from LPS-activated B cells is suppressed upon TCDD exposure in humans (Zhang *et al.*, 2010). In addition to these studies, several reports from our laboratory have also suggested that B cells are one of the sensitive targets of TCDD-mediated immunosuppression with respect to the primary IgM response. As mentioned, μ gene expression produces the IgM antibody and *in vitro* TCDD treatment suppressed μ gene expression in a concentration-dependent manner, while TCDD congeners of lower affinity for AHR were unable to downregulate this expression, suggesting that the affinity for AHR also affects the primary antibody response (Sulentic *et al.*, 2000). Most studies describing the effect of TCDD on the IgM response have been performed in mouse models. However, studies conducted with purified human B cells have also shown that TCDD-mediated IgM suppression occurs in a concentration-dependent manner and the magnitude of this response is consistent with the effect seen in the mouse model (Lu *et al.*, 2010).

Additional evidence has also supported the notion that AHR is required for TCDD mediated immunosuppression. In the absence of an exogenous ligand, activation of primary lymphocytes increased expression of AHR, enhanced AHR-ARNT DNA binding, and induced CYP1A1 expression (Crawford *et al.*, 1997). These effects are all hallmarks of TCDD induced AHR activation and also explains the sensitivity of B cells to TCDD. Few endogenous AHR ligands have been discovered which include bilirubin,

bilivirdin, lipoxin A4, and several tryptophan metabolites such as 6-formylindolo[3,2-b]carbazole (FICZ) and 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid (ITE) (Fujii-Kuriyama and Mimura, 2005; Wincent *et al.*, 2009; Yoshida *et al.*, 2012). It was found that the endogenous AHR agonist ITE suppressed the primary antibody response in purified mouse activated B cells (Yoshida *et al.*, 2012). In addition to the ITE-mediated suppression of the antibody response, the mRNA levels of CYP1A1 were found to be elevated in ITE treated purified mouse B cells. The mouse B cell lymphoma cell lines CH12.LX and BCL-1 have been demonstrated to be a useful *in vitro* model to elucidate the mechanism of TCDD mediated immunosuppressive effects as these cells could be activated with LPS into IgM secreting plasma cells. They also allow for determining the role AHR has in modulating the IgM antibody response as the CH12.LX expresses AHR where as the BCL-1 cell line lacks AHR. Using these two cell lines, it was found that LPS is able to induce a primary IgM response in both CH12.LX and BCL-1 cells but treatment with TCDD only induced a concentration-dependent suppression in the IgM response of CH12.LX cells (Sulentic *et al.*, 1998). An AHR murine knockout model supports this finding as the primary antibody response in AHR knockout (AHR^{-/-}) mice remained unaffected with TCDD treatment where as wild type mice with the same genetic background demonstrated suppression (Vorderstrasse *et al.*, 2001). These two pieces of evidence suggest that AHR is required for eliciting the toxic effects of TCDD and may play a role in modulating the IgM antibody response.

Several studies have also identified that a window of sensitivity exists for the humoral immune system suppression by TCDD. Time of addition studies conducted *in vivo* showed that TCDD was unable to suppress the IgM response if added 3 hours

post LPS activation (Holsapple *et al.*, 1986), and 24 hours post-sRBC stimulation (Tucker *et al.*, 1986). This window of sensitivity was also shown *in vitro* in CH12.LX cells, where TCDD added 24 hours post-LPS activation was unable to elicit suppressive effects on the IgM antibody response (Crawford *et al.*, 2003). Based on the establishment of a window of sensitivity it is likely that in addition to the alteration in effector function, TCDD also alters early activation signaling events in the B cell.

B cell differentiation into a plasma cell is partly dependent on a bistable transcription factor network and much of the early focus was on elucidating mechanisms by which TCDD mediated immunosuppression was dependent on gene network expression modifications. Pax5 is responsible for the maintenance of the resting naïve B cell and acts as a transcriptional repressor on BLIMP-1 (Yasuda *et al.*, 2012). From mouse *in vitro* and *in vivo* studies it has been determined that TCDD mediates concentration- and dose-dependent increases in mRNA and protein expression of Pax5 (North *et al.*, 2009; Schneider *et al.*, 2008; Yoo *et al.*, 2004). The TCDD-mediated increase in Pax5 expression and its inverse relationship with BLIMP-1 led to additional studies using CH12.LX cells and primary mouse splenocytes to evaluate the effects of TCDD on BLIMP-1 and its target transcription factors. It was found that TCDD suppressed mRNA and protein expression of BLIMP-1 in LPS-activated B cells (North *et al.*, 2009; Schneider *et al.*, 2009). The study conducted by Schneider *et al.*, also determined that TCDD reduced BLIMP-1 binding to the promoter region of the Pax5 gene. These studies along with others have also detected a reduction in AP-1 expression upon exposure of TCDD in LPS-activated B cells (Suh *et al.*, 2002). Similar to Pax5, LPS-activated CH12.LX cells showed TCDD elicited concentration-dependent

increases in Bach2 (De Abrew *et al.*, 2011). With the recent advances in stochastic modeling, it was hypothesized that these gene expression changes occur in an all-or-none fashion and modeling has predicted that TCDD induced modifications in AP-1 and Bach2 expression can result from an all or none mode of suppression (Zhang *et al.*, 2013).

Despite the alterations in the B cell differentiation regulatory gene network, the mechanisms by which TCDD can affect the early events in B cell activation have yet to be elucidated. The relationship between the key transcription factors AP-1 and BCL-6 that mediate B cell differentiation and upstream targets are currently being evaluated to help link perturbations in the activation of early signaling events and perturbations in the primary antibody response upon TCDD exposure. LPS activation of B cells is mediated by TLR4 and the receptor signaling converges on the JNK and ERK signalling cascades. JNK has been shown to phosphorylate c-Jun which is a component of AP-1, thereby inducing the transcription of AP-1 (Smeal *et al.*, 1991). ERK has been shown to regulate B cell differentiation by phosphorylating BCL-6 targeting it for proteosomal degradation thus lifting its repression on AP-1 and BLIMP-1 (Niu *et al.*, 1998; Yasuda *et al.*, 2011). TCDD has been shown to inhibit the phosphorylation of ERK and JNK in both mouse and human *in vitro* studies suggesting that ERK and JNK activation was inhibited (Lu *et al.*, 2011; North *et al.*, 2010). This bottom up approach is additional evidence that TCDD plays a role in mediating alterations of the early events that occur in B cell activation in addition to perturbing effector function.

Most of the knowledge of TCDD-mediated immunosuppression have been obtained from studies that utilize mouse and human models but some studies have also

been conducted in several rat strains with similar findings to the mouse and human of TCDD-mediated suppression of the primary antibody response. A study performed in mouse and Fischer 344 rats observed that TCDD suppressed the primary antibody response to TNP-LPS in both species (Smialowicz *et al.*, 1996). While this study found TCDD could mediate suppression of the antibody response in rats, it was noted that the dose required to induce the immunosuppressive effects in the rat (30µg/kg TCDD) was higher than the dose necessary to induce immunosuppression in the mouse (10µg/kg TCDD), suggesting that the mouse is more sensitive than the rat to TCDD. In female Brown Norway rats, serum IgE and IgG in response to house dust mite allergens was decreased upon exposure to TCDD (Luebke *et al.*, 2001). TCDD-mediated suppression has also been shown in Sprague-Dawley rats as the primary antibody response to sRBC was significantly suppressed in rats dosed with 100µg/kg TCDD (Pazdernik and Rozman, 1985). In addition, it has also been demonstrated in Wistar albino rats that TCDD exposure decreased IgM secretion and the suppression of IgM secretion could be partly recovered by the anti-oxidant curcumin, a spice found in turmeric (Çiftçi, 2011).

While TCDD-mediated suppression of the primary antibody response has been shown in mouse, human and rat, the rat model is of particular interest as TCDD-mediated enhancement of the primary antibody response have also been observed. One of the first studies conducted in the rat compared the effects of TCDD between mouse and Fischer 344 and Long-Evans rats by using the plaque forming cell (PFC) assay, which measures IgM secreting cells. This study demonstrated TCDD decreased the PFC response to sRBC in the mouse in a concentration-dependent manner;

however, in the Fischer 344 rat PFC response to sRBC was enhanced in a concentration dependent manner with TCDD treatment and an increase in the PFC response in Long-Evans rats treated with 1µg/kg TCDD. (Smialowicz *et al.*, 1994). Another early study performed in Fischer 344 rats demonstrated that TCDD enhanced the humoral immune response to a *Trichinella spiralis* infection by observing an increased number of encysted larvae; larvae encystment occurs after the parasite has been destroyed in an antibody-dependent manner (Luebke *et al.*, 1995). The IgG antibody response has also been found to be enhanced by TCDD as IgG secretion in response to sRBC was increased in a concentration-dependent manner in Sprague-Dawley rats treated with TCDD (Fan *et al.*, 1996). An additional study which exposed rats to TCDD over a longer time period has also confirmed the enhancement of the primary antibody response in rats. In rats dosed with 0.1µg/kg TCDD for four weeks, it was observed that TCDD increased the expression of Igλ1 and 2 chain C-regions which are components of the Ig light chain (Son *et al.*, 2003). Although, the mechanism by which TCDD mediates enhancement and suppression of the antibody response is unknown, it is clear that TCDD is capable of modulating B cell function in the rat.

E. AHR plays a role in development and homeostasis

Although several targets of B cell activation and effector function are known to be affected by TCDD, the specific mechanisms by which TCDD elicits toxicity have not yet been elucidated. In both rodents and humans, a range in sensitivity to the toxic effects of TCDD has been observed and has been attributed in part to genetic variations such as single nucleotide polymorphisms (SNPs) in AHR (Hao and Whitelaw, 2013; Nebert,

1989). Recent studies using microarray technology have allowed for the detection of species differences across human, mouse, and rat species. In microarray comparisons between primary mouse, rat, and human hepatocytes treated *in vitro* with TCDD, it was found that AHR activation via TCDD elicits species specific alterations in global gene expression and the sensitivity to TCDD-mediated differential gene expression was found markedly divergent among species (Black *et al.*, 2012; Forgacs *et al.*, 2013). With the increasing evidence that TCDD produces differential toxic effects, several AHR^{-/-} animal models have been constructed to understand the function of AHR in greater detail.

Using AHR^{-/-} mouse models, it became evident that AHR also plays a role in tissue development and homeostasis. AHR^{-/-} mice on C57/B6 backgrounds with targeted disruptions in exon 1 or exon 2 have been generated in three separate laboratories (Esser, 2009). Using zinc finger technology, an AHR^{-/-} rat has also recently been created by the targeted deletion of exon 2 which contains the bHLH domain that is involved in DNA binding and dimerization with ARNT (Harrill *et al.*, 2013). The targeted deletion of AHR exon 2 by zinc finger technology uses specifically engineered zinc finger proteins that are coupled to the *FokI* restriction enzyme to induce DNA double stranded breaks that are then repaired by homology directed repair (HDR) or nonhomologous end joining (NHEJ) resulting in the deletion of specific areas in the genome allowing for genomic disruptions (Urnov *et al.*, 2010). The targeted deletion of AHR in the knockout rat was confirmed at both the mRNA and protein level. Western blot analysis of liver tissue detected the 96kDa AHR protein in wild type rats and the

same band was absent in the AHR^{-/-} rats (Harril *et al.*, 2013). Furthermore, a reduction in AHR mRNA was detected by three separate qPCR assays (Harril *et al.*, 2013). Additionally, the wild type and AHR^{-/-} rats received a single dose of 25µg/kg TCDD. In wild type rats an increase in expression levels of CYP1A1, CYP1A2, and CYP1B1 was detected and this increase of metabolic enzyme expression was absent in the TCDD-dosed AHR^{-/-} rats (Harril *et al.*, 2013). Another hallmark of TCDD exposure is an increase in liver weight and a decrease in thymic weight (Bunger *et al.*, 2008). As expected, wild type rats dosed with TCDD showed increases in liver weight and decrease in thymic weight while AHR^{-/-} rats displayed no liver or thymic weight changes. Taken together, the targeted deletion of AHR in rat has been confirmed creating the AHR^{-/-} rat animal model and this model is resistant to some of the known toxic effects of TCDD.

Despite the AHR^{-/-} rat resistance to TCDD the phenotypic characterizations of this newly developed animal model have not yet been fully evaluated and phenotypic characterizations in organ development has recently come to light. Comparisons between the three AHR^{-/-} mouse models have been made however since the focus of this thesis is on B cell function in the AHR^{-/-} rat, the AHR^{-/-} mouse model developed by the Bradfield group will be used for comparisons in phenotype since both knockout models contain the targeted deletion exon 2 in the AHR gene. In characterizing the phenotype of the AHR^{-/-} rat several phenotypic differences between the AHR^{-/-} rat and

mouse were detected. In all three knockout mouse models, the most notable phenotype is a reduction in liver weight which has been attributed to a patent ductus venosus that results in a reduction of blood flow to the liver (Harstad *et al.*, 2006; Schmidt *et al.*, 1996). The perfusion of AHR^{-/-} mouse and rat with contrast media was used to determine if a patent ductus venosus was present in the AHR^{-/-} rat and a distinct color change of the liver as well as of outflow from the vena cava was seen in the AHR^{-/-} rat with the color change and outflow absent in AHR^{-/-} mouse (Harril *et al.*, 2013). This data indicates that the portal vasculature is unaffected in the AHR^{-/-} rat and they do not display the patent ductus venosus seen in the AHR^{-/-} mouse. Another phenotypic characteristic seen in the AHR^{-/-} mouse is the presence of a persistent hyaloid artery of the eye, this characteristic is absent in the AHR^{-/-} rat (Harril *et al.*, 2013). With regards to immune function, it has been found in AHR^{-/-} mice that there is a decrease in the percentage of mature B cells and an increase in the rise of B cell precursors compared to wild type (Thurmond *et al.*, 2000). Preliminary characterizations of the immune system in male AHR^{-/-} rats were evaluated by complete blood counts (CBC) and no alterations in the number of red blood cells or white blood cells was detected in female AHR^{-/-} rats. However, male AHR^{-/-} rats displayed a decrease in the number of platelets and leukocytes with no alteration in platelet function detected, while the number of erythrocytes, monocytes, neutrophils, and eosinophils remained unaffected. The one effect of the targeted deletion of AHR that is specific to the AHR^{-/-} rat are renal

abnormalities including hydronephrosis, hydroureters and bilateral lesions in the ureters and renal pelvis. These tissue abnormalities are absent in the $AHR^{-/-}$ mouse (Harril *et al.*, 2013). Histology performed on the urinary tract of the $AHR^{-/-}$ rats detected histopathological changes in the kidney and ureters and are thought to be secondary to the hydronephrosis and hydroureters (Harril *et al.*, 2013). Harril *et al.*, suggests that the renal abnormalities are due to a dysregulation in the alternative splicing of Wilm's tumor suppressor 1 (WT1) as the alternative splicing of WT1 mRNA has been shown to be regulated by AHR and play a role in kidney development.

Experimental Design

The majority of TCDD-mediated immunosuppressive effects on B cell activation and effector function that have been studied have been conducted with mouse and human B cells. With both model systems in place the mechanism by which TCDD mediates its immunosuppressive effects has yet to be determined. Using the newly developed AHR knockout rat animal model may identify possible mechanisms that have yet to be observed in the mouse and human. The purpose of this study was to evaluate the involvement of AHR and understand the effects TCDD plays on the humoral immune system with a focus on B cell activation and effector function using a AHR^{-/-} rat model. It was hypothesized that the targeted deletion of the aryl hydrocarbon receptor (AHR) results in the loss of sensitivity of the B cell to suppression by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and conversely the augmentation of the primary IgM antibody response, which is mediated in part, through a greater magnitude in B cell activation in the absence of TCDD.

A. Specific Aim 1:

To identify whether the targeted deletion of AHR affects the magnitude of B cell activation and reduces the sensitivity of the B cell to TCDD-mediated suppression by detecting increases in surface activation marker CD80, CD86, and MHC II expression. Activation with LPS upregulates the expression of CD80, CD86, and MHCII on the B cell surface and signifies early B cell activation. The level of expression can provide information concerning the magnitude of activation. To date, there has been no study

evaluating the effect of targeted deletion of AHR on rat B cell activation but it has been shown that the targeted deletion in mice results in a down regulation of CD80 and CD86 in antigen presenting cells (Jux *et al.*, 2009; Singh *et al.*, 2011). Flow cytometry will be used to identify the basal expression patterns and expression pattern changes upon activation with LPS in wild type and AHR^{-/-} rats dosed with the AHR agonist TCDD.

B. Specific Aim 2:

To evaluate whether the targeted deletion of AHR affects the magnitude of the IgM antibody response and reduces the sensitivity of the B cell to TCDD-mediated suppression by detecting increases in IgM. The downstream effector function of B cells, signified by the primary IgM antibody response, may be indirectly related to the magnitude of B cell activation. Increases in the expression of CD80, CD86, and MHCII by the targeted deletion of AHR may suggest similar changes in magnitude of the IgM antibody response. Wild type and AHR^{-/-} rats dosed with TCDD will be activated with LPS to induce the IgM antibody response. IgM secretion in addition to intracellular IgM will be evaluated by ELISA and flow cytometry, respectively, to determine the magnitude to which targeted deletion of AHR can affect B cell effector function.

Materials and Methods

A. Animals

5-8 week old female Sprague-Dawley rats purchased from Charles River (Wilmington, MA) were used in the optimization of B cell activation studies and euthanized with 100mg/kg phenobarbital. All animals were used in accordance to the guidelines set by the Michigan State University Institutional Animal Care and Use Committee. This study was performed in collaboration with the laboratory of Dr. Russell Thomas at The Hamner Institute for Health Sciences, Institute for Chemical Safety Sciences (Research Triangle Park, NC) and all AHR^{-/-} rats and wild type control rat spleens used in this study were received from that facility. Female AHR^{-/-} and wild type control animals were housed and cared for as described previously (Harril *et al.*, 2013).

B. Optimization of B cell Activation

To determine the appropriate activation for rat B cells, preliminary studies using Sprague-Dawley rats were performed to identify the conditions that will be used during the study. AHR^{-/-} rats and wild type controls were shipped from the Hamner Institute for Health Sciences and to mimic shipping conditions spleens were placed in 5mL of RPMI 1640 supplemented with 10% bovine serum overnight at 4°C. Splenocytes were isolated via aseptic mechanical disruption and split into two aliquots; one aliquot was reserved for erythrocyte lysis by ammonium chloride potassium (ACK) buffer and is referred to as the lysed group, while the second aliquot remained untouched and is

referred to the non-lysed group, each aliquot was put into a single cell suspension at 5×10^6 cells/mL. Lysed and non-lysed cells were plated in duplicate with a naïve control and splenocytes were activated with either 15µg/mL pokeweed mitogen (PWM), 15µg/mL lipopolysaccharide (LPS), 30µg/mL PWM or 30µg/mL LPS. Cells were cultured at 37°C with 5% CO₂ in RPMI 1640 supplemented with 10% bovine serum and 100 units/mL penicillin, 100 units/mL streptomycin. Supernatants for IgM sandwich enzyme linked immunoabsorbent assay (ELISA) were collected at days three and four of culture and frozen at -80°C in bovine calf serum supplemented with 10% dimethyl sulfoxide (DMSO) until ready for analysis.

To further evaluate the proper culture conditions for B cell activation the concentrations of LPS used were expanded. For this preliminary study one rat spleen was incubated overnight as above to mimic shipping conditions. Erythrocytes were not lysed and single cell suspensions containing 5×10^6 cells/mL were plated in triplicate. Cells were activated with 10, 15, 30, or 60µg/mL LPS; a non-activated naïve control group was also included. Cells were cultured at 37°C with 5% CO₂ for three days and the supernatants were collected for IgM ELISA as described below.

C. AHR^{-/-} and Wild Type Rat Dosing and Spleen Collection

All dosing and tissue collection was performed by the laboratory of Dr. Russell Thomas at The Hamner Institute for Health Sciences, Institute for Chemical Safety Sciences. Female wild type and AHR^{-/-} rats were dosed with TCDD at 3, 22, 100, 300,

or 1000 ng/kg/day for 5 days a week for four weeks. TCDD was suspended in corn oil and control rats received this vehicle, the delivery volume was 5mL/kg and dosing solutions were prepared as previously described (2006). After four weeks of dosing rats were euthanized as described in Harril et al. 2013, the spleens were collected and cut into two segments sagittally. One half of the spleen was shipped in 1640 RPMI mediate supplemented with 10% bovine serum on wet ice overnight and received for immediate processing the next day.

D. Lymphocyte Isolation and Culture

Spleens received from the Hamner Institute for Health Sciences in two separate shipments and are denoted as group one and two respectively. Splenocytes were isolated via mechanical disruption aseptically to produce a single cell suspension (1×10^7 cells/mL) in RPMI 1640 supplemented with 10% bovine serum, 100 units/mL penicillin, 100 units/mL streptomycin, and 2.1 g/L sodium bicarbonate. Splenocytes were plated at 0.5×10^6 cells/well in a 48 well plate. Cells were treated with LPS (15µg/mL) or vehicle to produce a non-activated control group and a LPS-activated group. Cells were cultured at 37°C with 5% CO₂. After 72 hours, plates were centrifuged at 3000 rpm for 5 minutes and the supernatant was collected in 200µL aliquots and frozen at -80°C for the IgM ELISA assay. The remaining cells were collected and washed once in protein free buffer 1 x Hank's Balanced Salt Solution (HBSS, pH 7.4; Invitrogen) for flow cytometry staining and analysis.

E. Flow Cytometry Analysis

Cells collected from group one were stained the same day while the cells collected from group two were frozen in bovine calf serum with 10% DMSO at -80°C until ready for assessment. Cells were first stained with Live/Dead Fixable Dead Cell Stain Kit near-infrared (Invitrogen, Grand Island, NY) for 15 minutes then washed twice with 1 x HBSS. Prior to surface and intracellular protein marker staining, the surface Fcγ receptor was blocked by incubation with mouse anti-rat CD32 (BD Pharmingen, San Jose, CA). All antibodies used for surface and intracellular protein staining are listed in appendix A and are conjugated to fluorescent dyes. All procedures were performed in the dark.

All surface markers were stained simultaneously in fluorescence-activated cell sorting (FACS) buffer which consisted of 1 x HBSS with 0.1% sodium azide (pH 7.6, Sigma-Aldrich, St. Louis, MO) and 1% bovine serum albumin (BSA, Calbiochem, Billerica, MA). In addition to the fluorescently labeled antibody for surface marker staining, a non-fluorescent mouse anti-Rat IgM (BD Pharmingen, San Jose, CA) was used to block the surface IgM for the detection of intracellular levels of IgM. All samples were incubated with the antibodies for 20 minutes and washed three times with FACS buffer. The cells were then incubated with CytoFix fixation buffer (BD Biosciences, San Jose, CA) for 10 minutes and washed once with FACS buffer.

Intracellular IgM staining was performed after surface marker staining. Cells were permeabilized by incubation in 1 x Perm/Wash solution (BD Biosciences, San Jose, CA) for 30-60 minutes. The antibody used for intracellular staining was diluted in

permeabilization buffer and incubated for 20 minutes. Cells were washed once with FACS buffer then reconstituted in a final volume of 200 μ L for analysis. All samples were processed with a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA) and evaluated with FloJo (Tree Star Inc., Ashland, OR).

F. IgM ELISA

96-well Immulon 4 HBX plates (Thermo Scientific, Waltham, MA) were assembled and coated with purified mouse anti-Rat IgM antibody (BD Pharmingen, San Jose, CA) at 1:100 in phosphate buffered saline (PBS) overnight at 4 $^{\circ}$ C. The plate was then blocked with PBS containing 3% BSA (3% BSA-PBS) for 30 minutes at 37 $^{\circ}$ C while the supernatants were allowed to thaw on ice. Supernatants were appropriately diluted in PBS and incubated at 37 $^{\circ}$ C for one hour, afterwards a secondary biotinylated mouse anti-rat IgM antibody was added at a 1:2000 dilution in 3% BSA-PBS and allowed to incubate for one hour at 37 $^{\circ}$ C. Streptavidin peroxidase (Sigma-Aldrich, St. Louis, MO) was diluted at 1:2000 in 3% BSA-PBS and incubated for 30 minutes at 37 $^{\circ}$ C. ABTS solution was prepared from ABTS tablets (Roche, Indianapolis, IN) and ABTS buffer (Roche, Indianapolis, IN) based on the manufacturers protocol and added to the plate. The plate was kinetically read at 405nm for 60 minutes at 60 second intervals with 3 seconds of shaking in between reads on a Bio-Teck Synergy HT (Winooski, VT) plate reader. The plates were washed three times with PBS containing 0.05% Tween20 (Sigma-Aldrich, St. Louis, MO) and three times with distilled water between each

addition of samples and reagents. Quantification of samples was prepared from a standard curve made from rat IgM standard (Rockland Immunochemicals, Gilbertsville, PA).

G. Statistical Analysis

The mean was determined for each treatment group ($n = 5$) in individual experiments, the error bars represent the $\pm$ SEM for each group. Homogenous data was analyzed using a one-way analysis of variance (ANOVA) followed by a Dunnett's two tailed t-test to compare treatment groups to non-activated control groups when significant differences were observed ($p < 0.05$). Two- way ANOVA was employed when two variables were involved and followed by a Bonferroni's post-hoc test to determine significant differences among groups. Data expressed as percent were transformed by using $Y = \log(Y)$ prior to statistical analysis. All statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

Results

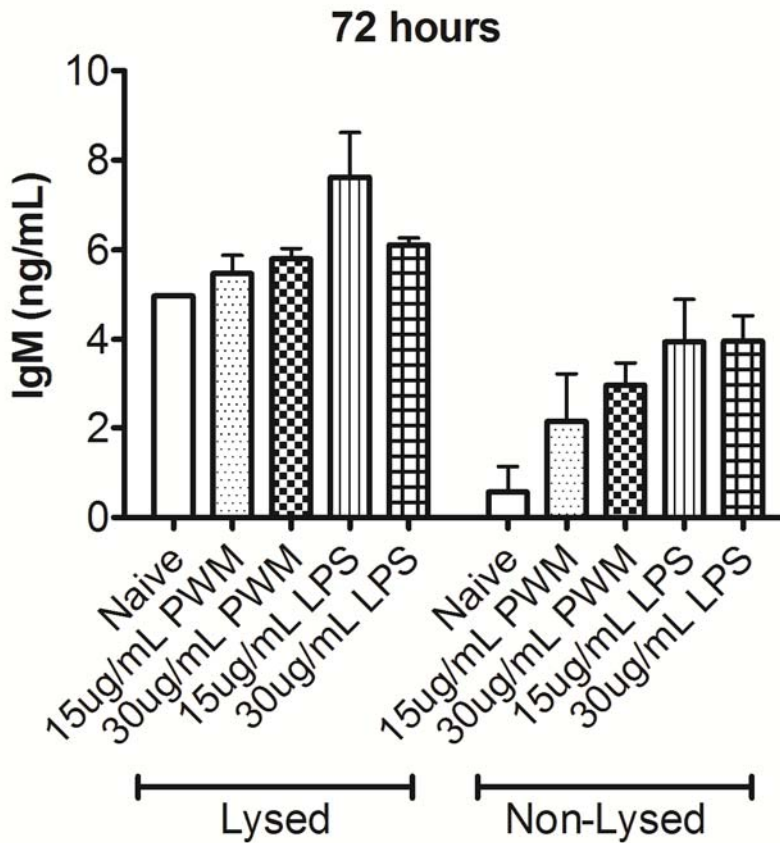
A. Optimization of Culture and Activation Conditions

The first step in this study was to determine the optimal stimulus that would induce B cell activation leading to an IgM antibody response in Sprague-Dawley rats. To induce B cell activation and differentiation into IgM secreting plasma cells the B cell polyclonal activators LPS and PWM were selected. To establish the optimal activator for this study, rat splenocytes were treated with 15µg/mL and 30µg/mL of LPS and 15µg/mL and 30µg/mL of PWM and the quantity of secreted IgM was measured by ELISA after 72 and 96 hours. The activator that yielded the highest IgM response was 15µg/mL LPS at both 72 and 96 hours of culture (Figure 1A and 1B). Rat B cells responded modestly to both 15µg/mL and 30µg/mL PWM and secreted a lower quantity of IgM in comparison to similar concentrations of LPS. Based on these data it was determined that LPS would be used in subsequent experiments and the dose of LPS was further optimized by expanding the LPS concentration response curve. An increase in the IgM antibody response of activated primary rat B cells was seen in comparison to naïve (inactivated) cells; however, there was no significant differences in IgM secretion observed with increasing concentrations of LPS above 10µg/mL (Figure 1C).

Red blood cells (RBCs) present in culture have been shown to enhance the responses of B cells to activators and prior to beginning the study we wanted to confirm this effect (Yachnin, 1972). Single cell suspensions of splenocytes were split into two aliquots; one aliquot containing erythrocytes (non-lysed group), and in the

second aliquot the erythrocytes were removed by lysis with ACK buffer (lysed group). The lysed group secreted more IgM compared to the non-lysed group at 72 and 96 hours (Figure 1A and 1B). This result is in contrast to previous studies that have reported an enhanced B cell response to activators in the presence of erythrocytes. Cell counts were performed at both 72 and 96 hours of culture and the lysed group contained a large number of dead cells making quantification of IgM and comparisons between naïve and activated B cells difficult. The removal of erythrocytes from splenocyte preparations allowed for a higher level of IgM secretion but due to the cell death that had taken place in the lysed naïve control, it was determined that primary cell cultures of wild type and AHR^{-/-} rat splenocytes would retain the erythrocytes.

A.



B.

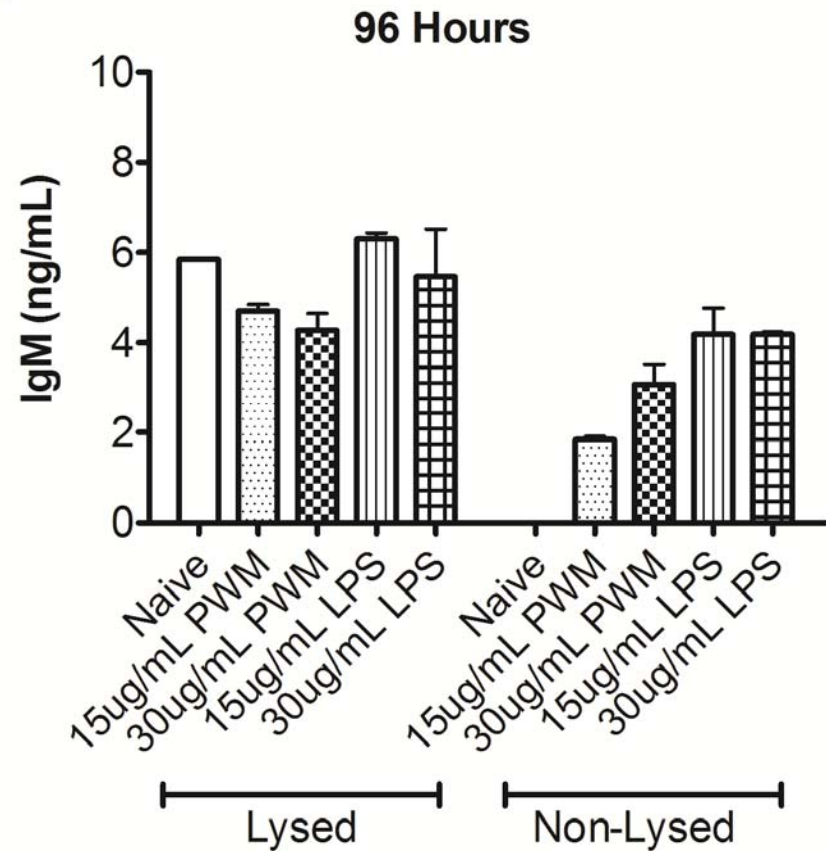
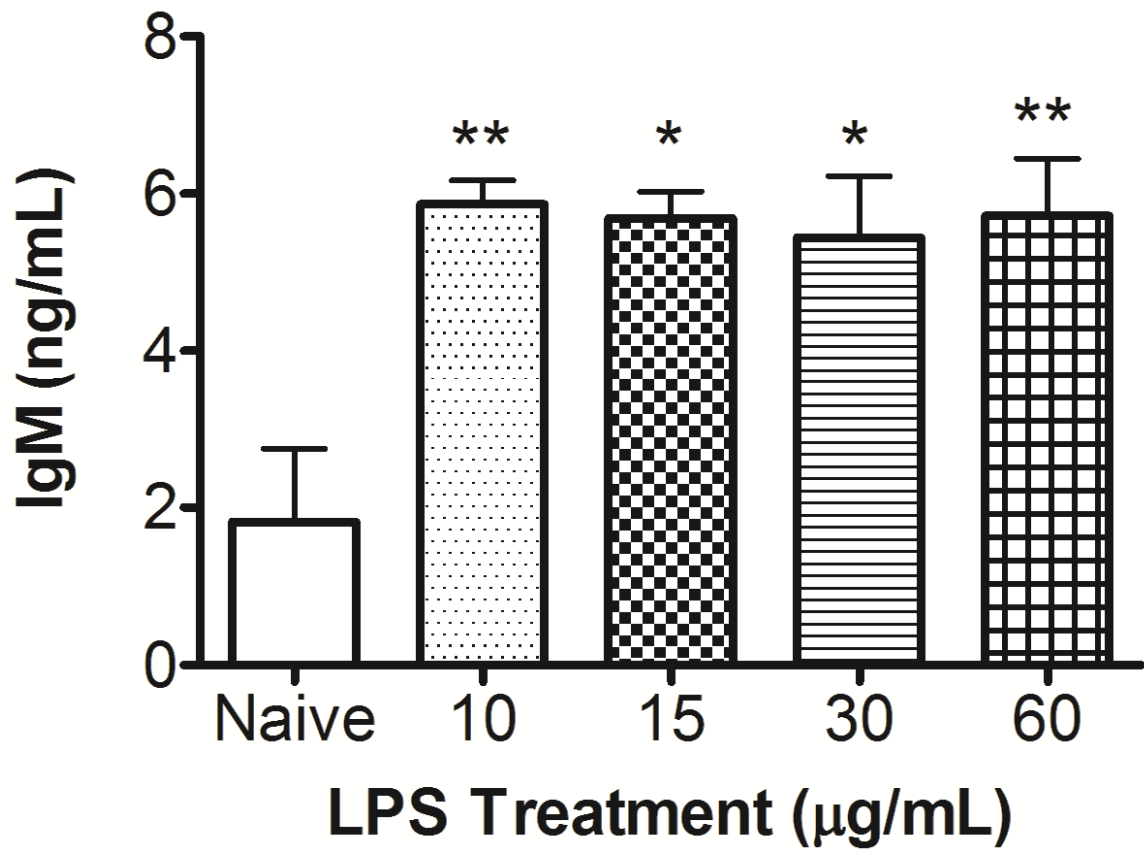


Figure 1. Optimization of primary Sprague-Dawley rat B lymphocyte culture and activation conditions. Splenocytes were isolated from one rat and erythrocytes were either lysed with ACK buffer or allowed to remain in culture (non-lysed) and activated with 15 μ g/mL or 60 μ g/mL of LPS or 15 μ g/mL or 60 μ g/mL of PWM. Supernatant were collected at 72 hours (A) and 96 hours (B) and an ELISA was performed to detect levels of secreted IgM. C depicts an ELISA at three days of culture with non-lysed primary rat B cells treated with 10, 15, 30, or 60 μ g/mL LPS. Panels A and B are results from one rat with each treatment group plated in duplicate. In C, data presented is the mean $\pm$ SEM from each treatment plated in triplicate. * P < 0.05 and ** P < 0.01 compared to naïve.

Figure 1 (cont'd)

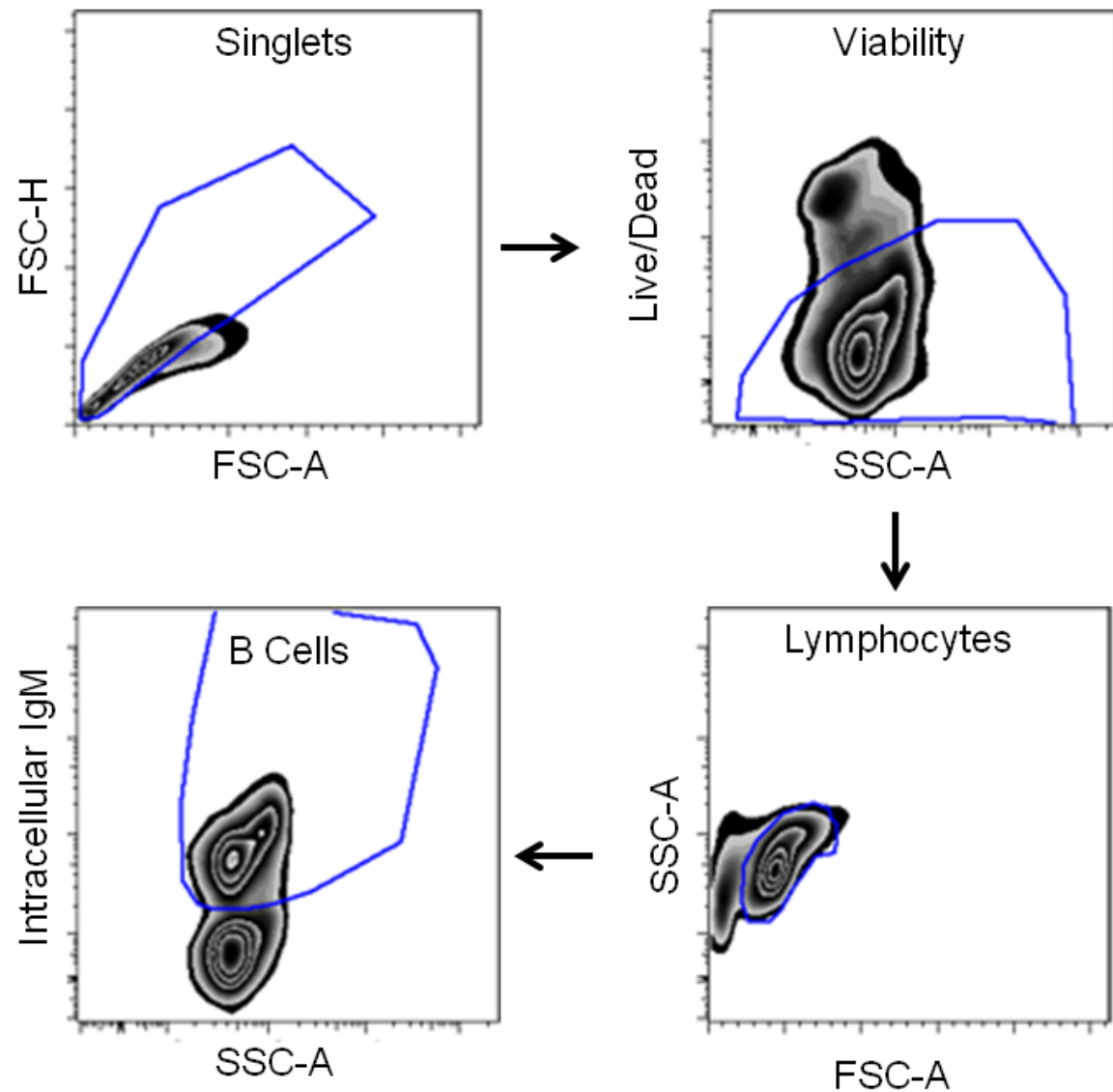
C.



B. Evaluation of Rat B cell Activation by the Measurement of Activation Markers

Wild type and AHR^{-/-} rat splenocytes were examined by flow cytometry and B cells were identified using the gating scheme shown in Figure 2. First, splenocytes were gated to identify single cells (singlet gate) to eliminate artifacts created by doublets in the analysis. Within the singlet gate, splenocytes were gated on viability (viable gate) to eliminate dead cells from analysis. Lymphocytes were identified within the viable gate based on cell size and granularity. The final gate identified B cells within the lymphocyte gate by the presence of IgM. The two groups were analyzed separately to prevent a loss in the detection of significant differences among the activated and TCDD treatments since the two groups were shipped separately and may have been handled under different shipping conditions. A second rationale for analyzing the groups separately was that group one was processed immediately after collection for flow cytometry analysis while the cells from group two were frozen at -80°C before processing for flow cytometry.

Figure 2. Schematic representation of the flow cytometry gating for the identification of B cells within $AHR^{-/-}$ and wild type splenocyte cultures. Samples were first gated on single cells noted as the singlet gate to eliminate doublets which produce artifacts in the sample analysis. This is done by sorting the cells by their size which is forward scatter-height (FSC-H) and forward scatter-area (FSC-A). Taking the singlet gate, a second gate was added to exclude the analysis of dead cells and include only the live cells by the live/dead dye and side scatter-area (SSC-A) which determines cellular granularity; this gate is noted as the viable gate. The lymphocytes were then identified in the viable gate by their size (FSC-A) and granularity (SSC-A). The B cells were then identified from the lymphocyte gate by the presence of intracellular IgM. The gating scheme was applied to all groups analyzed by flow cytometry. “For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.”



I. AHR Deletion Effects on the B cell Activation Marker CD80

To evaluate the potential AHR-mediated alteration of CD80 expression in the rat, basal levels of non-activated and LPS-activated vehicle control B cells from both group one and group two were compared separately. In group one, activation with LPS significantly increased the percentage of CD80⁺ B cells in both wild type (from 4.6% to 8.1%) and AHR^{-/-} (from 5.1% to 16.1%) rats with the AHR^{-/-} having a more robust response to LPS activation when compared to wild type (Figure 3). In group two, LPS activation of wild type B cells increased the percentage of CD80⁺ cells from 5.7% to 13.5% but this upregulation of CD80 was not significant (Figure 4). AHR^{-/-} had a more robust response and significantly increased the percentage of CD80⁺ cells from 6.7% in non-activated B cells to 20.7% in LPS-activated B cells (Figure 4). The observed increase in the percentage of CD80⁺ cells of AHR^{-/-} rats in group one was not seen in group two.

In addition to evaluating the basal expression, the percentage and mean fluorescence intensity (MFI) was also used to determine the density of CD80 expressed on the surface in TCDD-dosed wild type and AHR^{-/-} rat B cells. The percentage of CD80⁺ cells determines the number of B cells expressing CD80 on the surface within a sample and the MFI determines the magnitude of surface expression of CD80 on a per cell basis. In group one; LPS activation significantly increased the percentage of CD80⁺

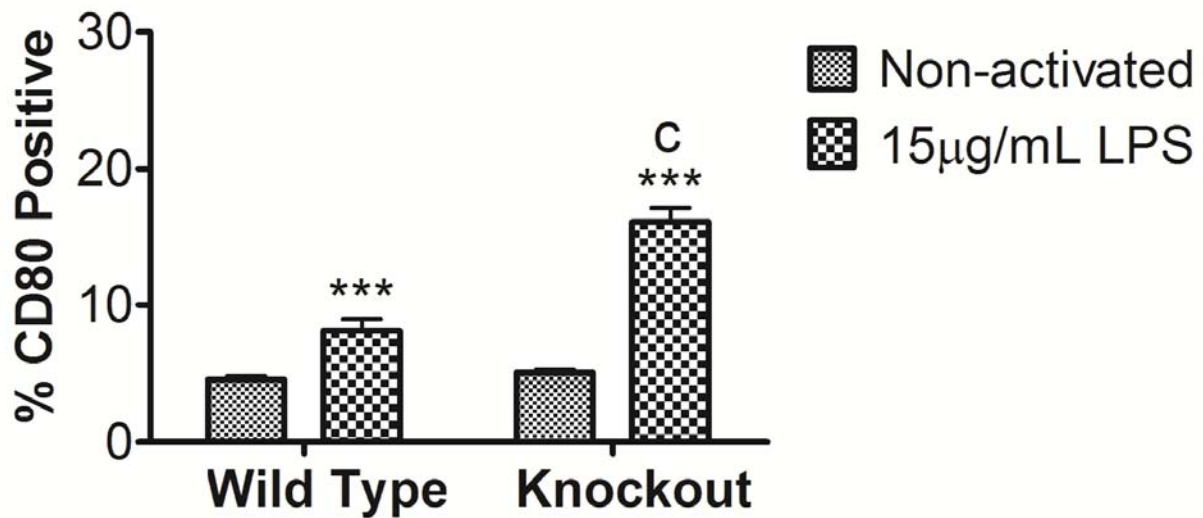


Figure 3. Targeted deletion of AHR leads to a higher percentage of CD80⁺ B cells upon LPS activation compared to wild type in group one. Vehicle control cells in group one were evaluated by flow cytometry for the basal expression of CD80 in wild type and AHR^{-/-} B cells. Cells were also treated with 15µg/mL LPS to activate B cells inducing an upregulation of CD80 expression. Comparisons were made between transformed percent positive data for non-activated and LPS-activated wild type and AHR^{-/-} B cells. Data is presented as the mean ± SEM from five rats per treatment group. *** P ≤ 0.001 compared to non-activated within the genotype. C indicates P ≤ 0.001 compared to LPS-activated wild type cells.

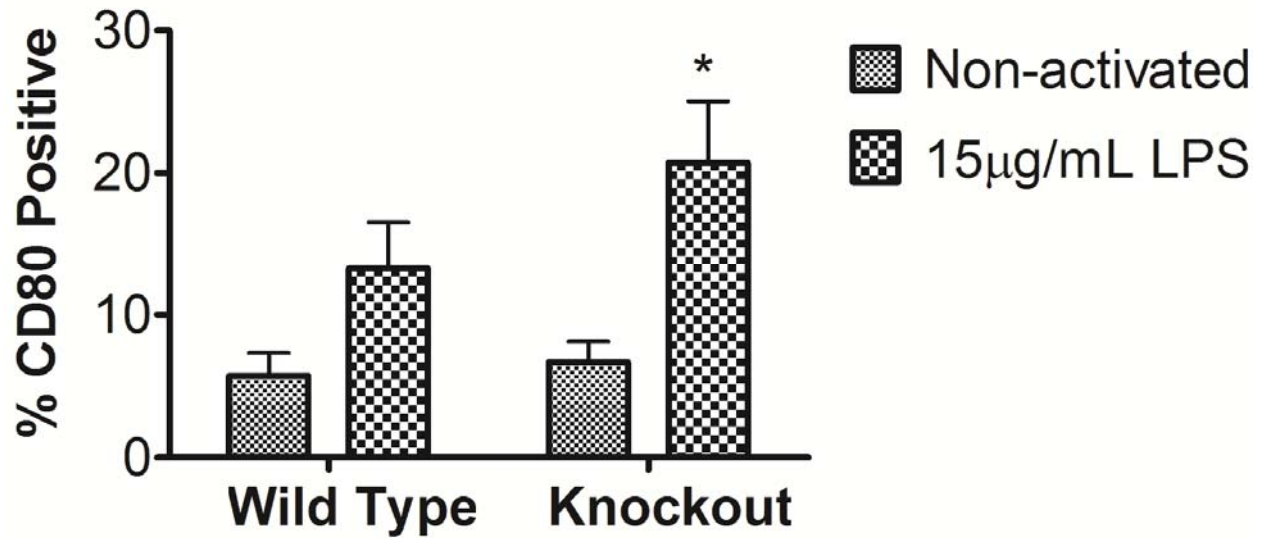


Figure 4. LPS activation increases the percentage of CD80⁺ B cells and the deletion of AHR leads to additional increases in CD80 expression in group two. Vehicle control sets in group two were evaluated by flow cytometry for the basal expression of CD80 in wild type and AHR^{-/-} B cells. Cells were also treated with 15µg/mL LPS to activate B cells inducing an upregulation of CD80 expression. Comparisons were made between transformed percent positive data for non-activated and activated wild type and AHR^{-/-} B cells. Data is presented as the mean ± SEM from five rats per treatment group. * P ≤ 0.05 compared to non-activated within the genotype.

B cells in wild type and AHR^{-/-} rats in comparison to non-activated splenocytes of their respective genotype (Figure 5A and 5B). AHR^{-/-} B cells had an increased response to LPS resulting in a higher percentage of CD80⁺ cells when compared to wild type. In wild type rats, the percentage of CD80⁺ non-activated B cells was significantly decreased at 300 and 1000ng/kg/day TCDD treatment compared to vehicle (Figure 5A). The percentage of CD80⁺ B cells from AHR^{-/-} rats treated with 22ng/kg/day TCDD decreased compared to the B cells from vehicle treated AHR^{-/-} rats (Figure 5B). Even though there is no significant dose-response in LPS-activated AHR^{-/-} B cells there is an overall trend towards a decrease with increasing doses of TCDD. The MFI in wild type and AHR^{-/-} rats of group one remained relatively unchanged upon LPS activation (Figure 5C and 5D). A significant increase in the CD80⁺ MFI was also observed in wild type LPS-activated B cells treated with 300ng/kg/day TCDD (Figure 5C). Whereas in the AHR^{-/-} there is a bell shaped expression pattern with a significant increase peaking at 100ng/kg/day TCDD then decreasing with increasing TCDD doses (Figure 5D). The only significant genotype effect in the MFI was a reduction in LPS-activated AHR^{-/-} B cells at 1000ng/kg/day TCDD treatment (Figure 5D).

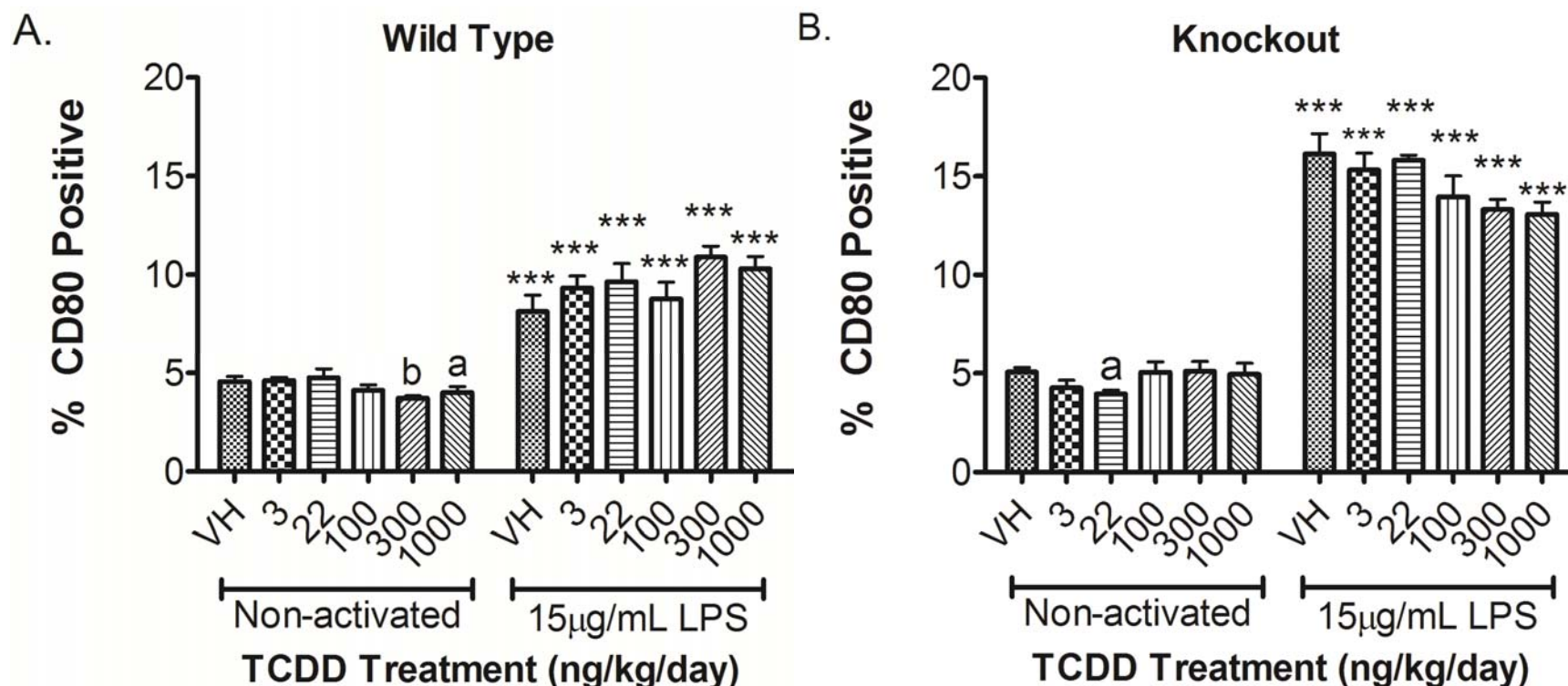
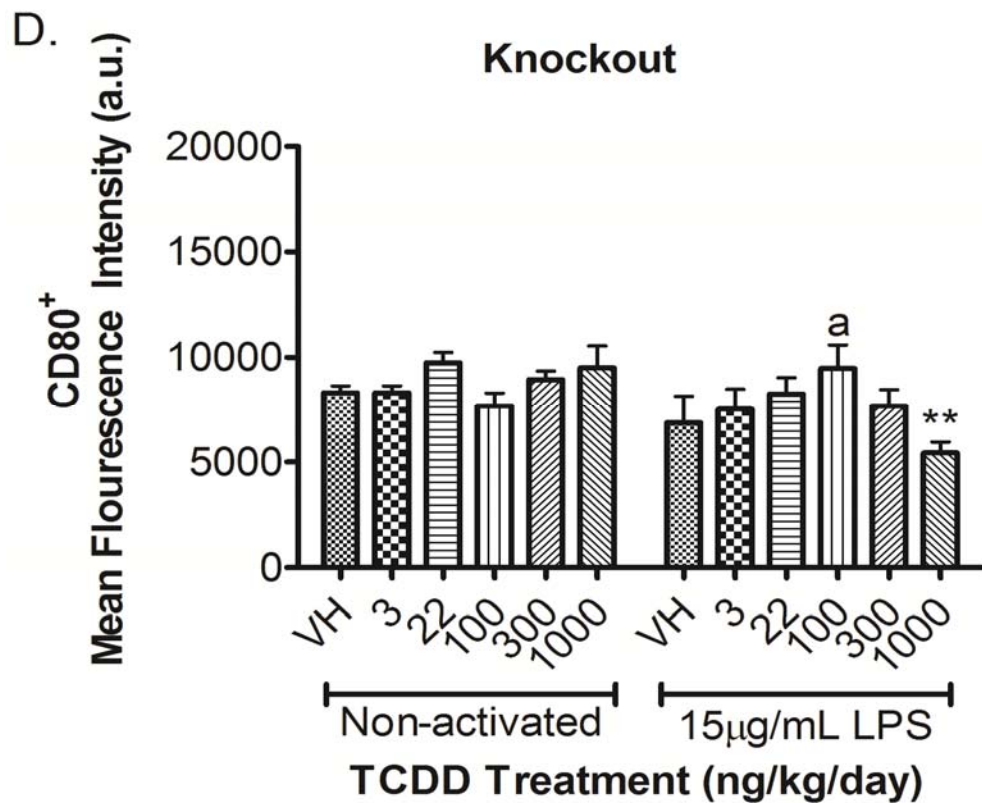
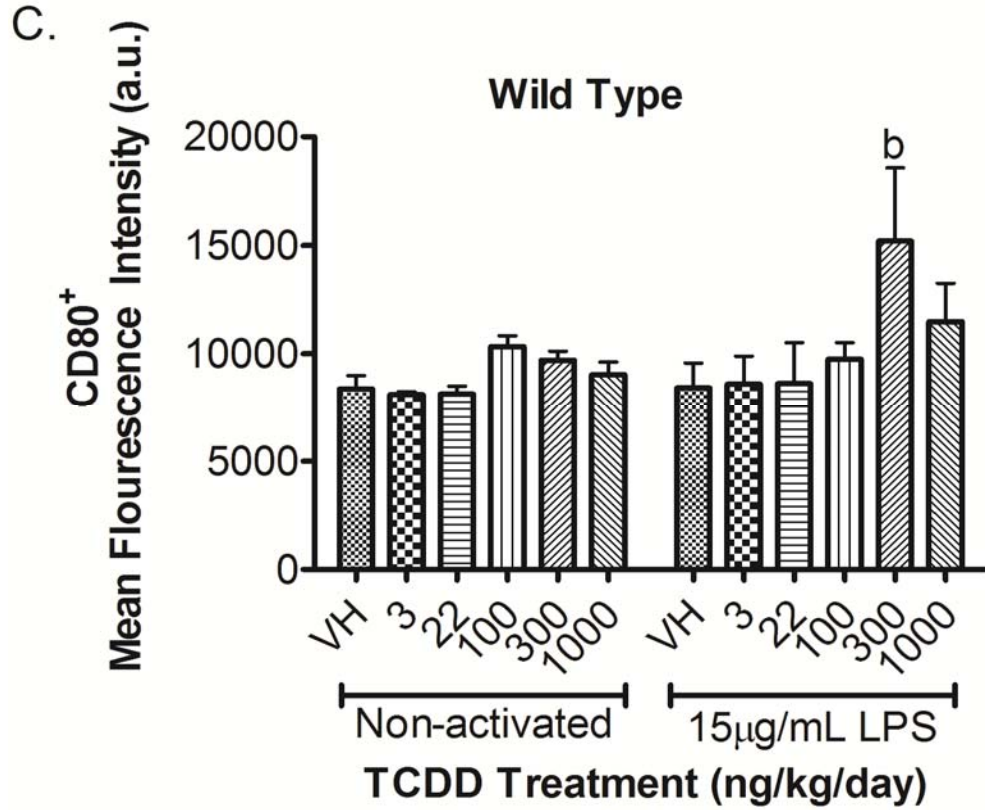


Figure 5. Deletion of AHR produces a higher percentage of CD80⁺ B cells compared to wild type with no significant TCDD suppression observed in either genotype. Data presented are from group one. Wild type and AHR^{-/-} rats were dosed with corn oil vehicle (VH) control and varying concentrations of TCDD. Splenocytes from these rats were activated with 15µg/mL LPS for 72 hours and compared to a non-activated control. Percentage of CD80⁺ B cells in wild type (A) and AHR^{-/-} (B) were measured by flow cytometry. The MFI of CD80⁺ B cells in wild type (C) and AHR^{-/-} (D) were obtained in the same flow cytometry analysis. Data is presented as the mean ± SEM from five rats per treatment. ** P ≤ 0.01 and *** P ≤ 0.001 compared to non-activated B cells within the genotype. a indicates p ≤ 0.05 and b indicates P ≤ 0.01 compared to VH control within the genotype.

Figure 5 (cont'd)



Similar evaluations were made for group two. No significant increase in the percentage of CD80⁺ B cells was observed in wild type upon activation with LPS despite an overall trend towards an upregulation of CD80 (Figure 6A). However, LPS activation led to significant increases in the percentage CD80⁺ B cells of AHR^{-/-} rats in the vehicle, 22 and 100µg/kg/day TCDD treatments, when compared to non-activated B cells of the same treatment (Figure 6B). It was also noticed that the overall response in the percentage of CD80⁺ B cells of AHR^{-/-} rats in group two was more varied compared to group one but the same trend towards a dose dependent decrease is still observed. No significant alterations in the MFI were observed for non-activated and LPS-activated wild type and AHR^{-/-} B cells (Figure 6C and 6D). In contrast to group one, no TCDD effects were detected in the percentage or MFI of CD80⁺ B cells of wild type or AHR^{-/-} rats.

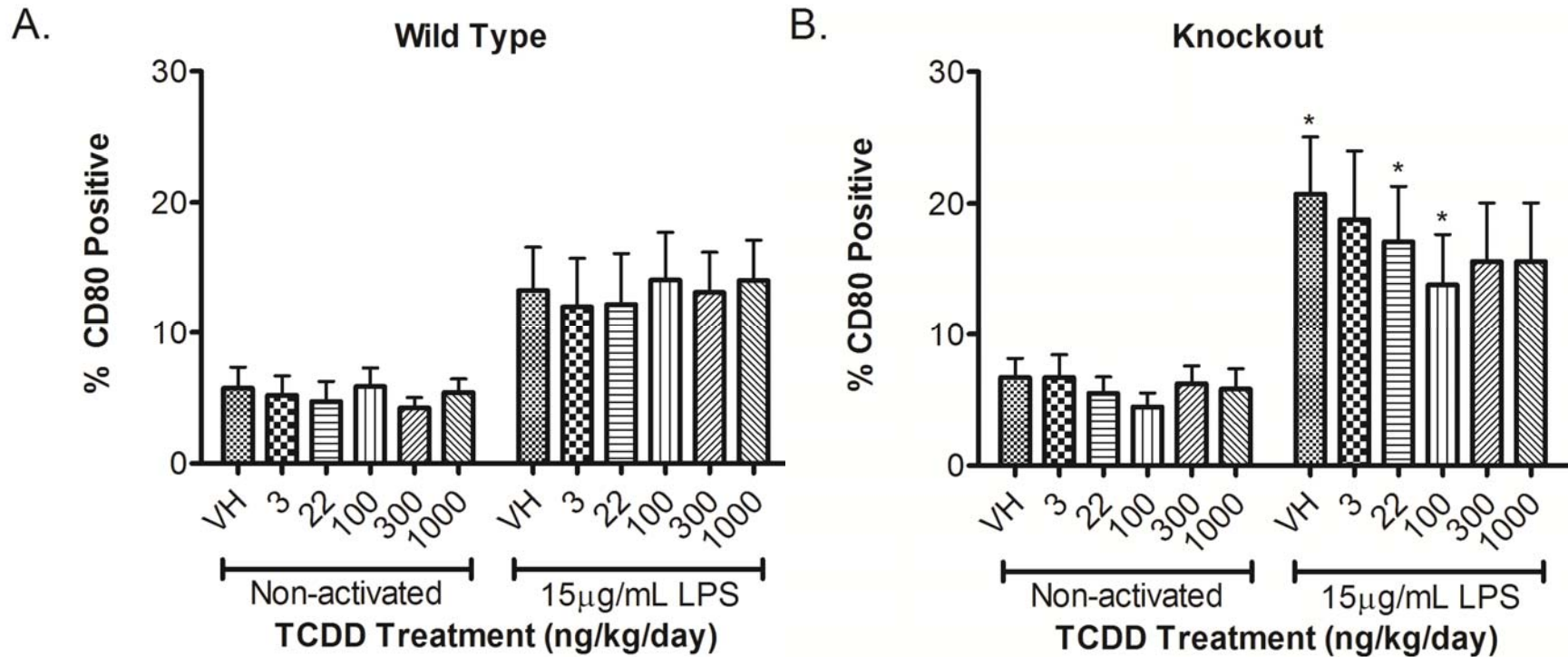
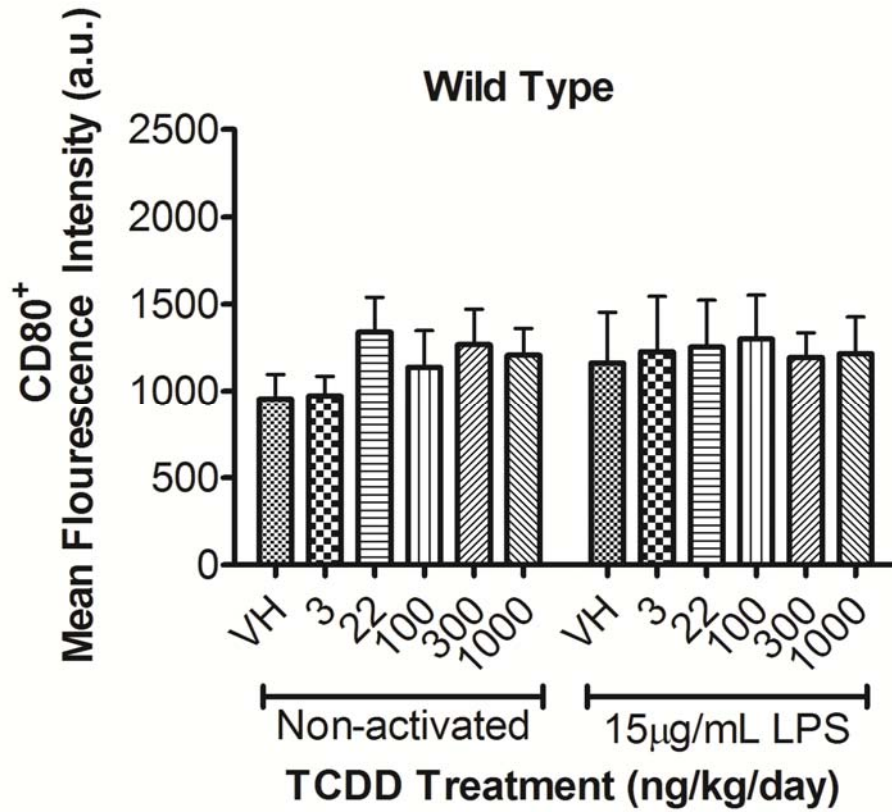


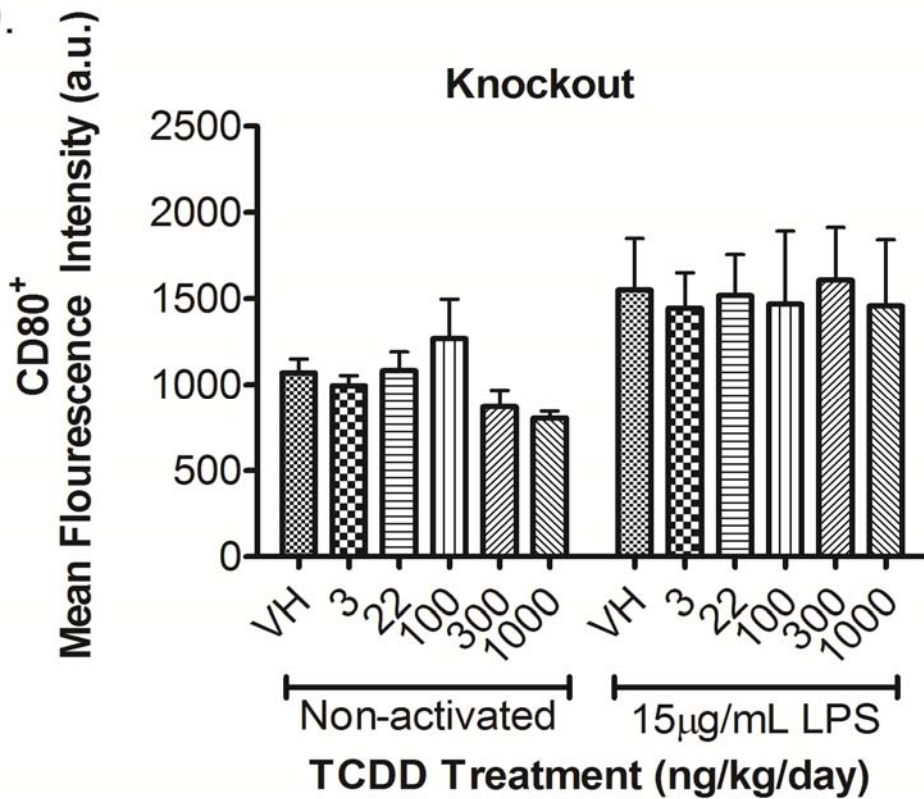
Figure 6. TCDD was unable to suppress the upregulation in the percentage of CD80⁺ B cells in wild type and AHR^{-/-} rat B cells upon LPS activation. Data presented are from group two. Wild type and AHR^{-/-} rats were dosed with corn oil vehicle (VH) control and varying concentrations of TCDD and splenocytes from these rats were treated with 15μg/mL LPS for 72 hours and compared to a non-activated control. Percentage of CD80⁺ B cells in wild type (A) and or AHR^{-/-} (B) were measured by flow cytometry. The MFI of CD80⁺ B cells in wild type (C) and or AHR^{-/-} (D) were obtained in the same flow cytometry analysis. Data is presented as the mean ± SEM from five rats per treatment group. * P ≤ 0.05 compared to non-activated B cells within the genotype.

Figure 6 (cont'd)

C.



D.



II. AHR deletion effects on CD86 Expression

The expression of CD86 was evaluated in non-activated and LPS-activated vehicle treated wild type and AHR^{-/-} B cells by flow cytometry; this evaluation was done for both groups. LPS activation induced a significant increase in the percentage of CD86⁺ B cells in wild type and AHR^{-/-} rats of group one (Figure 7). This significant upregulation was also detected in group two (Figure 8). A difference was observed between the two genotypes of group one as the AHR^{-/-} B cells had a stronger response to LPS by the significant increased the percentage of CD86⁺ B cells compared to LPS-activated wild type B cells. No difference in the percentage of CD86⁺ cells between the two genotypes was observed for group two in either non-activated or LPS-activated B cells.

As previously described for CD80, the percentage and MFI of CD86 was evaluated to detect changes in the magnitude of activation in TCDD-dosed wild type and AHR^{-/-} rat B cells. In group one, activation with LPS upregulated the percentage of CD86⁺ B cells in wild type and AHR^{-/-} rats (Figure 9A and 9B). Also observed in group one is a significant increase in the CD86⁺ MFI in both wild type and AHR^{-/-} LPS-activated B cells in comparison to non-activated cells within their respective genotype (Figure 9C and 9D). No significant TCDD effects were observed in the percentage of CD86⁺ B cells of wild type and AHR^{-/-} rats however, a trend towards a decrease was observed in LPS-activated AHR^{-/-} B cells (Figure 9A and 9B). In group one LPS-

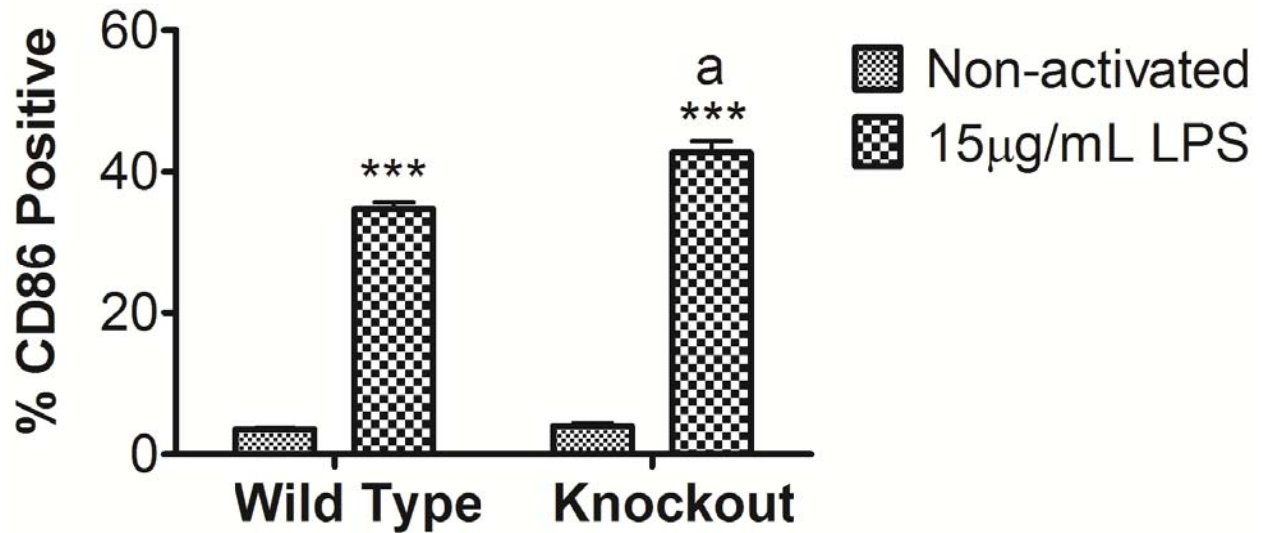


Figure 7. Targeted deletion of AHR in rats leads to higher percentage of CD86⁺ B cells upon LPS activation compared to wild type in group one. Vehicle treatment control in group one was evaluated by flow cytometry for the basal expression of CD86 in wild type and AHR^{-/-} B cells. Cells were also treated with 15µg/mL LPS to activate B cells inducing an upregulation of CD86 expression. Comparisons were made between transformed percent positive data for non-activated and activated wild type and AHR^{-/-} B cells. Data is presented as the mean ± SEM from five rats per treatment group. *** P ≤ 0.001 compared to non-activated within the genotype. a indicates P ≤ 0.05 compared to LPS-activated wild type B cells.

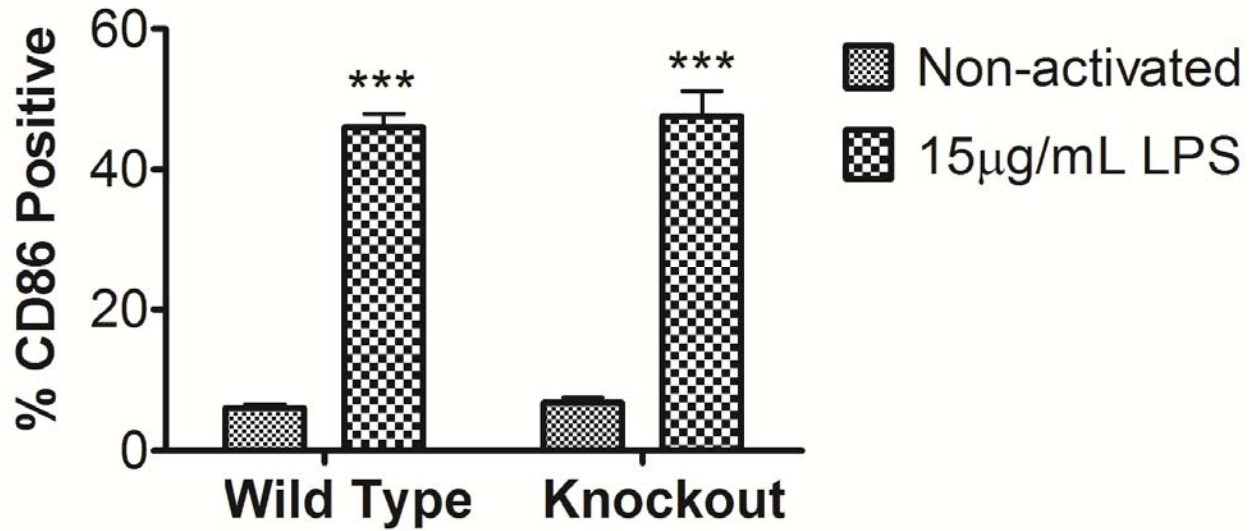


Figure 8. Activation with LPS leads to higher percentage of CD86⁺ B cells but the deletion of AHR in rats does not produce a further increase in CD86 expression of group two. Vehicle control treatment of group two was evaluated by flow cytometry for the basal expression of CD86 in wild type and AHR^{-/-} B cells. Cells were also treated with 15µg/mL LPS to activate B cells inducing an upregulation of CD86 expression. Comparisons were made between transformed percent positive data for non-activated and LPS-activated wild type and AHR^{-/-} B cells. Data is presented as the mean ± SEM from five rats per treatment group. *** P ≤ 0.001 compared to non-activated within the genotype.

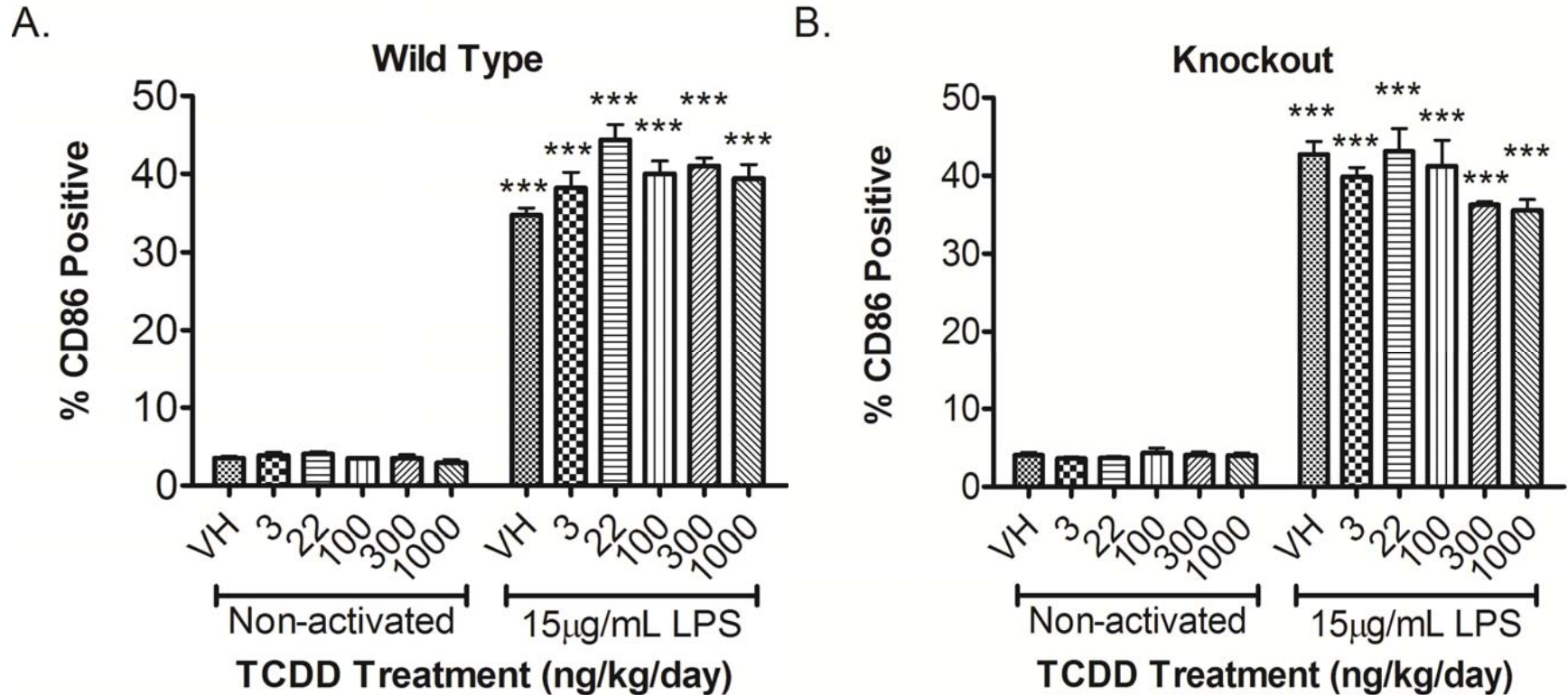
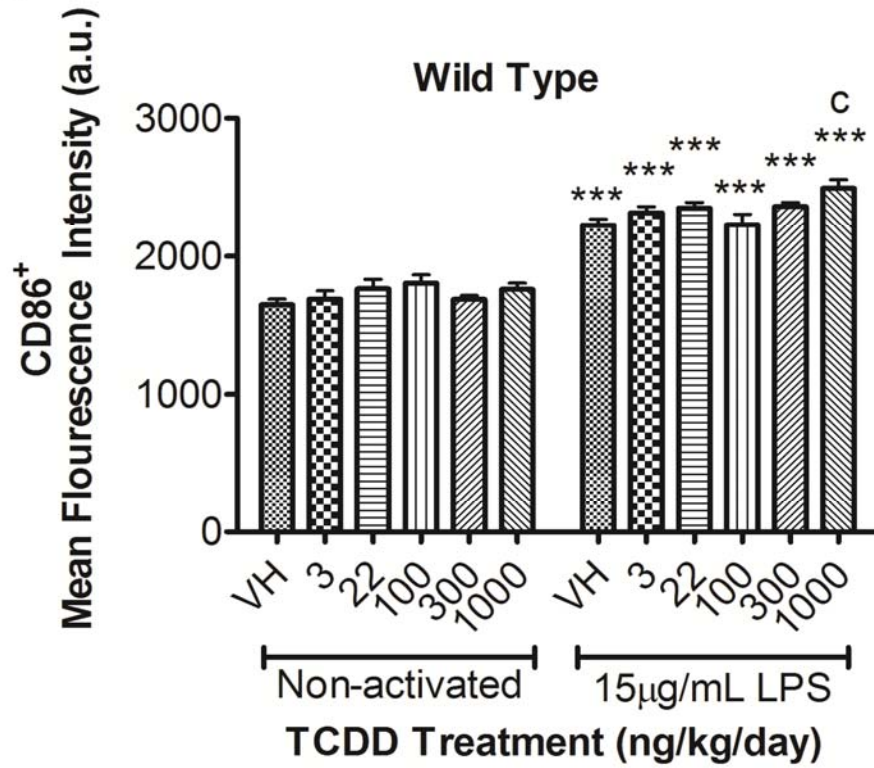


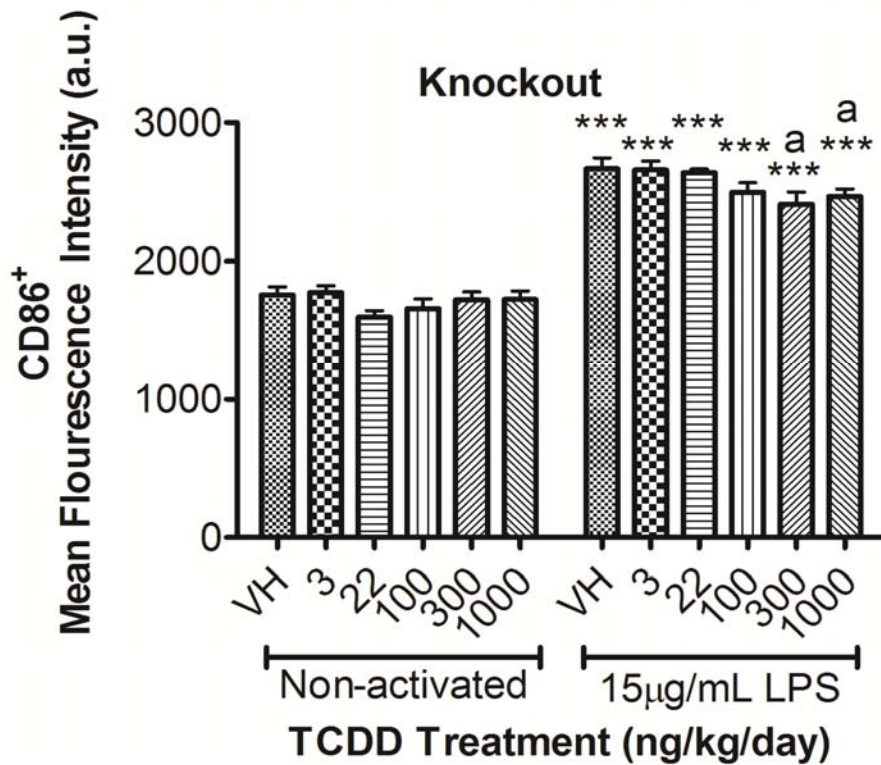
Figure 9. LPS activation upregulates CD86 expression in wild type and $AHR^{-/-}$ rat B cells similarly and no significant dose dependent TCDD-mediated suppression of CD86 was observed. Data presented are from group one. Wild type and $AHR^{-/-}$ rats were dosed with corn oil vehicle (VH) control and varying concentrations of TCDD and splenocytes from these rats were treated with 15µg/mL LPS for 72 hours and compared to a non-activated control. Percentage of CD86⁺ B cells in wild type (A) and $AHR^{-/-}$ (B) were measured by flow cytometry. The MFI of CD86⁺ B cells in wild type (C) and $AHR^{-/-}$ (D) were obtained in the same flow cytometry analysis. Data is presented as mean ± SEM from five rats per treatment group. *** $P \leq 0.001$ compared to non-activated B cells within the genotype. a indicates $P \leq 0.05$ and c indicates $P \leq 0.001$ in comparison to vehicle control within the genotype.

Figure 9 (cont'd)

C.



D.



activated wild type B cells a significant increase was observed in the CD86⁺ MFI at 1000ng/kg/day TCDD treatment (Figure 9C). However, in LPS-activated AHR^{-/-} B cells a significant decrease in the CD86⁺ MFI was observed at 300 and 1000ng/kg/day TCDD treatment (Figure 9D). These trends are not seen in the percentage or MFI of CD86⁺ B cells in wild type rats. In group two the same significant increase in both the percentage (Figure 10A and 10B) and MFI (Figure 10C and 10D) of CD86⁺ cells was detected in the LPS-activated wild type and AHR^{-/-} rats. No significant TCDD effect was observed in group two and the trend towards a decrease in CD86 expression observed in group one was absent.

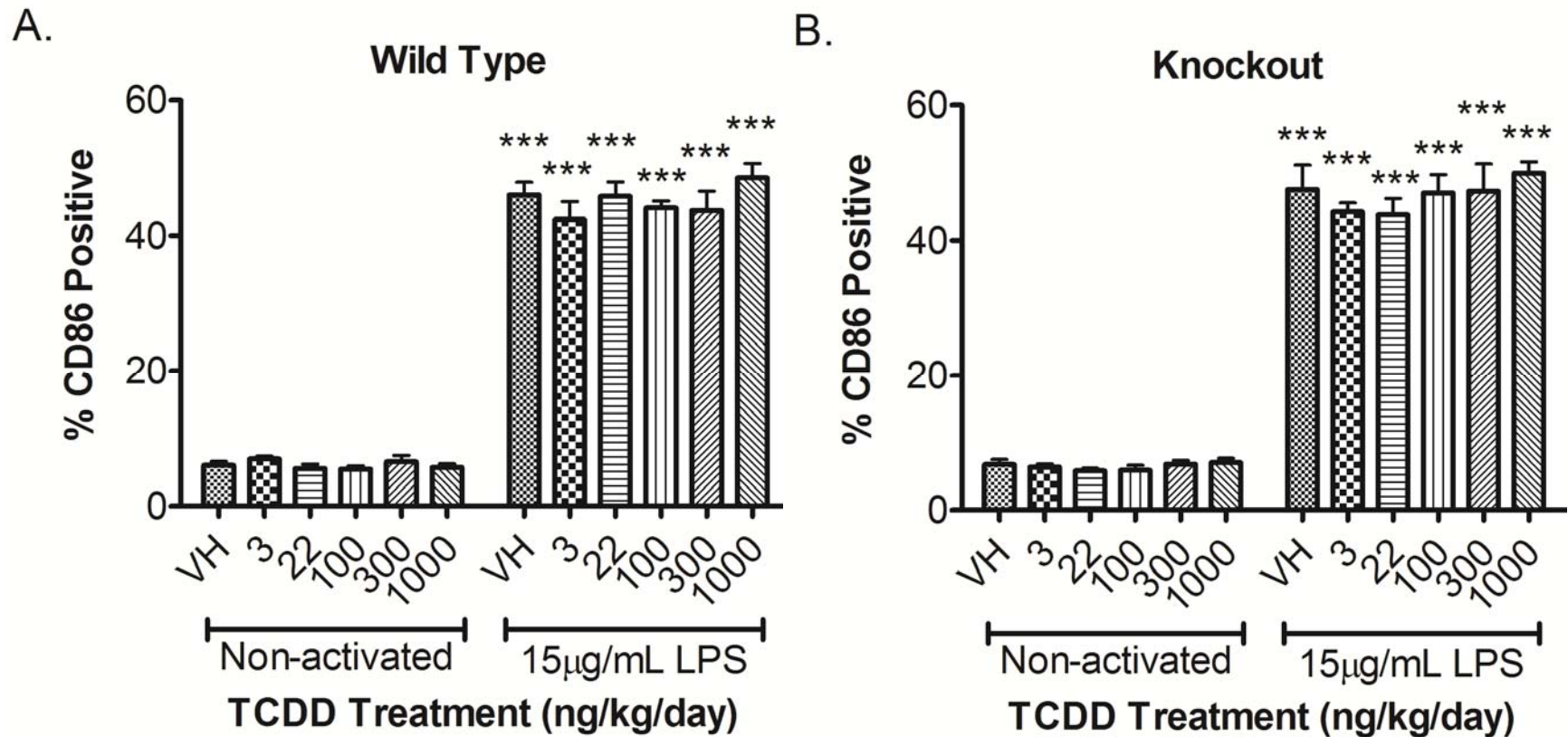
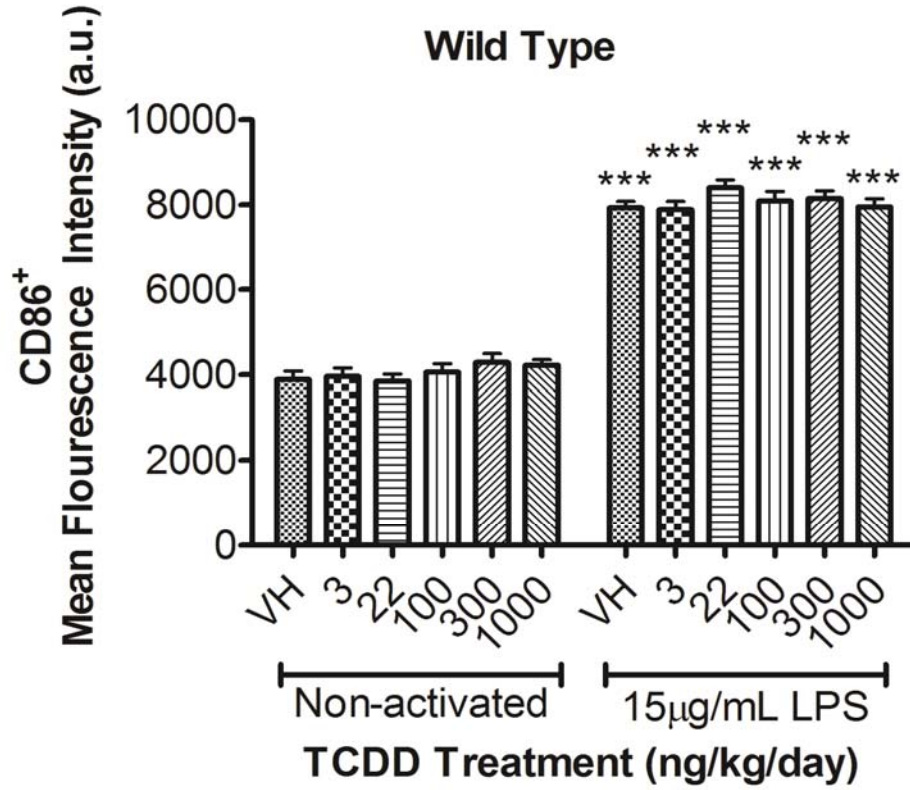


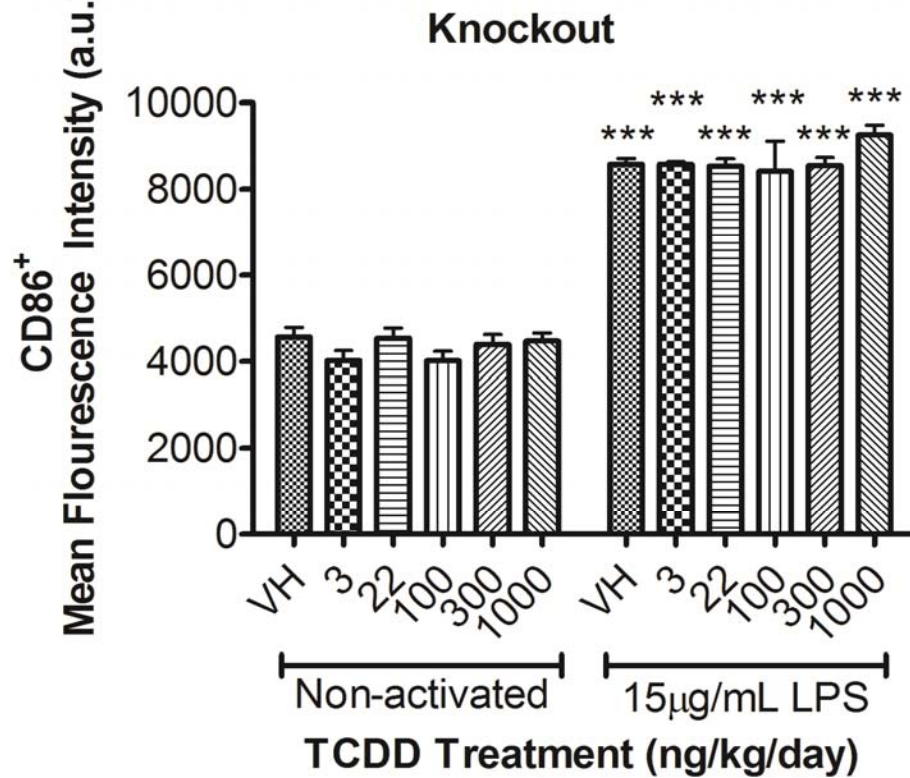
Figure 10. LPS activation upregulates CD86 expression in wild type and $AHR^{-/-}$ rat B cells similarly and no significant TCDD-mediated suppression of CD86 was observed. Data presented are from group two. Wild type and $AHR^{-/-}$ rats were dosed with corn oil vehicle (VH) control and varying concentrations of TCDD and splenocytes from these rats were treated with 15 μ g/mL LPS for 72 hours and compared to a non-activated control. Percentage of CD86⁺ B cells in wild type (A) and $AHR^{-/-}$ (B) were measured by flow cytometry. The MFI of CD86⁺ B cells in wild type (C) and $AHR^{-/-}$ (D) were obtained in the same flow cytometry analysis. Data is presented as the mean $\pm$ SEM from five rats per treatment group. *** $P \leq 0.001$ compared to non-activated B cells within the genotype.

Figure 10 (cont'd)

C.



D.



III. Assessing the role AHR has on regulating MHC II Expression

To demonstrate that B cell activation is modulated by AHR, basal MHC II expression and expression of MHC II upon LPS activation in vehicle treated rats were evaluated. In contrast to what was previously observed with CD80 and CD86 in group one, LPS activation resulted in a significant decrease in the percentage of MHC II⁺ B cells in AHR^{-/-} rats compared to wild type. LPS activation resulted in a two-fold decrease from 48% in wild type B cells to 22.6% in AHR^{-/-} B cells of group one (Figure 11). In group two, LPS activation resulted in an increased percentage of MHC II⁺ B cell in wild type and AHR^{-/-} rats, no significant differences were observed between the two genotypes. Also observed in group two was a significant decrease in the percentage of MHC II⁺ AHR^{-/-} non-activated B cells compared to non-activated wild type B cells (Figure 12). There was a two-fold decrease in the percentage of MHC II⁺ non-activated B cells in the AHR^{-/-} rats (4.6%) in comparison to non-activated wild type B cells (8.6%). Interestingly, the difference between the two genotypes observed in group two was detected in the non-activated B cells, whereas the genotype effect of group one was seen in the LPS-activated B cells.

The percentage and MFI of MHC II⁺ B cells were analyzed in wild type and AHR^{-/-} rats as previously done with CD80 and CD86. In contrast to the previous two activation markers examined, MHC II displayed different trends between wild type and

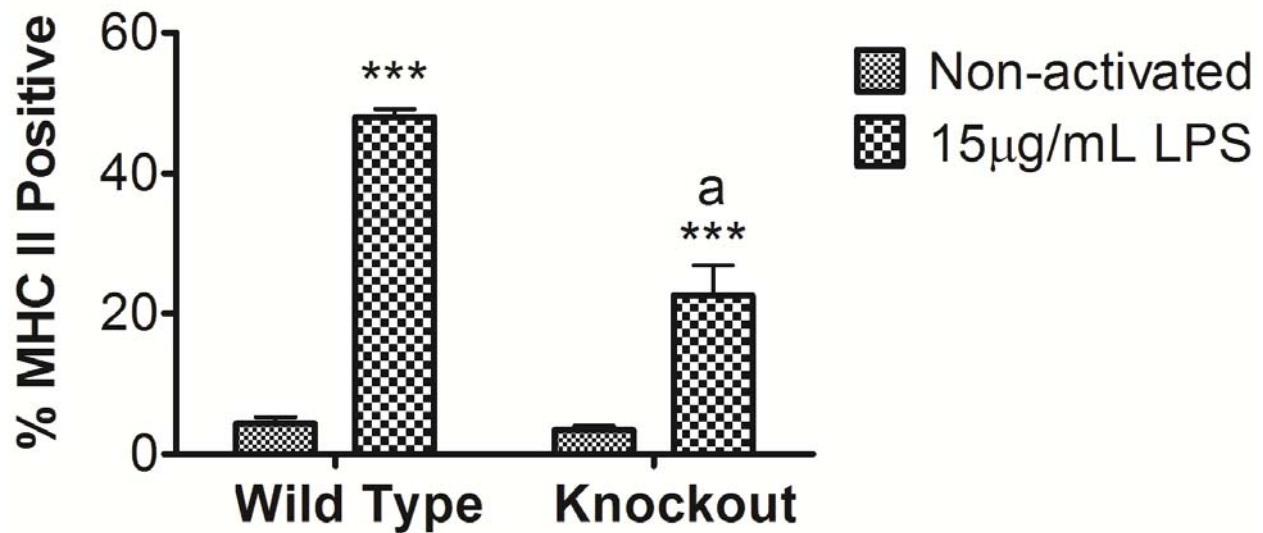


Figure 11. LPS activation results in a down regulation of MHC II⁺ expression in AHR^{-/-} B cells compared to LPS-activated wild type B cells. Vehicle control in group one was evaluated by flow cytometry for the basal expression of MHC II in wild type and AHR^{-/-} B cells. Cells were also treated with 15µg/mL LPS to activate B cells to induce an upregulation of MHCII expression. Comparisons were made between transformed percent positive data for non-activated and LPS-activated wild type and AHR^{-/-} B cells. Data is presented as the mean $\pm$ SEM from five rats per treatment group. *** $P \leq 0.001$ compared to non-activated within the genotype. a indicates $P \leq 0.05$ compared to LPS-activated wild type splenocytes.

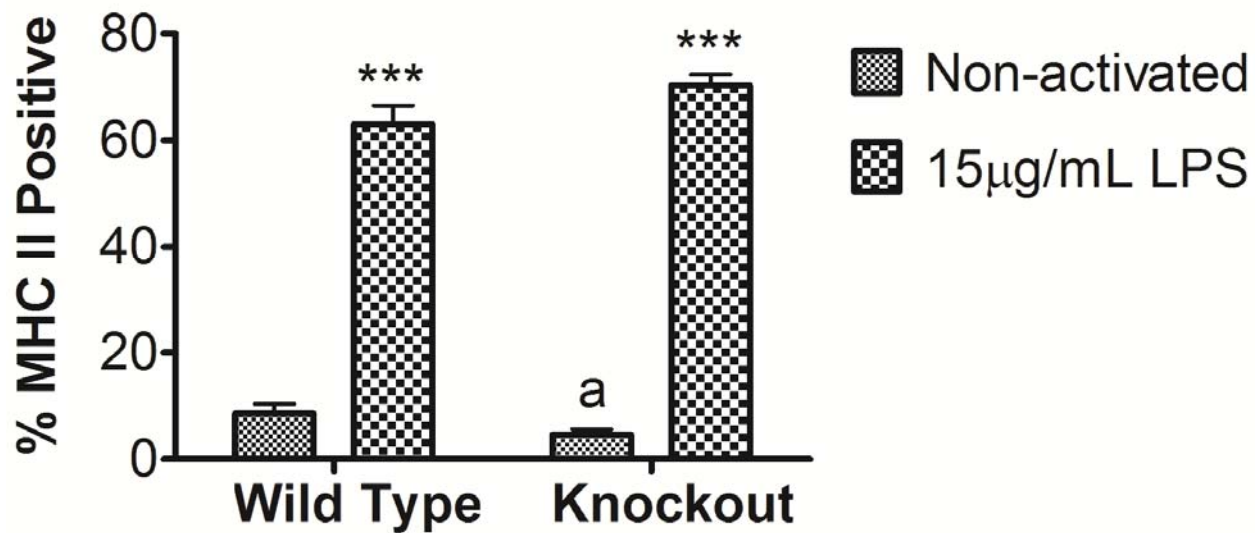


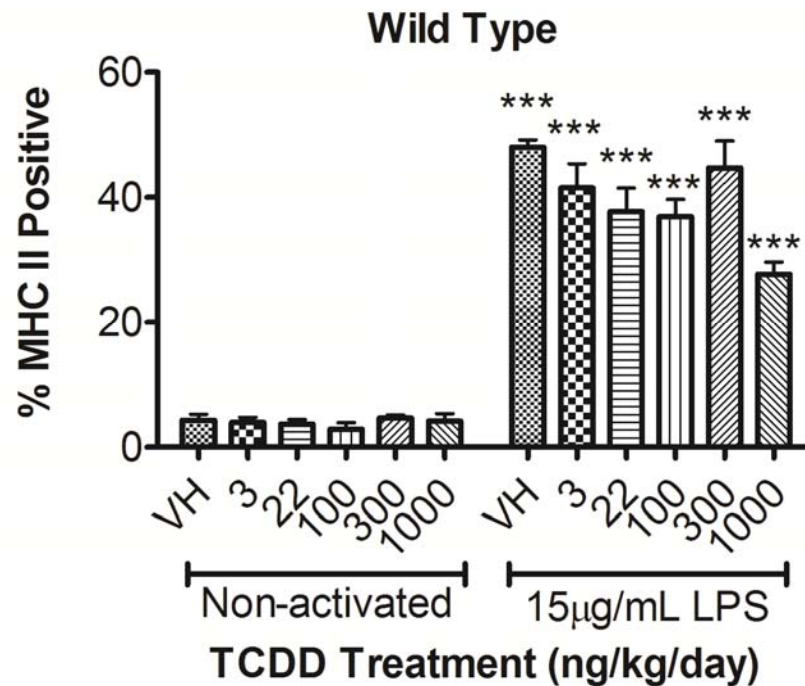
Figure 12. Non-activated $AHR^{-/-}$ rat B cells have a lower basal expression of MHC II while the level of MHC II upon LPS activation is similar to LPS-activated wild type B cells. Vehicle control in group two was evaluated by flow cytometry for the basal expression of MHC II in wild type and $AHR^{-/-}$ B cells. Cells were also treated with 15µg/mL LPS to activate B cells to induce an upregulation of MHC II expression. Comparisons were made between transformed percent positive data for non-activated and LPS-activated wild type and $AHR^{-/-}$ B cells. Data is presented as the mean $\pm$ SEM from five rats per treatment group. *** $P \leq 0.001$ compared to non-activated within the genotype. a indicates $P \leq 0.05$ compared to non-activated wild type splenocytes.

AHR^{-/-} rat B cells. For group one, LPS activation significantly increased the percentage of MHC II⁺ B cells in wild type and AHR^{-/-} rats at all TCDD doses (Figure 13A and 13B). The MHC II⁺ MFI differs from the percentage of MHC II⁺ cells in that LPS activation significantly increased MHC II in wild type B cells treated with vehicle, 300 and 1000 ng/kg/day TCDD doses (Figure 13A) and AHR^{-/-} B cells treated with 100 and 1000 ng/kg/day TCDD (Figure 13D). TCDD-mediated upregulation in the percentage of MHC II⁺ B cells at 100 ng/kg/day in LPS-activated AHR^{-/-} rats (Figure 13B). An increase in the MHC II⁺ MFI of the AHR^{-/-} B cells was also observed at 100 ng/kg/day treatment (Figure 13D). In wild type rats the only TCDD-mediated effects observed were in the MFI of non-activated B cells treated with 22 and 100 ng/kg/day TCDD (Figure 13C).

In group two wild type B cells, LPS activation resulted in an increase in the percentage of MHC II⁺ cells (Figure 14A). No significant TCDD effects were detected; however, a U-shaped dose-response curve was observed and not seen in any other treatment (Figure 14A). The LPS-activated AHR^{-/-} B cells had a similar increase in the percentage of MHC II⁺ cells; although, the trend towards an increase with increasing doses of TCDD observed in group one was not seen (Figure 14B). In contrast to wild type, there was a significant decrease in the percentage of MHC II⁺ LPS-activated B cells of AHR^{-/-} rats when treated with 3, 22, and 1000 ng/kg/day TCDD (Figure 14B).

The MHC II⁺ MFI for both wild type and knockout B cells increased with LPS activation but remained unaltered upon TCDD exposure (Figure 14C and 14D).

A.



B.

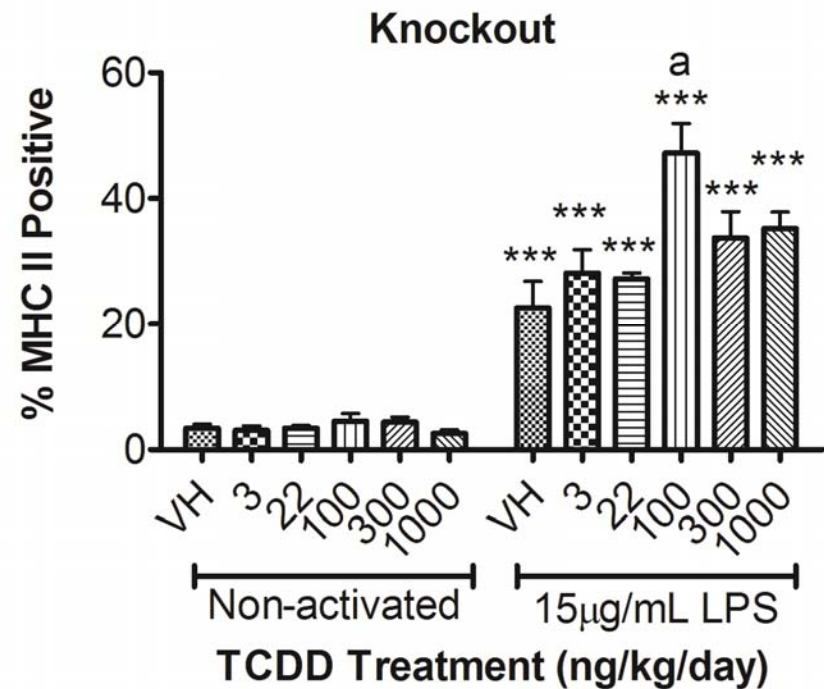
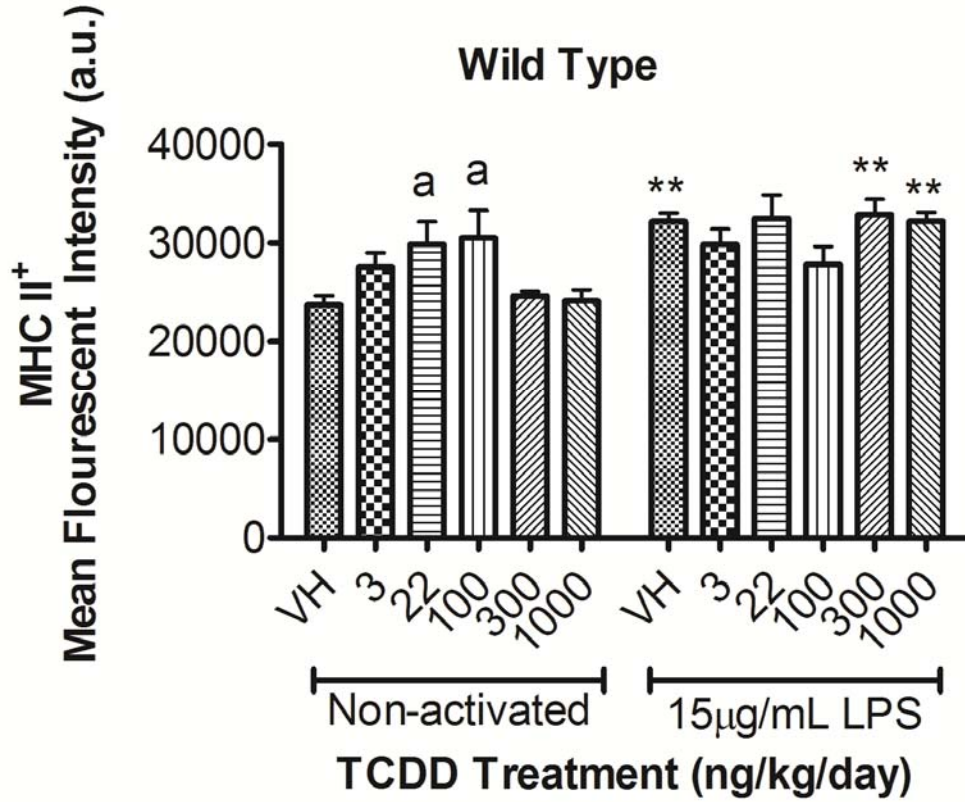


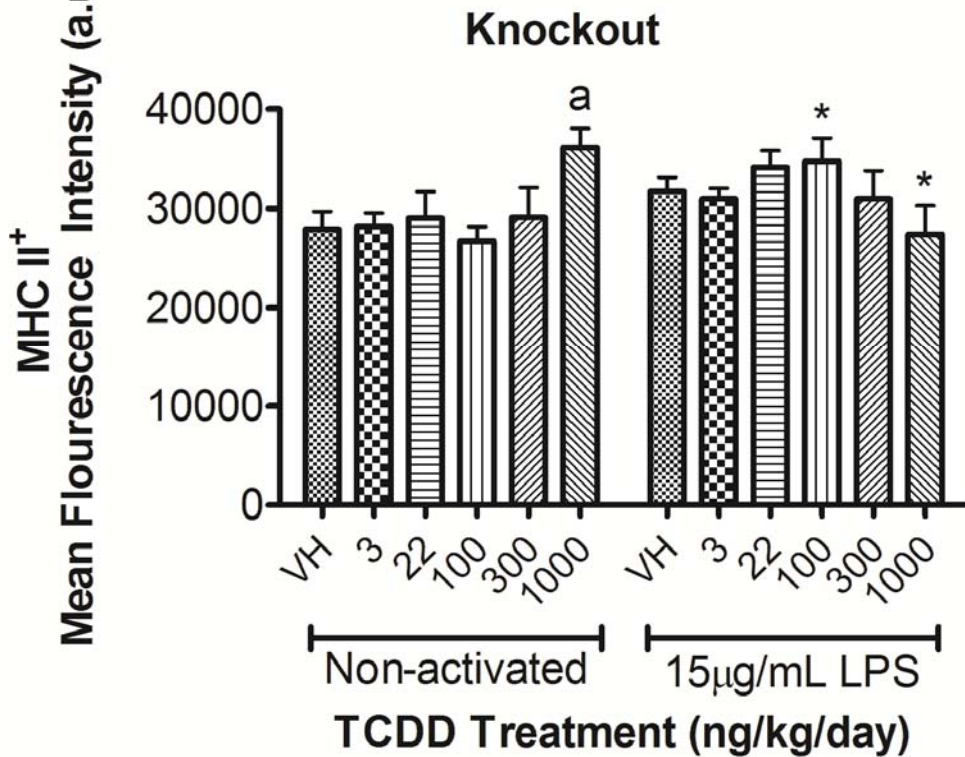
Figure 13. Targeted deletion of AHR in rats leads to the down regulation of MHC II when activated with LPS and TCDD produces opposing trends in the percentage of MHC II of wild type and $AHR^{-/-}$ rats. Data presented are from group one. Wild type and $AHR^{-/-}$ rats were dosed with corn oil vehicle (VH) control and varying concentrations of TCDD and splenocytes from these rats were treated with 15µg/mL LPS for 72 hours and compared to a non-activated control. Percentage of MHC II⁺ B cells in wild type (A) and $AHR^{-/-}$ (B) were measured by flow cytometry. The MFI of MHC II⁺ B cells in wild type (C) and $AHR^{-/-}$ (D) were obtained in the same flow cytometry analysis. Data is presented as the mean ± SEM from five rats per treatment group. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$ compared to non-activated B cells within the genotype. a indicates $P \leq 0.05$ compared to vehicle control within its respective genotype and activation set.

Figure 13 (cont'd)

C.



D.



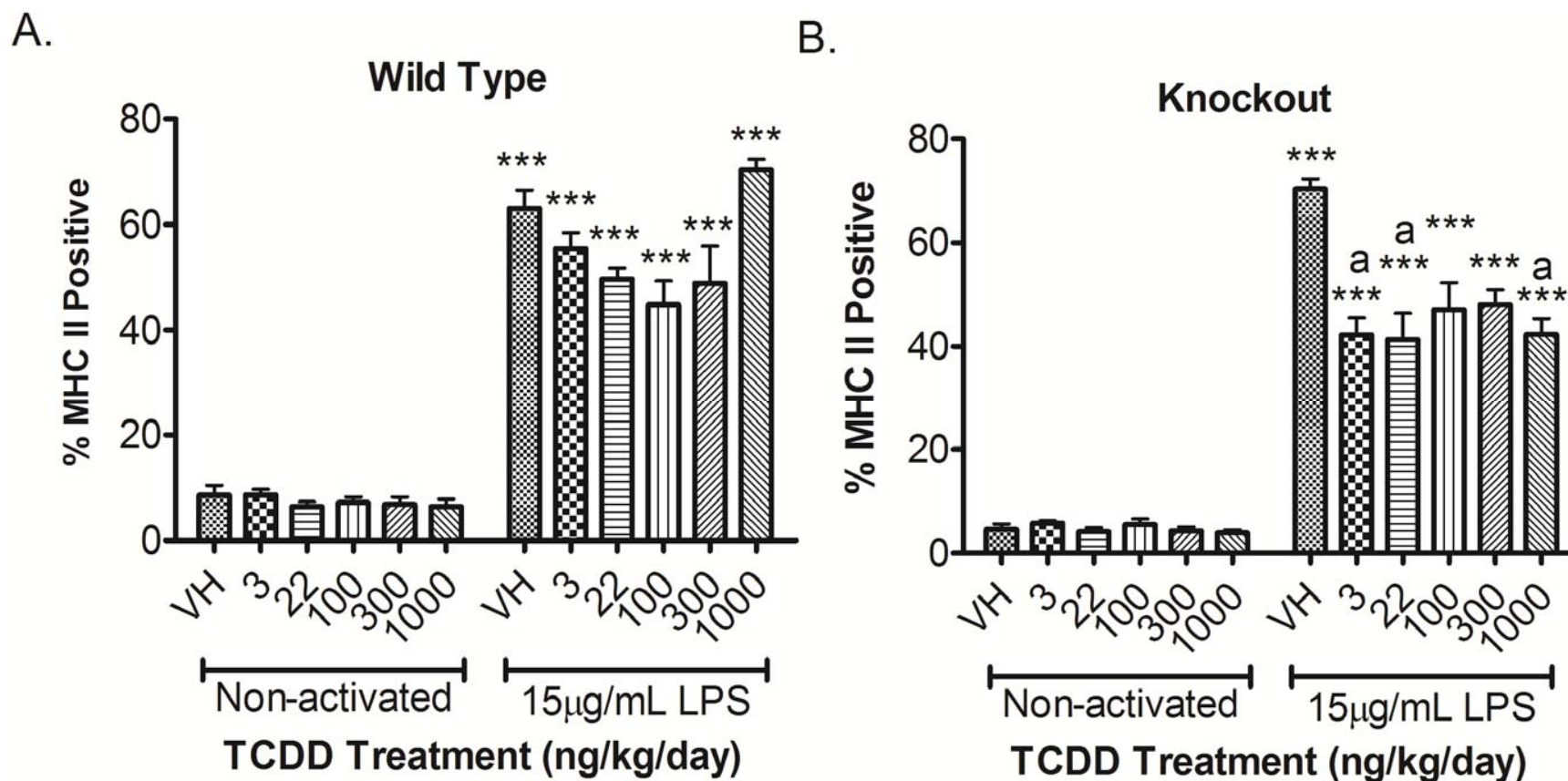


Figure 14. Targeted deletion of AHR in rats leads to the down regulation of MHC II when activated with LPS. Data presented are from group two. Wild Type and AHR^{-/-} rats dosed with varying concentrations of TCDD and splenocytes from these rats were activated with 15µg/mL LPS for 72 hours and compared to a non-activated control. Percentage of MHC II⁺ B cells in wild type (A) and AHR^{-/-} (B) were measured by flow cytometry. The MFI of MHC II⁺ B cells in wild type (C) and AHR^{-/-} (D) were obtained in the same flow cytometry analysis. Data is presented as the mean ± SEM from five rats per treatment group. *** P ≤ 0.001 compared to non-activated B cells within the genotype. a indicates P ≤ 0.05 compared to vehicle control within its respective genotype and activation set.

C.



C. Modulation of the IgM Primary Antibody Response

The second aim of this study was to determine the magnitude of change in the IgM antibody response in the absence of AHR. Two approaches were taken; the level of intracellular IgM and the level of IgM secreted into the external environment were determined by flow cytometry and ELISA, respectively. It was observed during flow cytometry analysis that a separate population of IgM⁺ B cells expressing high levels of IgM was detected in both genotypes and this population was denoted as IgM⁺ high. The IgM⁺ high B cell population was used for comparisons between treatment groups. In group one, LPS activation increased the percentage of intracellular IgM⁺ high wild type B cells from 1.79% (non-activated) to 3.28% (LPS) (Figure 15A and 15B), suggesting the B cells were only modestly activated. However, in group one AHR^{-/-} rats the percentage of intracellular IgM⁺ high B cells increased from 1.83% (non-activated) to 7.27% (LPS), a two-fold increase compared to the activated wild type rats (Figure 15B and 15D). In group two, similar observations were made but a more robust response was seen in splenocytes from the LPS-activated AHR^{-/-} rats with an increase in intracellular IgM⁺ high B cells from 1.26 % (non-activated) to 9.28% (LPS) (Figure 16B and 16D). This is in contrast to group two wild type B cells which only increased in the percentage of intracellular IgM⁺ high cells from 1.03% (non-activated) to 3.95% (LPS), a two-fold decrease in comparison to LPS-activated AHR^{-/-} B cells (Figure 16A and 16C).

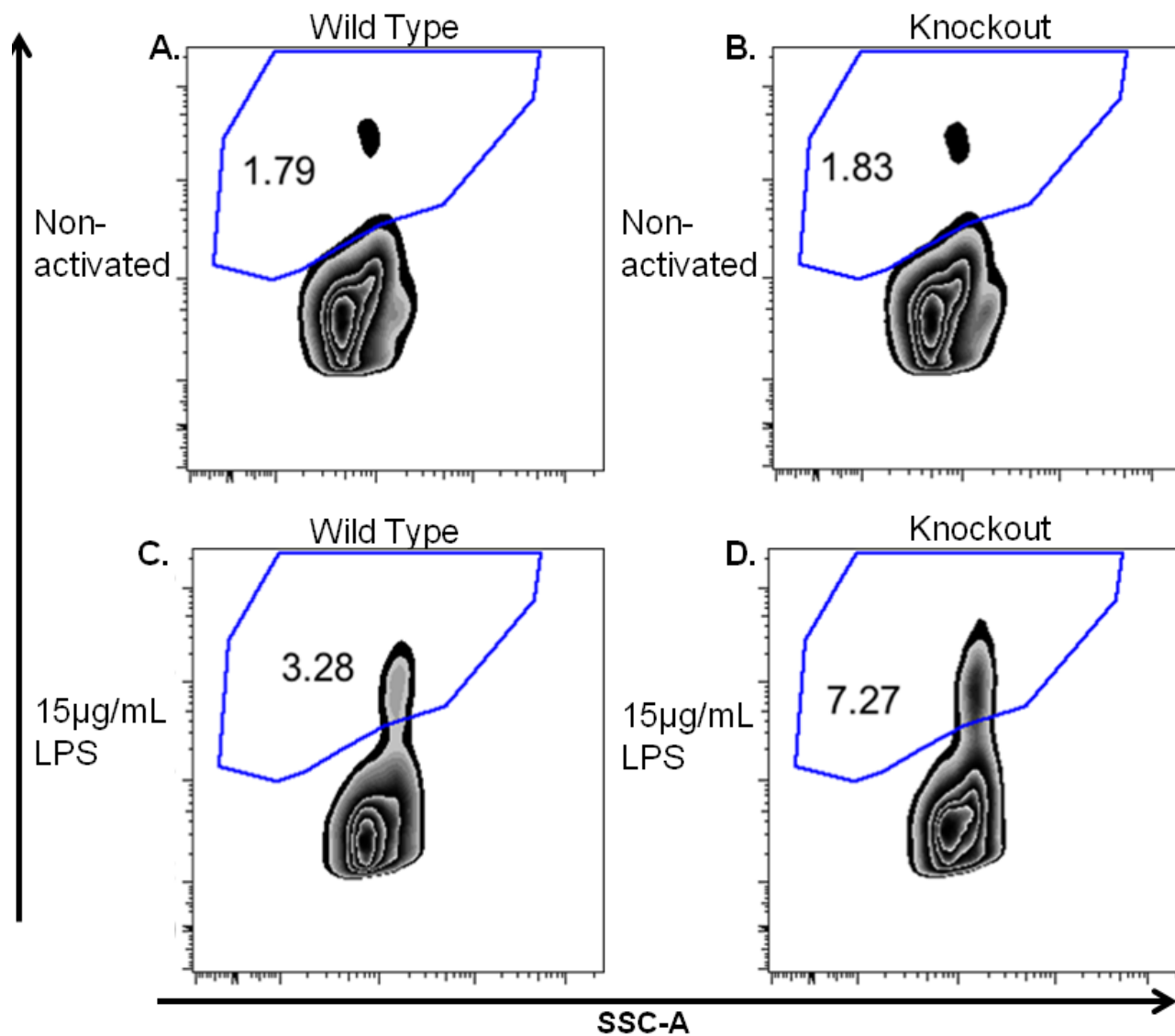


Figure 15.
Activated AHR^{-/-} B cells upregulate intracellular IgM over activated wild type B cells. Corn oil vehicle control in group one was evaluated by flow cytometry for the basal expression of intracellular IgM in wild type and AHR^{-/-} B cells. Comparisons were made between non-activated control and splenocytes activated with 15µg/mL LPS which induces an IgM antibody response. Data is presented as the mean percentage of IgM⁺ high B cells from five rats per treatment.

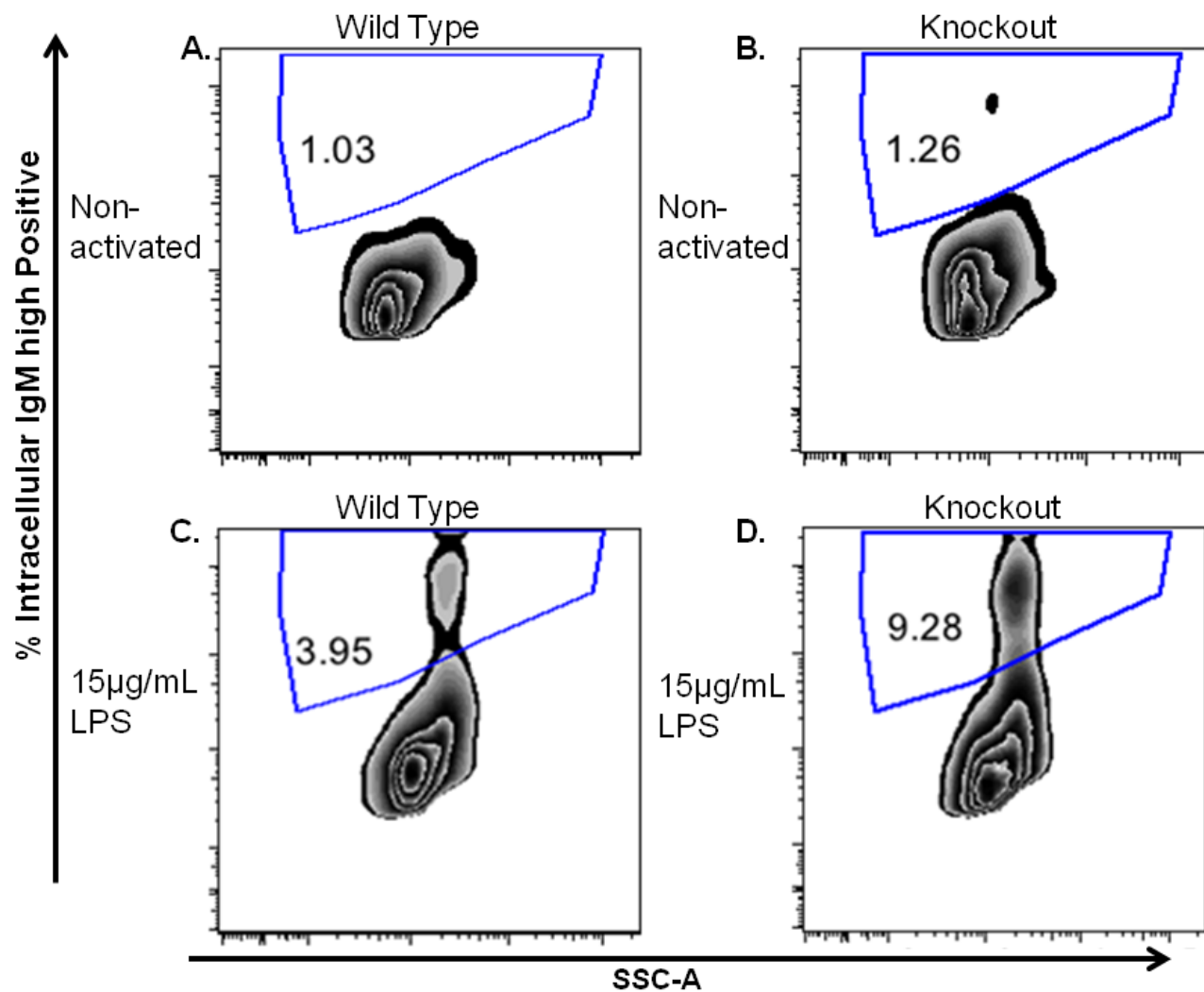
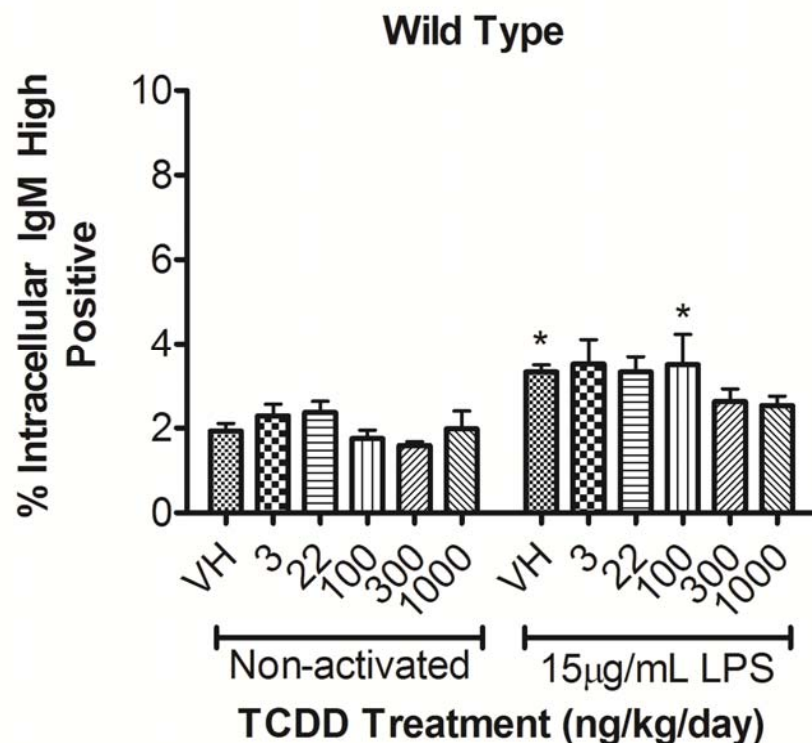


Figure 16. Targeted deletion of AHR in rat B cells results in an enhanced response of intracellular IgM over activated wild type B cells. Corn oil vehicle control in group one was evaluated by flow cytometry for the basal expression of intracellular IgM in wild type and AHR^{-/-} B cells. Comparisons were made between non-activated control and splenocytes activated with 15µg/mL LPS which induces an IgM antibody response. Data is presented as the mean percentage of IgM⁺ high B cells from five rats per treatment.

The magnitude of change in intracellular IgM in vehicle and TCDD treated wild type and AHR^{-/-} rats for group one and group two were examined next. In the splenocytes of group one wild type rats, LPS activation moderately upregulated the percentage of intracellular IgM⁺ high B cells and no significant TCDD-mediated suppression was observed; however, a trend towards a decrease was seen (Figure 17A). The targeted deletion of AHR in group one led to a significant increase in the percentage of intracellular IgM⁺ high B cells (Figure 17B). No TCDD-mediated immunosuppressive effects were detected in the AHR^{-/-} B cells. These data show that the percentage of rat intracellular IgM⁺ high B cells increased upon the targeted deletion of AHR.

The same comparisons were made for vehicle and TCDD treated wild type and AHR^{-/-} rats of group two and the increase in the percent intracellular IgM⁺ high B cells was found to be dissimilar to group one. Group two LPS-activated wild type B cells significantly increased the percentage of IgM⁺ high B cells, which indicated that group two responded more strongly to LPS when compared to group one (Figure 18A). Similar to group one, no significant TCDD-mediated suppression in IgM was observed but a trend towards a decrease was observed at higher TCDD doses in group two. The targeted deletion of AHR in the LPS-activated knockout B cells resulted in a two-fold increase in the percentage of intracellular IgM⁺ high B cells compared to wild type (Figure 18B).

A.



B.

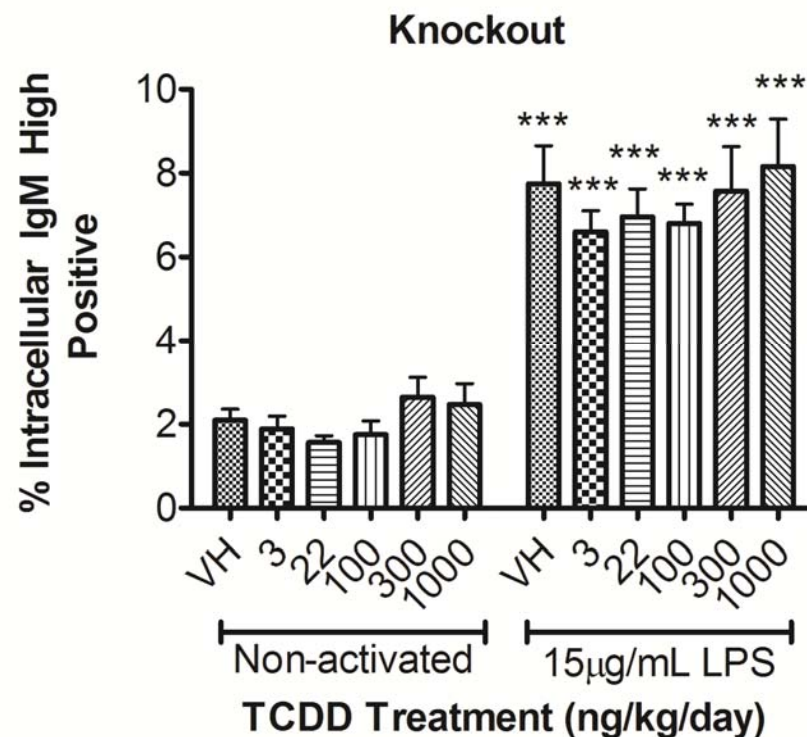


Figure 17. Targeted deletion of AHR results in an increase in the percentage of intracellular IgM⁺ high B cells. Data presented are from group one. Wild type and AHR^{-/-} rats were dosed with corn oil vehicle (VH) control and varying concentrations of TCDD and splenocytes from these rats were treated with 15µg/mL LPS for 72 hours and compared to a non-activated control. Percentage of intracellular IgM⁺ high B cells in wild type (A) and AHR^{-/-} (B) were measured by flow cytometry. Data is presented as the mean ± SEM from five rats per treatment group. * P ≤ 0.05 and *** P ≤ 0.001 compared to non-activated B cells within the genotype.

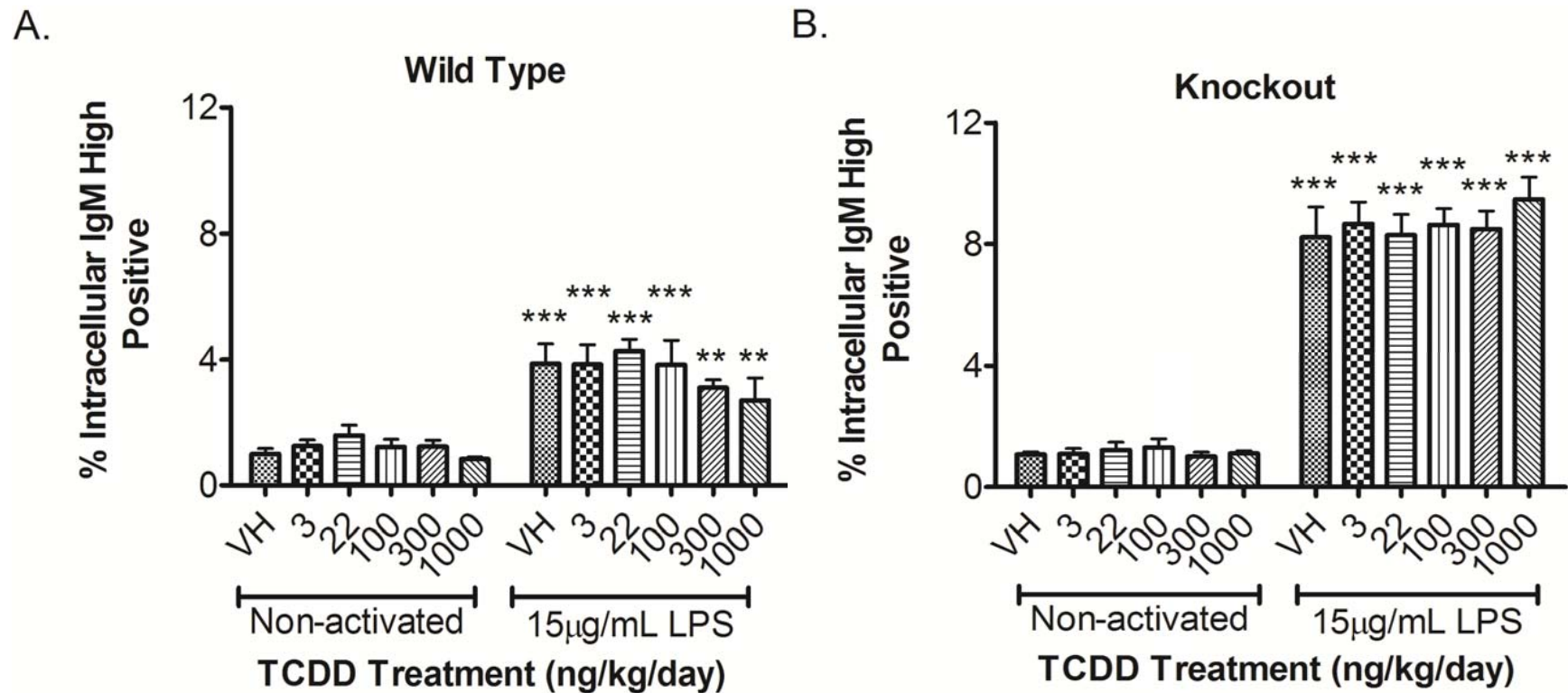


Figure 18. Targeted deletion of AHR produces an upregulation of intracellular IgM⁺ high B cells and TCDD is unable to suppress this upregulation. Data presented are from group two. Wild type and AHR^{-/-} rats were dosed with corn oil vehicle (VH) control and varying concentrations of TCDD and splenocytes from these rats were treated with 15µg/mL LPS for 72 hours and compared to a non-activated control. Percentage of intracellular IgM⁺ high B cells in wild type (A) and AHR^{-/-} (B) were measured by flow cytometry. Data is presented as the mean ± SEM from five rats per treatment group. ** P ≤ 0.01 and *** P ≤ 0.001 compared to non-activated B cells within the genotype.

The modifications in IgM secretion were also determined using an ELISA assay. Activation with LPS in wild type B cells only produced a modest increase in secreted IgM of group one and no TCDD-mediated suppression was observed (Figure 19A). LPS activation in AHR^{-/-} B cells resulted in a significant increase in secreted IgM when compared to non-activated and no TCDD-mediated suppression of secreted IgM was observed (Figure 19B). Similar to CD80, CD86 and MHC II expression, vehicle treated non-activated and LPS-activated wild type and AHR^{-/-} B cells were used to determine the basal quantity of secreted IgM in group one. AHR^{-/-} B cells responded more robustly to LPS compared to wild type which only moderately increased IgM secretion when activated with LPS (Figure 19C). Also, a difference between the two genotypes was observed as LPS-activated AHR^{-/-} B cells secreted a greater quantity of IgM when compared to LPS-activated wild type B cells.

Similar to the intracellular IgM results described, the quantity of IgM secretion in group two was found dissimilar to group one. In group two, LPS-activated wild type B cells significantly increased the secretion of IgM in rats treated with vehicle, 100 and 300ng/kg/day TCDD (Figure 20A). No TCDD-mediated IgM suppression was observed; however, a bell-shaped trend which peaked at 100ng/kg/day TCDD was detected. In group two, LPS activation of AHR^{-/-} B cells significantly increased IgM secretion except for the 100ng/kg/day TCDD dose (Figure 20B). TCDD treatment did not lead to an

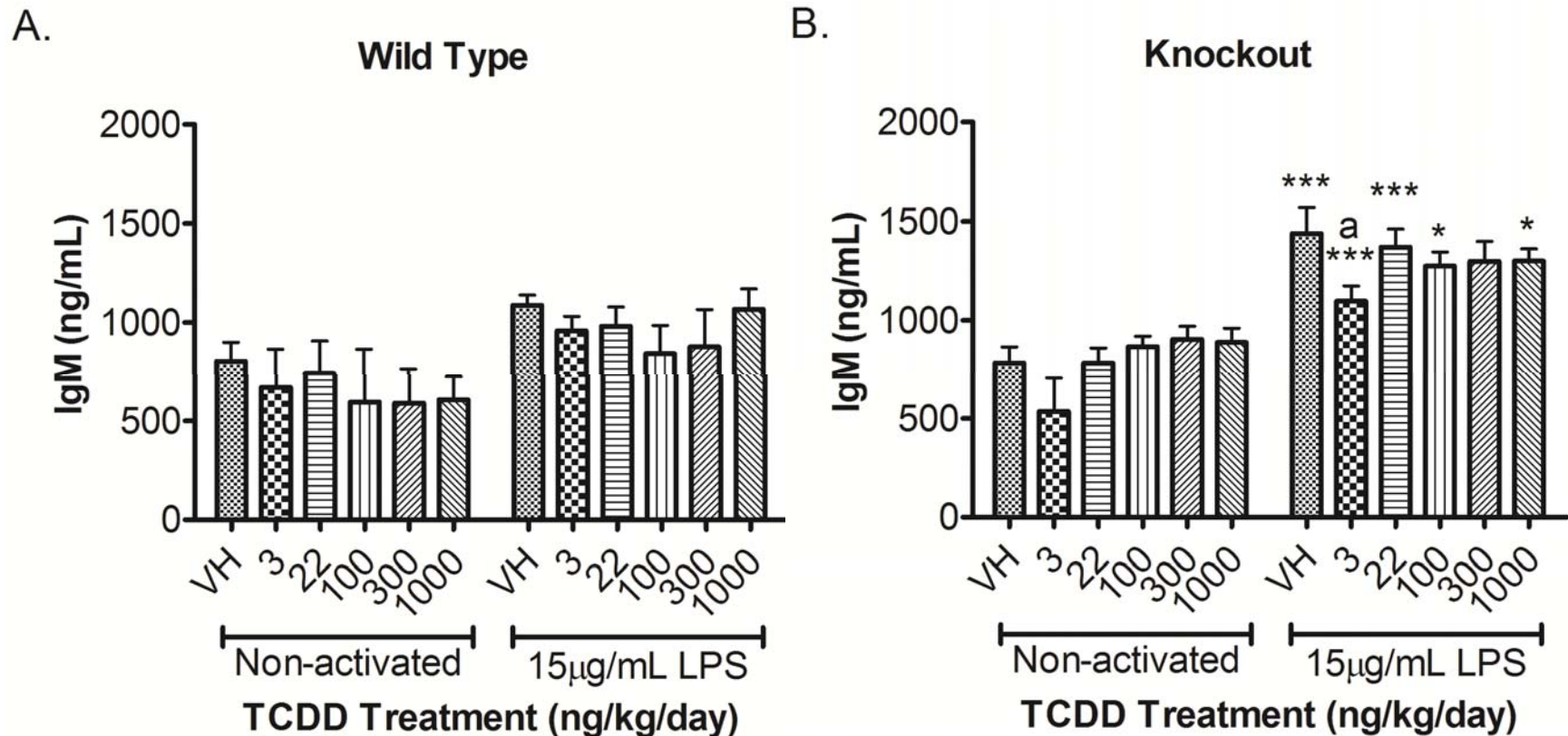
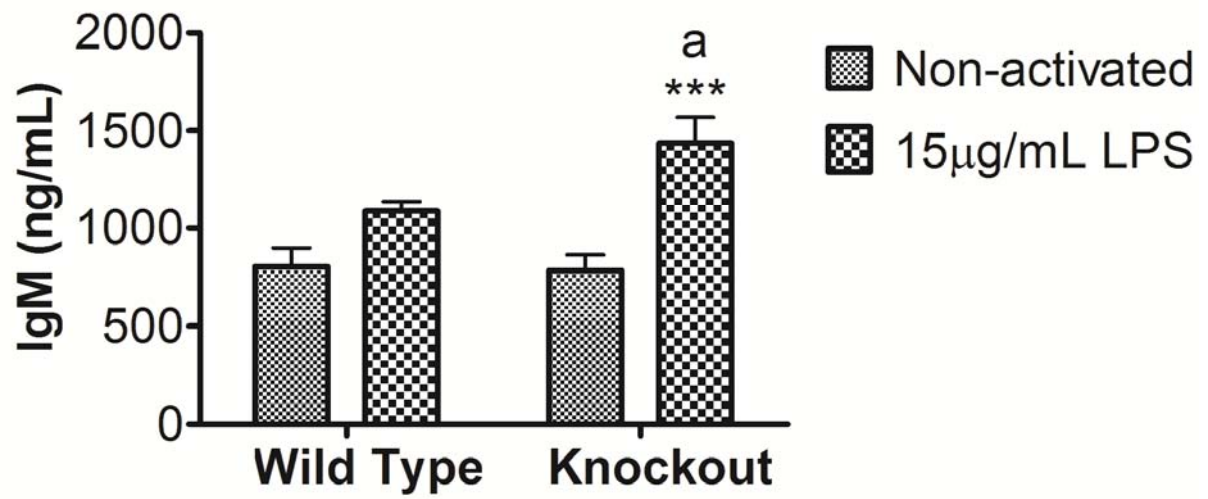


Figure 19. LPS-activated $AHR^{-/-}$ rat B cells secrete higher quantity of IgM over their wild type counterparts with no TCDD-mediated suppression of the antibody response. Data presented are from group one. Wild type and $AHR^{-/-}$ rats were dosed with corn oil vehicle (VH) control and varying concentrations of TCDD and splenocytes from these rats were treated with 15μg/mL LPS for 72 hours and compared to a non-activated control. Quantity of secreted IgM measured in ng/mL from wild type (A) and $AHR^{-/-}$ (B) activated B cells were measured by ELISA. Panel C compares the basal secretion of IgM in vehicle treated wild type and $AHR^{-/-}$ B cells obtained in the same ELISA assay. Data is presented as the mean $\pm$ SEM from five rats per treatment group. * $P \leq 0.05$ and *** $P \leq 0.05$ compared to non-activated B cells within the genotype. a in panel B indicates $P \leq 0.05$ compared to vehicle control in its respective genotype and activation set. a in panel C indicates $P \leq 0.05$ compared to LPS-activated wild type.

Figure 19 (cont'd)

C.



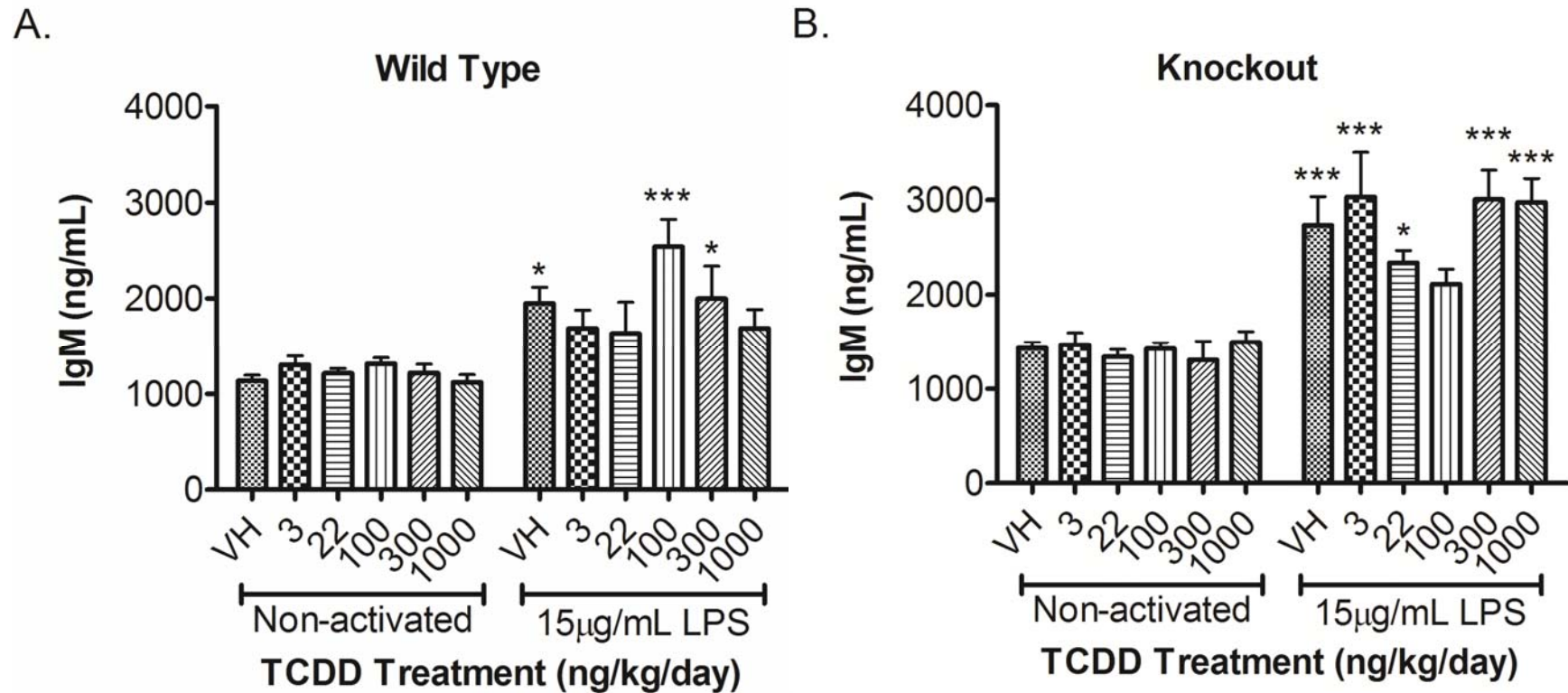
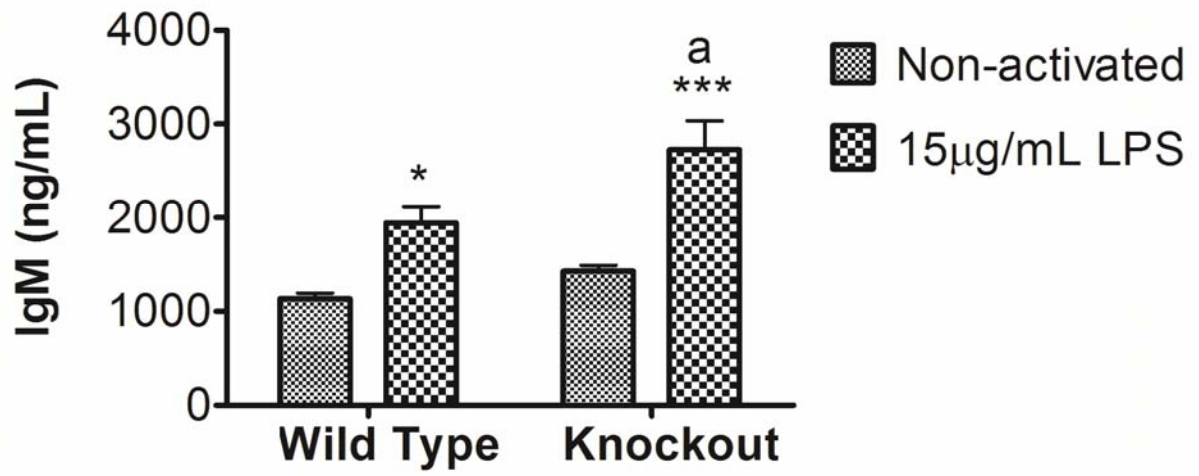


Figure 20. The targeted deletion of AHR leads to a greater quantity of IgM secretion in LPS-activated B cells. Data presented are from group two. Wild type and $AHR^{-/-}$ rats were dosed with corn oil vehicle (VH) control and varying concentrations of TCDD and splenocytes from these rats were treated with $15\mu\text{g/mL}$ LPS for 72 hours and compared to a non-activated control. Quantity of secreted IgM measured in ng/mL from wild type (A) and $AHR^{-/-}$ (B) activated B cells were measured by ELISA. Panel C compares the basal secretion of IgM in vehicle treated wild type and $AHR^{-/-}$ B cells obtained in the same ELISA assay. Data is presented as the mean $\pm$ SEM from five rats per treatment group. * $P \leq 0.05$ and *** $P \leq 0.001$ compared to non-activated B cells within the genotype. In panel C, a indicates $P \leq 0.05$ compared to LPS-activated wild type.

Figure 20 (cont'd)

C.



effect in AHR^{-/-} B cells but an opposite trend occurred from wild type in that a U-shaped trend was seen with the lowest IgM quantity at 100ng/kg/day TCDD. In comparing the basal IgM secretion in vehicle treated wild type rats, the mean IgM secretion significantly increased from 1114ng/mL to 1946ng/mL in LPS-activated B cells (Figure 20C). The vehicle treated AHR^{-/-} B cells had a stronger response to LPS compared to wild type as the mean IgM secretion significantly increased from 1429ng/mL to 2730ng/mL, a 1.9 fold change upon activation with LPS (Figure 20C). The targeted deletion of AHR did result in a difference between the two genotypes as LPS-activated AHR^{-/-} B cells secreted a greater quantity of IgM compared to LPS-activated wild type B cells.

Discussion

This AHR^{-/-} rat study was conducted in collaboration with the group of Dr. Russell Thomas at the Hamner Institute for Health Sciences and was primarily designed as a model to evaluate the carcinogenic effects of TCDD. The doses of TCDD that were selected by those at the Hamner Institute for Health Sciences were based on previous studies that demonstrated increased hepatocellular adenoma and cholangiocarcinomas incidence in female Sprague-Dawley rats over a range from 22 ng/kg/day to 100ng/kg/day (2006). Although, the specific mechanism in which TCDD induces liver tumors is unknown, it is hypothesized that inflammation is a key event in the mode of action by which TCDD promotes liver cancers. Inflammation can result from exposure to allergens, microbial infections, viral infections, and toxic chemicals. Chronic inflammation has been associated with several steps in carcinogenesis including tumor initiation and promotion, cancerous cell survival, proliferation, and metastasis (Reuter *et al.*, 2010). Inflammation results from the recruitment of several immune cell types, such as neutrophils and monocytes, to the site of damage where they act to remove the damaging stimulus. In the rat exposure to TCDD results in inflammatory cell infiltration and exposure to 50µg/kg TCDD has been demonstrated to increase liver inflammation in Long-Evans and Hans/Wistar rats (Pohjanvirta *et al.*, 1989; Pohjanvirta and Tuomisto, 1994). Inflammation can lead to the production of reactive oxygen species (ROS) which is thought to be a potential link between inflammation and the development of cancer as ROS can induce DNA damage which promotes neoplastic transformations (Reuter *et al.*, 2010). In primary Wistar rat hepatocytes, treatment of TCDD induced the production of ROS and 8-oxo-Dg, a hallmark of oxidative DNA damage (Knerr *et al.*, 2006). The

induction of DNA damage by ROS has been shown to be AHR-dependent as a study performed in the mouse hepatoma cell line Hepa1c1c7, demonstrated that AHR activation by the agonist B[a]P-7,8-dione increased 8-oxo-Dg production whereas B[a]P-7,8-dione failed to induce the production of 8-oxo-Dg in Hepa1c1c2, a mouse hepatoma cell line that is deficient in AHR (Park *et al.*, 2009). The evidence that AHR plays a role in carcinogenesis led to the development of the AHR^{-/-} rat to further evaluate the mechanism of TCDD-mediated tumor development.

While this study did not focus on the liver tumor promoting activities of TCDD, we had the opportunity to use spleens from AHR^{-/-} rats to assess TCDD-mediated perturbations in several immune cell types with this study focusing on B cell activation and effector function. Using flow cytometry, the resting B cell expressions of surface markers CD80, CD86, and MHC II were evaluated to determine if the targeted deletion of AHR affects the magnitude of B cell activation. No change was observed in the percentage of CD80⁺ B cells between non-activated wild type and AHR^{-/-} B cells. It was also determined that the percentage of CD86⁺ B cells were similar between wild type and AHR^{-/-} rats. The expression of MHC II in non-activated B cells of wild type and AHR^{-/-} rats was similar in group one; however, MHC II expression in non-activated AHR^{-/-} B cells of group two was significantly decreased when compared to non-activated wild type B cells. The difference in MHC II expression between the groups may be due to individual or shipment variability as group two was found to be more

variable than group one. Taken together, the targeted deletion of AHR does not affect the B cell in its resting state.

To determine whether the targeted deletion of AHR alters the magnitude of B cell activation, comparisons were also made between LPS-activated wild type and LPS-activated AHR^{-/-} B cells. LPS was a sufficient stimulus to induce B cell activation as the percentage of CD80⁺, CD86⁺, and MHC II⁺ cells were increased in LPS-treated wild type and AHR^{-/-} B cells when compared to non-activated wild type and AHR^{-/-} B cells. The targeted deletion of AHR led to a discrepancy in the expression of MHC II between group one and two. There was no difference between LPS-activated wild type and LPS-activated AHR^{-/-} B cells in group two; however, a decrease in the percentage of MHC II⁺ B cells in the AHR^{-/-} rat was observed in group one. The decrease in MHC II expression in the LPS-activated B cells of the AHR^{-/-} rat is in contrast to another study in which MHC II expression was upregulated in Langerhans cells isolated from AHR^{-/-} mice (Jux *et al.*, 2009). The targeted deletion of AHR produced differential effects on MHC II expression in mice and rats and suggests that the transcription of MHC II is regulated in a species specific manner. Due to the discrepancy in the effects of MHC II expression in the LPS-activated B cell of the AHR^{-/-} rat, no conclusion can be made as to whether the targeted deletion of AHR alters the magnitude of MHC II expression. In contrast to MHC II, clear alterations in the expression of CD80 and CD86 were observed in the absence of AHR. In LPS-activated B cells of AHR^{-/-} rats, the

percentage of CD80⁺ cells were upregulated compared to LPS- activated wild type B cells. Similarly, the percentage of CD86⁺ cells were upregulated in LPS-activated AHR^{-/-} B cells compared to LPS-activated wild type B cells. The upregulation in CD80 and CD86 activation marker expression suggests that AHR plays a role in regulating the magnitude of B cell activation. In the absence of AHR there is an augmentation of B cell activation in the AHR^{-/-} rat.

In determining whether the targeted deletion of AHR augments B cell effector function, the IgM antibody response to LPS was compared in wild type and AHR^{-/-} rats. Using flow cytometry, it was observed that LPS treatment increased the percentage of intracellular IgM⁺ high B cells of AHR^{-/-} rats when compared to LPS-activated B cells of wild type rats. In addition, IgM secretion as measured by ELISA was also increased in the LPS-activated AHR^{-/-} B cells, when compared to LPS-activated wild type B cells. The targeted deletion of AHR results in an augmentation in B cell effector function in the rat as demonstrated by the increase in intracellular IgM and IgM secretion in the AHR^{-/-} rat. The augmentation of B cell activation and effector function in the absence of AHR suggests that AHR regulates B cell activation and effector function at the transcriptional level.

It is evident by the presence of the ductus venosus in the AHR^{-/-} mouse and the renal abnormalities in the AHR^{-/-} rat that the AHR is an important ligand activated transcription factor for developmental homeostasis. The developmental perturbations in

the AHR^{-/-} mouse and rat may arise from the lack of AHR activation by an endogenous ligand. AHR activation by an endogenous ligand also suggests that AHR activation may be responsible for regulating B cell homeostasis. Removal of the AHR in the rat prevents the transcription factor from becoming activated by endogenous ligands such that it leads to the increase in CD80, CD86, MHC II, and IgM expression. Essentially, the augmentation of the B cell activation and effector function in the AHR^{-/-} rat occurs because the brake in the regulation of activation marker and IgM expression is absent.

Plasma cell development begins with the activation of signaling cascades mediated through the engagement of TLR on the surface of the B cell. The early signaling event within the B cell converges on several protein kinases, including JNK, AKT, and ERK, which leads to activation of the bistable network of transcription factor responsible for B cell differentiation. JNK regulates the activity of AP-1 as JNK phosphorylates c-Jun, a constituent of AP-1, to increase the transcriptional activity of AP-1 (Smeal *et al.*, 1991). ERK and AKT are also important controllers of plasma cell development as they regulate the abundance and transcription of BCL-6 respectively (Omori *et al.*, 2006; Niu *et al.*, 1998). B cell activation by LPS can converge on JNK, ERK, and AKT signaling to initiate B cell differentiation. In addition, studies in AHR^{-/-} mice have demonstrated that the deletion of AHR results in an increased sensitivity to LPS (Sekine *et al.*, 2009). A plausible explanation for the augmentation in B cell activation and IgM response in the AHR^{-/-} rat may be an increase in the sensitivity of the B cell to LPS which would allow for a more robust activation of JNK, ERK, and AKT.

The increased kinase activity may lead to further increases in the transcriptional activity of AP-1 and decrease in BCL-6 levels such that plasma cell development is enhanced.

In addition to LPS, B cells can also become activated by cytokines secreted from various immune cells such as macrophages and T cells. Studies performed in AHR^{-/-} mice have demonstrated that cytokine secretion by macrophages is also regulated in an AHR-dependent manner. It has been observed that LPS-activation of bone marrow derived macrophages of AHR^{-/-} mice resulted in an increased secretion of interleukin-1 β (IL-1 β) when compared to wild type mice (Sekine *et al.*, 2009). A second study conducted in AHR^{-/-} mice had demonstrated that LPS-activation of bronchoalveolar lavage cells, comprised of >99% macrophages, resulted in an increase in IL-6 secretion compared to wild type mice (Thatcher *et al.*, 2007). The cytokines IL-1 β and IL-6 play an important role in B cell function as IL-1 β can promote B cell activation and IL-6 is an important cytokine in promoting B cell differentiation. The deletion of AHR in the rat may also affect macrophages and lead to an increase in IL-1 β and IL-6 secretion. It may be possible that the elevated IL-1 β and IL-6 secretion by macrophages could promote enhanced B cell activation and differentiation and be partially responsible for the augmentation in B cell activation and effector function observed in the AHR^{-/-} rat.

While both wild type and AHR^{-/-} rat B cells were activated in response to LPS, the magnitude of B cell activation and the primary antibody response were not as strong when compared to other B cell studies previously reported by our laboratory. The increased expression of rat CD80, CD86, and MHC II in response to LPS is lower compared to previous mouse *in vitro* and *in vivo* studies from our laboratory (North et

al., 2009 et al. North 2010). Also, the increased expression of rat CD80 and CD86 in response to LPS is lower compared to the increase of CD80 and CD86 in activated primary human B cells previously reported by our laboratory (Lu et al., 2009; Lu et al., 2011). The decrease in the magnitude of B cell activation in response to LPS in the rat may be due to a reduction in the sensitivity of the rat B cell to LPS. It has been reported that the sensitivity to LPS can vary among species with mouse being one of the most responsive species to LPS while the rat has been found to be one of the least sensitive species to LPS (Brade, 1999). It has also been reported that there are intra-strain differences in the sensitivity to LPS with the Wistar rat being more resistant to LPS in comparison to the Sprague-Dawley rat (Brade, 1999). While rats are one of the most resistant strains to LPS, the strain used in this study (Sprague-Dawley) is one of the more responsive strains within the rat species.

As mentioned, the response of B cells to LPS is mediated through activation of TLR4 and species specific differences in tissue distribution and function may be partly responsible for the reduced sensitivity to LPS in the rat. Since the LPS response in wild type and AHR^{-/-} rats was lower compared to what has been observed in the mouse it is possible that the tissue distribution of TLR4 in the rat differs from the mouse. In the mouse, lung, heart, leukocytes and muscle tissue express high levels of TLR4 and in humans TLR4 expression in B cells is very low (Ganley-Leal *et al.*, 2010; Rehli, 2002). The rat B cell may have similar levels of TLR4 expression to that of the human B cell leading to the reduction of sensitivity to LPS. Also, functional differences in TLR4 have been determined between human and mouse (Werling *et al.*, 2009). LPS treatment increased TLR4 expression in human macrophages whereas LPS downregulated the

expression of TLR4 in murine macrophages (Rehli, 2002). The species specific functional differences shown in mouse and human suggest that there could be additional functional differences of TLR4 in the rat.

While the magnitude of B cell activation and effector function was compared in LPS-activated wild type and AHR^{-/-} rats, this study also evaluated the sensitivity of the B cell to TCDD to determine if TCDD alters B cell function in the presence and absence of AHR. TCDD produced modest alterations in B cell activation of AHR^{-/-} and wild type rats but no common trend toward suppression or enhancement was observed between the two groups and no single dose of TCDD elicited more effects than other doses used. The TCDD-mediated alterations in B cell activation observed were mainly on the expression of MHC II and TCDD-mediated upregulation in the percentage and MFI of MHC II⁺ B cells in both wild type and knockout rats of group one. The upregulation of MHCII observed in the rat is similar to previous reports that TCDD increases MHC II expression in mouse dendritic cells (Bankoti *et al.*, 2010). However, in group two TCDD treatment suppressed the percentage of MHC II⁺ B cells in AHR^{-/-} rats. The variability in the effect of TCDD on the expression of MHC II makes it difficult to conclude that TCDD-mediate a specific effect on MHC II expression.

Despite the variability in the effect of MHC II, clear TCDD-mediated alterations were made on the expression of CD80. TCDD did not significantly affect the percentage of CD80⁺ in wild type or AHR^{-/-} rats; however, a trend towards a decrease was observed in the AHR^{-/-} rats and a trend towards an increase was observed in wild

type rats. The TCDD-mediated increase in CD80 of wild type rats has also been demonstrated in a recent study conducted in wild type and AHR^{-/-} mice in which TCDD-mediated upregulation of CD80 and CD86 expression in dendritic cells of wild type mice (Vogel *et al.*, 2013). In this study, TCDD did not affect the expression of CD86 in wild type or AHR^{-/-} rats. The increase in CD80 expression of wild type rats demonstrated in this study is in divergence from other studies performed previously by our laboratory in which TCDD-mediated suppression of CD80 and CD86 in mice and humans (Lu *et al.*, 2011; North *et al.*, 2009). Though CD80 was moderately affected by TCDD, no significant TCDD-mediated alterations in B cell activation were observed and suggest that the doses selected were suboptimal for inducing immune effects. The doses of TCDD that were selected were appropriate for a cancer model and it should be noted that *in vivo* doses typically used for immunological studies are done in the µg/kg range and are only given as a single dose rather than several ng/kg doses over the course of several weeks. For example, studies conducted in the rat have demonstrated that TCDD-mediated suppression of the IgM response resulted from single *in vivo* doses ranging from 1-30µg/kg TCDD. The dosing scheme may have been a possibility for the lack of significant TCDD effects on B cell activation.

Several studies have demonstrated significant TCDD-mediated effects on the primary antibody response in the rat and interestingly both TCDD-mediated suppression and enhancement of IgM has been observed. Reports from several laboratories have demonstrated TCDD-mediated suppression of the primary antibody response in the Fischer 344, Brown-Norway, Sprague-Dawley, and Wistar albino rat strains under a variety of experimental conditions. It should be noted that although the B cell activators

and doses of TCDD used varied from study to study, the commonality among the studies was that the rats were dosed with TCDD prior to being immunized. In the Fischer 344 rat study, 30µg/kg TCDD was given as a single intraperitoneal injection and seven days after dosing rats were immunized with TNP-LPS (Smialowicz *et al.*, 1996). Three days post immunization it was observed that the PFC response to TNP-LPS was suppressed (Smialowicz *et al.*, 1996). At five days post immunization with sRBC; the Sprague-Dawley rat also demonstrated suppression in the PFC response when dosed with a single intraperitoneal injection of 100µg/kg TCDD given one week prior to immunization (Rozman *et al.*, 1984). In the studies performed in the Brown-Norway rat, TCDD was given as a single intraperitoneal dose seven days prior to sensitization to house dust mite allergen (Luebke *et al.*, 2001). Two week post sensitization, Brown-Norway rats were challenged with house dust mite allergens and the IgG and IgE antibody response was suppressed upon exposure to 10 and 30µg/kg TCDD (Luebke *et al.*, 2001). The study performed in the Wistar albino rat differs from the other rat strain studies in that Wistar albino rats were orally gavaged with 2µg/kg/week TCDD for 8 weeks (Çiftçi 2011). TCDD-mediated a suppression of IgM in the Wistar albino rat and this suppression could be partially recovered when the rats were also administered with 100mg/kg/day curcumin concurrently with TCDD (Çiftçi 2011). The dosing scheme in this study differs from the other studies performed in the rat as lower doses of TCDD (3-1000ng/kg/day) were given over an extended period of time (4 weeks). Also, this study differs from previous reports as LPS was used to activate B cells. Although the experimental conditions were different from earlier studies performed in the rat, similar results were observed as TCDD-mediated a trend towards suppression in the

percentage of IgM⁺ high B cells of wild type rats. The observed trend towards a suppression of the IgM response was not significant which may have been due to low doses of TCDD used in this study. To allow for the detection of significant TCDD-mediated suppression of the IgM response to LPS in wild type rats, it would be beneficial to increase the dose of TCDD as this would allow for a more accurate estimation of TCDD-mediated alterations of the immune response in the rat.

It has also been demonstrated in Fischer 344, Long-Evans, and Sprague-Dawley rats that TCDD treatment increased the primary antibody response under different experimental conditions. Fischer 344 and Long-Evans rats were dosed with 1, 3, 10, and 30µg/kg TCDD by a single intraperitoneal injection and immunized with sRBC one week post TCDD treatment (Smialowicz *et al*, 1994). Four days after immunization it was demonstrated that there was a dose-dependent increase in the PFC response to sRBC starting at 3µg/kg TCDD in the Fischer 344 rat and an increase of the PFC response at 1, 3, and 10µg/kg TCDD in the Long-Evans rat. (Smialowicz *et al*, 1994). In the Sprague-Dawley study, rats were orally gavaged with 10, 20, and 40µg/kg TCDD and immunized sRBC five days post TCDD treatment (Fan *et al.*, 1996). At 7 and 14 days post immunization, TCDD-mediated a dose-dependent increase in serum IgG levels with no change in IgM levels occurring in Sprague-Dawley rats (Fan *et al.*, 1996). While doses of TCDD and the B cell activator used in this study differed from the previous reports it was also observed that TCDD treatment resulted in a trend toward an increase in the percentage of IgM⁺ high B cells in AHR^{-/-} rats, however the increase was not significant. ELISA was also used to evaluate TCDD-mediated alterations in the magnitude of B cell effector function and no TCDD effect was observed in wild type or

AHR^{-/-} rats. The changes observed in the magnitude of the primary antibody response were only observed when the treatments were evaluated by flow cytometry and this is due to flow cytometry having a greater sensitivity in the detection of IgM compared to ELISA. Although no significant effects of TCDD treatment were observed, the trend towards TCDD-mediated enhancement of IgM in AHR^{-/-} B cells suggests it is possible that the deletion of AHR does not result in the loss of sensitivity of the B cell to TCDD. As mentioned, the lack of a significant increase of the IgM antibody response in the AHR^{-/-} rats may be due to the lower doses of TCDD used in this study.

The differential effects of TCDD on the primary antibody response may also be a result of the rat strain used in each of the studies as not all rat strains are equally sensitive to TCDD. The Hans-Wistar rat is one of the most resistant strains to TCDD with an LD₅₀ of over 9600µg/kg while the Long-Evans rat is one of the more sensitive strains with an LD₅₀ of 10-20µg/kg (Tuomisto *et al.*, 1998). This study utilized the Sprague-Dawley rat strain and this strain is known to be one of the more sensitive strains to TCDD as the AHR cDNA sequence between the Long-Evans and Sprague-Dawley rats are similar while the cDNA sequence of AHR in the Hans-Wistar rat differs (Tuomisto *et al.*, 1998). In addition to the differences in AHR sequence between rat strains and the modest upregulation of IgM in AHR^{-/-} rat observed in this study, the toxic effects of TCDD in the rat suggests that AHR may interact with other factors such as NF-κB in eliciting the effects of TCDD-mediated toxicity as well as altering the magnitude of B cell activation and effector function.

The NF- κ B family of transcription factors regulate the expression of over 200 genes that correspond to various immune functions such as inflammation, cell growth, and B cell development (Aggarwal, 2004; Feng *et al.*, 2004) and is composed of NF- κ B proteins such as p50 and Rel proteins that include p65 (RelA), RelB, and c-Rel. Many proteins are involved in NF- κ B signaling and the proteins involved in the canonical and noncanonical pathways can be reviewed in (Gilmore, 2006). The involvement of NF- κ B in immune cell function has lead to the discovery that AHR associates with several members of the NF- κ B family including p65 and RelB in several cell types (Barouki *et al.*, 2007; Ruby *et al.*, 2002). Some effects of NF- κ B that have been demonstrated in immune cell activation include studies in primary human dendritic cells by which inhibition of NF- κ B downregulated the expression of CD80, CD86, and MHC II (Yoshimura *et al.*, 2001). Similar results were observed in primary human monocytes in that blocking NF- κ B translocation to the nucleus inhibited LPS-mediated induction of CD80, CD86 and MHC II (Ardeschna *et al.*, 2000).

A requirement of NF- κ B transcriptional activity includes the association with co-activator proteins and it has been suggested that AHR and NF- κ B share the transcriptional co-activator steroid co-activator 1 (SRC-1) in addition to NF- κ B and ARNT sharing the co-activator p300/CBP (Tian *et al.*, 2002). The sharing of co-activator proteins may create a competition between AHR and NF- κ B for binding to transcriptional co-activators. The deletion of AHR in the rat may alleviate the competition between AHR and NF- κ B such that NF- κ B can bind to SRC-1 and p300/CBP to increase the expression of CD80, CD86, and MHC II compared to wild type rats. Additional evidence for supporting competition between AHR and NF- κ B was

found in a study using the Hepa1c1c7 cell line which demonstrated that AHR suppressed NF- κ B DNA binding and TCDD treatment enhanced the inhibition of AHR on NF- κ B, preventing the binding of NF- κ B to its promoter regions within the DNA (Tian *et al.*, 1999). However, a previous report demonstrated that TCDD induced DNA binding of NF- κ B family members p65, RelB, and c-Rel in CH12.LX cells which contain AHR (Sulentic *et al.*, 2000). The TCDD-mediated increase in binding of NF- κ B in the presence of AHR suggests that activation of NF- κ B in B cells is AHR-independent. In studies using AHR^{-/-} mice, it has been observed that there is an increase in NF- κ B activity in AHR^{-/-} mice compared to wild type (Thatcher *et al.*, 2007). The augmentation of the magnitude of B cell activation in the AHR^{-/-} rat may suggest that AHR and NF- κ B interact in the rat and these interactions could account for the differences in what has been observed in the wild type and AHR^{-/-} B cells. In the presence of AHR, it may be possible that AHR inhibits NF- κ B activation by LPS such that B cell activation is suppressed resulting in decreased expression of CD80, CD86, and MHC II which could subsequently decrease the primary IgM antibody response. In contrast, the increased CD80, CD86, and MHC II expression of the AHR^{-/-} rat observed in this study may have resulted from the AHR-mediated repression of NF- κ B being lifted.

In addition to modifications to the magnitude of B cell activation, the suppression of NF- κ B by AHR could also mediate perturbations in the primary IgM antibody response as NF- κ B has been shown to interact with some of the transcription factors in the bistable network of transcription factors that is required for B cell differentiation. One mouse study has demonstrated that inhibition of NF- κ B in LPS-activated B cells

abates BLIMP-1 expression (Morgan *et al.*, 2009). Using a human pancreatic cell line, another study observed that a significant downregulation of c-fos, a component of AP-1, occurred upon the inhibition of NF- κ B (Fujioka *et al.*, 2004). The AHR-mediated suppression of NF- κ B could potentially reduce the expression of transcription factors that promote B cell differentiation leading to a reduction in plasma cell development subsequently affecting the IgM response to LPS. The increased IgM response observed in the AHR^{-/-} rat may have resulted from increased NF- κ B activity which upregulated AP-1 and BLIMP-1 expression to promote B cell differentiation. The evidence that NF- κ B activity affects CD80, CD86 and MHC II expression and transcription factors required for plasma cell development suggest that NF- κ B could serve as a link between B cell activation and effector function in the rat.

Conclusion

TCDD-mediated suppression of the immune response have been evaluated over the course of several decades and studies have utilized a variety of models including cell lines derived from several species, primary rat B cells, primary mouse B cells, and primary human B cells. The advance in scientific technology has allowed for the creation of knockout animal models and the AHR^{-/-} mouse has been an important tool in determining the mechanism of TCDD-induced toxicity. Despite the multitude of models available the mechanisms by which TCDD mediates its immunotoxic effects have not yet been discovered. Also, the manner in which AHR is able to modulate B cell function in response to TCDD is unknown. We had the opportunity to utilize spleens from the newly developed AHR^{-/-} rat as a novel model system to evaluate the role the AHR plays in modulating B cell activation and effector function in the presence and absence of TCDD.

This thesis demonstrates for the first time that the AHR is capable of regulating the magnitude of B cell activation as the deletion of the AHR led to the increase in CD80 and CD86 when compared to wild type rats activated *in vitro* with LPS. MHC II was also used to measure the magnitude of B cell activation but due to the variability between groups it was excluded in the conclusion that the targeted deletion of AHR augments B cell activation. It was also discovered that AHR augments the IgM antibody response to LPS as B cells from AHR^{-/-} rats have an increased percentage of IgM⁺ high B cells as well as an increase in secreted IgM when compared to B cells of wild type rats.

The augmentation in B cell activation and effector function in the AHR^{-/-} rat as well as the sensitivity of the B cell to TCDD remaining intact are novel findings which illustrate that AHR not only mediates TCDD-induced toxicity but in part is also responsible for regulating B cell physiology. The concomitant increase in B cell activation marker expression and IgM response upon LPS activation in the absence of AHR also suggests that these two B cell events are correlated with one another. While the mechanisms in which the magnitude of B cell activation are correlated with the magnitude of effector function was not determined, it would be of interest to evaluate alterations of the MAPK and NF-κB pathway in the AHR^{-/-} rat as the MAPK family members JNK, ERK, and AKT in addition to NF-κB are known to interact with AHR as well as regulating the expression of activation markers and transcription factors that are responsible for B cell differentiation.

The NF-κB signaling cascade is composed of a number of proteins and future studies could be performed to determine which members of the NF-κB pathway associate with AHR in rat as well as the effects of NF-κB regulation on transcription in the presence and absence of AHR. Future studies could also evaluate the possibility of a direct effect of TCDD on NF-κB since this study has observed modest TCDD-mediated alterations in B cell activation and effector function in the absence of AHR. In addition to the future studies evaluating the effect of TCDD and the targeted deletion of AHR on NF-κB, it would be interesting to repeat the studies conducted in this thesis using an alternate B cell activator rather than LPS since the response to LPS was not as robust when compared to the mouse and human studies performed previously in our laboratory. It may be beneficial to activate the rat B cell using an alternate T-cell

independent activator or T-cell dependent method to allow for more accurate comparisons between species. Moreover, the doses of TCDD used in this study were suboptimal for producing significant changes in B cell activation and effector function and it would be beneficial to use doses of TCDD that range from 30-1 μ g/kg which are similar to the historical studies performed in the rat which have established TCDD-mediated alterations in B cell effector function, this would allow for more accurate comparisons of TCDD-mediated toxicity among species.

In summary, significant scientific advances in this study have been made not only in addressing the question whether AHR modulates the magnitude of B cell activation and effector function but also in providing new evidence of TCDD-mediated effects that occur in the absence of AHR. The establishment of the novel AHR^{-/-} rat model has led to the discovery of species specific alterations in B cell activation and effector function not previously observed in the mouse and human and can serve as a useful tool in the exploration of new possibilities to determine the mechanism of TCDD-induced immunotoxicity.

APPENDIX

APPENDIX

A. Antibodies

<u>Target</u>	<u>Fluorophore</u>	<u>Host</u>	<u>Reactivity</u>	<u>Type</u>	<u>Supplier</u>
CD80	PE	Mouse	Rat	Monoclonal; 3H5	BD Pharmingen
CD86	Alexa Flour 647	Mouse	Rat	Monoclonal; 24F	Biologend
MHC II	PerCP	Mouse	Rat	Monoclonal; Ox-6	BD Pharmingen
IgM	None	Mouse	Rat	Monoclonal; G53-238	BD Pharmingen
IgM	FITC	Mouse	Rat	Monoclonal; G53-238	BD Pharmingen

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