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Research Article

Suppression of the IgM Response by Aryl Hydrocarbon Receptor Activation in Human Primary B Cells Involves Impairment of Immunoglobulin Secretory Processes

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ABSTRACT

Aryl hydrocarbon receptor (AHR) activation by 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) is well established at suppressing humoral immunity. Previous studies in mouse B cells revealed that decreased IgM production was due to a significant suppression in the mRNA levels of the immunoglobulin M components (*IgH, IgJ*, and *Igκ* chains) and subsequent decrease in IgM synthesis. In contrast, the current study shows that activation of AHR in human B cells also results in a significant suppression of the number of IgM-secreting cells, but this is not due to a decrease in the transcription or translation of *IgH, IgJ*, and *Igκ* chains. Instead, the reduced humoral response is due to the impairment of IgM secretion. This is further evidenced by an accumulation of intracellular IgM in human B cells, which indicates that activation of AHR alters distinct regulatory pathways in human and mouse B cells leading to the suppressed primary IgM response. Collectively, these results demonstrate that although AHR activation mediates suppression of humoral immune responses across many different animal species, the mechanism of action is not necessarily conserved across species.

Key words: AHR; human B cells; immunoglobulin; secretion, 2; 3, 7, 8-tetrachlorodibenzo-*p*-dioxin.

The aryl hydrocarbon receptor (AHR) is a cytoplasmic protein activated by xenobiotics including dioxin and dioxin-like compounds. AHR is critical for key cell processes such as: cell growth; proliferation; differentiation; and the regulation of autoimmunity and inflammation (Budinsky *et al.*, 2014). Likewise, AHR plays a critical role in homeostasis of hepatic, reproductive, and vascular systems (Abbott *et al.*, 1999; Bunger *et al.*, 2003; Schmidt *et al.*, 1996). For the immune system, AHR also plays a role in the expansion, maturation (Thurmond *et al.*, 2000) and differentiation of hematopoietic stem cells (Smith *et al.*, 2013). Additionally, the activation of AHR is key for the differentiation of Th_{17} cells, induction of IL-22, and generation of T_{reg} from $CD4^+$ T cells (Veldhoen *et al.*, 2008, 2009). The identification of endogenous AHR ligands (ie, indoles, indigoids, and tryptophan metabolites) has changed the understanding and perception of AHR into a key regulator of cell cycle regulation, cell development, and homeostasis (Barouki *et al.*, 2007; Nguyen and Bradfield, 2008; Nguyen *et al.*, 2013).

Persistent AHR activation is associated with toxicity and is a consequence of exposure to high-affinity AHR ligands like the halogenated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls. These compounds are highly lipophilic, resistant to metabolism and their toxicity is critically dependent on AHR. The involvement of the AHR in mediating the toxicity of these compounds is evidenced by the resistance of hepatotoxicity, the induction of AHR gene battery, and suppression of humoral immune responses upon TCDD exposure in AHR-null mice and rats (Fernandez-Salguero *et al.*, 1997; Phadnis-Moghe *et al.*, 2016; Vorderstrasse *et al.*, 2001). However, the AHR is also necessary for proper immune responses as previous studies demonstrated that exposure to inflammatory stimuli results in substantial defects in mucosal immunity of AHR-null mice

(Stockinger *et al.*, 2014). From a toxicological standpoint, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) serves as a prototypical high-affinity AHR ligand and is the most potent agonist within the family of dioxin-like compounds. TCDD-mediated activation of the AHR can produce a wide range of biological and toxicological responses including hepatotoxicity and immune suppression in different animal species (Harper *et al.*, 1991; Holsapple *et al.*, 1986; Kerkvliet, 2002; Poland and Knutson, 1982; Sulentic and Kaminski, 2011). Previous studies have shown the B lymphocyte to be a sensitive target to suppression by high-affinity AHR ligands (Holsapple *et al.*, 1991).

Toxicity by dioxin-like compounds is primarily mediated through the AHR canonical pathway. AHR is a ligand-activated transcription factor belonging to the Per-AHR nuclear translocator (ARNT)-Sim superfamily of proteins. In the absence of ligand, AHR remains quiescent as part of a multiprotein complex composed of heat shock proteins 90 (hsp90), Ah-associated protein-9 (Ara9), and co-chaperone protein (p23) in the cytosol (Whitlock, 1990). Upon ligand binding, the AHR undergoes a conformational change, exposing the nuclear localization sequence at the N-terminal followed by translocation into the nucleus (Perdew, 1988). In the nucleus, the ligand-receptor complex heterodimerizes with ARNT (Mimura *et al.*, 1999). Thereafter, the AHR-ARNT heterodimer binds to dioxin response elements (DREs) in the promoter region of various genes and acts as a transcription factor. Negative regulation of the AHR occurs after dissociation from the DRE, followed by export to the cytoplasm and subsequent degradation through the ubiquitin-26S proteasome pathway or by a secondary pathway involving repression of AHR transcriptional activity by AHR repressor (Reyes *et al.*, 1992).

Humoral immunity is mediated through antibodies which are produced by B lymphocytes (Myers, 1991). IgM is the first wave of antibody produced after activation of mature naïve B cells (Myers, 1991). IgM monomers are heterodimeric proteins consisting of 2 heavy (H) and 2 light (L) chains (Schroeder and Cavacini, 2010). Secreted IgM has a pentameric structure of 5 IgM monomers linked by a joining (J) chain (Vollmers and Brandlein, 2006). The secreted IgM pentamers are critical in recognizing microbial pathogens and regulating B cell homeostasis (Baumgarth, 2011; Ehrenstein and Notley, 2010). IgM molecules are synthesized and assembled in the endoplasmic reticulum (ER) and IgM monomers are assembled into pentamers with the assistance of chaperones in the intermediate compartment between the ER and Golgi (Haas and Wabl, 1983; Melnick *et al.*, 1992). Correctly assembled IgM pentamers then detach from the chaperone proteins and continue through the secretory pathway in the Golgi (Zheng *et al.*, 2013) and are secreted via vesicular transport to the extracellular space.

Comprehensive studies in mouse models have demonstrated that AHR activation suppresses antibody production. In mice, impairment of the IgM response is caused by decreases in *Blimp*-1, *Pax*-5, and *Bach2* expression, transcription factors which play an important role in controlling B cell differentiation into antibody-secreting cells (Delogu *et al.*, 2006; North *et al.*, 2010). Previous studies using an *in vitro* human system has also demonstrated a marked decrease in the number of IgM-secreting B-cells upon AHR activation by persistent high-affinity ligands (Lu *et al.*, 2011), further supporting a putative role for the AHR in immunological modulation.

Since AHR activation by high-affinity ligands results in suppression of the IgM response in virtually every animal species investigated, the general assumption has been that the molecular mechanism of action is likely similar across species. However, our studies show that suppression of the primary antibody response by AHR activation in human primary B cells is mechanistically different compared with the mouse, one of the most widely studied immunotoxicological models of AHR biology.

MATERIALS AND METHODS

Chemicals and reagents. A 99.1% pure TCDD in dimethylsulfoxide (DMSO) was purchased from Accustandard Inc (New Haven, Connecticut). DMSO (Sigma Aldrich St Louis, Missouri) was used to dilute TCDD. The AHR antagonist (CH-223191) was purchased from Sigma Aldrich (St. Louis, Missouri).

Cell culture. CD40 ligand (CD40L) is highly conserved between human and mouse and therefore human CD40L is capable of robustly activating both human and mouse B cells (Spriggs *et al.*, 1992). CD40L-L cells are a mouse fibroblast line stably transfected with human CD40L (generous gift from Dr David Sherr, Boston University). The cells were maintained in Dulbecco's Modified Eagle's Medium (Thermo-Fisher Scientific, Waltham, Massachusetts) supplemented with 10% bovine calf serum (Thermo Scientific, Lafayette, Colorado), 50 μM of 2-mercaptoethanol, and HT supplement (Invitrogen, Carlsbad, California). The expression of human CD40L on the surface of the cells was monitored routinely to select for high CD40L-expressing cells. CD40L-L cells were thawed 4 days before irradiation with 3500 rad of x-rays using X-Rad 320 (Precision X-Ray, Inc, North Branford, Connecticut) 1 day before being co-culture with human primary B cells. CD40L-L cells were seeded at 1×10^4 cells/ml in 500 μl of media per well in 48-well tissue culture plates (Costar, Corning, New York). Human peripheral blood B cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% human AB serum (Valley Biomedical, Virginia), and 50 μM of 2-mercaptoethanol. In all cases, cells were cultured in 5% CO_2 incubator at 37°C.

Mice. Pathogen-free female C57BL/6 mice (6 weeks of age) were purchased from Charles River (Portage, Michigan). Mice were randomized, transferred to plastic cages containing sawdust bedding (5 mice per cage), and quarantined for 1 week. Mice were provided food (Purina certified laboratory chow) and water *ad libitum* and were not used for experimentation until their body weight was 17–20 g. Animal holding rooms were maintained at 21°C–24°C and 40%–60% humidity with a 12-h light/dark cycle. The Michigan State University Institutional Animal Care and Use Committee approved all animal procedures used in this investigation.

Purification of human B cells from leukocyte packs. Leukocyte packs collected from anonymous platelet donors were obtained from Gulf Coast Regional Laboratories (Houston, Texas). All human leukocyte packs were tested for presence of Human Immunodeficiency Virus (HIV), Hepatitis B/C Virus (HBV/HCV) and Human T-cell lymphotropic Virus (HTLV) before shipment. For each experiment, blood packs were diluted with Hanks Balanced Salt Solution (HBSS) and overlaid on Ficoll-Paque Plus density gradient (GE Healthcare, Piscataway, New Jersey) and centrifuged at 1300 × g for 25 min with low acceleration and brake. The peripheral blood mononuclear cells were isolated post-centrifugation, washed, counted and subjected to magnetic column-based isolation that enriched $CD19^+CD27^-$ naïve human B cells (>95% purity). This negative selection was conducted using the MACS Naïve Human B cell isolation kits

(Miltenyi Biotec, Auburn, California) following manufacturer's instructions. Purified human B cells at the concentration of 1×10^6 cells/ml were then treated with either 0.02% DMSO vehicle control (VH) or various concentrations of TCDD. Treated B cells were then activated by co-culture with sub lethally irradiated CD40L-L cells (1×10^4 cells/ml) in a 48-well cell culture plate in the presence of recombinant human cytokines IL-2 (1 ng/ml), IL-6 (1 ng/ml) (Roche Applied Science, Indianapolis, Indiana), and IL-10 (4 ng/ml) (Biovision Inc, Milpitas, California) for total 7 days.

Quantification of mRNA levels by real-time PCR. RNA was isolated using Qiagen RNeasy kits (Germantown, Maryland) per the manufacturer's instructions. The RNA concentrations were determined by Nanodrop ND-1000 Scientific spectrophotometer (Thermo-Fisher Scientific, Wilmington, Delaware) and 500 ng of RNA was reverse-transcribed using High Capacity cDNA RT-PCR kit by Applied Biosystems (Foster City, California). The cDNA was amplified using Applied Biosystems Taqman Gene Expression Assays. All quantitative real-time PCR reactions were performed on an Applied Biosystems model ABI Prism 7900 Sequence Detection System. Human 18S ribosomal RNA (Applied Biosystems, Foster City, California) was used as an internal control gene. The fold change in mRNA expression was calculated using the $\Delta\Delta C_t$ method. The probes used for human B cells were IGHM (Hs00385741_m1), IgJ (Hs00376160_m1) and Igk (Hs02384840_gH). The probes used for mouse B cells were Ighm (Mm01718956_m1), IgJ (Mm00461780_m1). SYBR Green system was used to quantify the level of *Igκ* mRNA in mouse B cells. The primers for *Igκ* were designed based on Schneider *et al.* (2008). The control used for SYBR Green reactions was mouse HPRT.

Enzyme-linked immunospot assay. The number of IgM-secreting cells was quantified by enzyme-linked immunospot (ELISPOT). Briefly, multiscreen 96-well filter plates (Millipore, Billerica, Massachusetts) were coated with anti-human IgM antibody (5 μg/ml) (Sigma Aldrich, St. Louis, Missouri) overnight and then blocked with 5% bovine serum albumin (Sigma Aldrich, St. Louis, Missouri) for 2 h. B cells were washed with RPMI 1640 twice, resuspended in RPMI 1640 containing 10% bovine calf serum (Thermo Scientific, Lafayette, Colorado) and incubated on primary antibody-coated plates overnight at 37°C with 5% CO_2. Biotin-conjugated anti-human IgM antibody (Sigma Aldrich, St. Louis, Missouri) and then streptavidin-horseradish peroxidase (HRP) (Sigma Aldrich, St. Louis, Missouri) were added for a 1 h incubation at 37°C with 5% CO_2. All incubations were followed by 3 washes with phosphate-buffered saline (pH 7.4) containing 0.1% Tween-20 (Sigma Aldrich, St. Louis, Missouri) and 3 washes with nanopure water. The spots were developed with an aminoethylcarbazole staining kit (Sigma Aldrich, St. Louis, Missouri). The number of spots per well between 0.0001 and 9.6372 mm^2 were quantified via the Immunospot Software (Cellular Technology, Ltd, Shaker Heights, Ohio) and normalized to the number of viable cells in each well.

Enzyme-linked immunosorbent assay. The amount of supernatant IgM present in cell culture medium was quantified using a sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, Immulon 4 HBX 96-well microtiter plates (VWR International, Radnor, Pennsylvania) were coated with anti-human IgM antibody (1 μg/ml; Sigma Aldrich) for overnight. Culture media collected from human B cells was incubated over primary antibody-coated plates for 90 min at 37°C with 5% CO_2 followed by addition of an antihuman IgM-HRP conjugate antibody (Sigma Aldrich). Incubations were followed with washes using phosphate-buffered saline (pH 7.4) containing 0.05% Tween-20 (Sigma Aldrich) and with nanopure water. 2, 2'- Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (Roche Diagnostics, Basel, Switzerland) was then added as a colorimetric substrate for the HRP. The rate of colorimetric change was quantified with a Synergy HT microplate reader (BioTek, Winooski, Vermont) at 405 nM for 1 h. The concentration of IgM in culture supernatants was calculated based on a standard curve created in each plate.

Western blotting. The antibodies used in the studies were HRP antihuman IgM A6907 (Sigma Aldrich, St. Louis, Missouri); antimouse IgM RMM-1 (Biolegend); HRP Goat-antirat IgG Poly4054 (Biolegend). Total cell lysates were prepared by lysing cells with Radioimmunoprecipitation assay containing protease inhibitor (Roche Applied Science, Indianapolis, Indiana). Cell lysates were stored at −20°C. The denatured sample was obtained by heating the cell lysates in 95°C for 10 min and was followed by loading the sample on a SDS-PAGE gel at the Mini-PROTEAN Precast System (Biorad, Hercules, California). The gel was then transferred to a nitrocellulose blotting membrane (GE healthcare, Little Chalfont, United Kingdom) on a semi-dry transfer system TE70 DWR (GE healthcare, Little Chalfont, United Kingdom) for 1 h. The membrane was then blotted with antibodies for 1 h followed by washes with Tris-buffered saline containing 0.1% Tween-20 (Sigma Aldrich, St. Louis, Missouri). SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Lafayette, Colorado) was used with the HRP. The membrane was developed in an image developer to visualize the protein distribution. The amount of IgM protein was quantified by densitometry and the total IgM levels were normalized to actin protein levels in each corresponding lane.

Flow cytometry. Antibodies used for flow cytometry were as follows: PE antihuman Ig light chain κ (Clone: MHK-49), PE/Cy7 anti-human CD19 (HIB19), FITC anti-mouse IgM (II/41), PE antimouse Igκ (RMK-45), anti-mouse IgJ (PA5-13486), Alexa Fluor Plus 647 anti-rabbit IgG (RJ243415). For staining, approximately 5×10^5 cells were harvested at the indicated time points and viable cells were identified by Fixable Live/Dead Near-IR dye (Life Technologies, Carlsbad, California) following manufacturer's instructions prior to cell surface or intracellular staining. Surface Fc receptors were blocked using human AB serum before staining for surface and intracellular proteins. Unconjugated anti-human IgH/Igκ was used to block the surface IgH/Igκ on cells before intracellular staining. For surface staining of CD19, cells were resuspended in FACS buffer (1× phosphate-buffered saline, 1% bovine serum albumin [BSA] (San Diego, California) and 0.1% sodium azide, pH: 7.6) in the presence of 20% human AB serum and the anti-human CD19 were added at the company specified concentrations and incubated at 4°C for 15–30 min. Following incubation with antibody, the cells were washed twice with FACS buffer to remove excess antibodies and then fixed by incubation in the BD Cytofix fixation buffer (BD Bioscience, Franklin Lakes, New Jersey) for 10 min and stored at 4°C until they were ready to be analyzed by the flow cytometry. For intracellular protein staining, cells that were previously fixed after surface staining were permeabilized with 1× BD PermWash buffer (BD Bioscience, Franklin Lakes, New Jersey) by washing twice and then incubated with PermWash buffer for 30 min at 4°C. Immediately after, cells were incubated for additional 30 min at 4°C with antibodies

specific to intracellular IgH, Igκ, and IgJ. In all cases, cells were analyzed on BD FACSCanto II using FACS Diva software (BD Bioscience, Franklin Lakes, New Jersey) and subsequently analyzed using FlowJo (Version 10, Treestar, LLC, Software Ashland, Oregon). Unless otherwise stated, cells were gated on singlets, live (as determined by Live/Dead dye) followed by gating on lymphocyte populations. Gates were drawn based on the unstimulated cells (resting human B cells, without CD40L and cytokine activation) or unstained cells as appropriate.

RESULTS

AHR-Mediated Suppression of the IgM Response in Mouse and Human Primary B Cells

Impairment of humoral immunity after exposure to various AHR ligands has been demonstrated in different animal species with the largest body of work conducted in mice. To gain further insight into the molecular mechanism by which AHR activation mediates suppression of the IgM response in primary human B cells, side-by-side comparisons were initially performed in human and mouse primary B cells using an *in vitro* CD40L activation model system. The IgM concentration in culture supernatant and the number of IgM-secreting B cells in both mouse (Figs. 1A and 1B) and human B cells (Figs. 1C and 1D) were significantly suppressed by AHR activation.

Previous studies using AHR null mice showed an absolute requirement for the AHR in suppression of humoral immune responses by TCDD, demonstrating the receptor is required for suppression (Fernandez-Salguero *et al.*, 1997; Vorderstrasse *et al.*, 2001). To further determine the involvement of AHR in the decrease of IgM secretion by human primary B cells, the AHR antagonist (CH-223191) was employed. Treatment of CD40L-activated human primary B cells with the AHR antagonist alone increased the number of IgM-secreting cells and enhanced the supernatant IgM concentration, compared with vehicle control (Figs. 1E and 1F). Treatment of human B cells with TCDD in combination with increasing concentrations of AHR antagonist produced a concentration-dependent reversal of the IgM response. (Figs. 1E and 1F).

AHR Activation by TCDD Suppressed IgH, IgJ, and Igκ Chains in Mouse but Not in Human Primary B Cells

Studies were performed to compare the effect of TCDD treatment on intracellular protein levels of IgH, IgJ, and Igκ in mouse and human B cells. The intracellular protein levels of IgH and IgJ were significantly suppressed by TCDD in a concentration-dependent manner in mouse primary B cells; whereas, Igκ was less sensitive to suppression and was only significantly decreased at the highest TCDD concentration (Figs. 2A–D). Western blotting of IgH also revealed a concentration-dependent decrease of total IgH protein in TCDD-treated mouse B cells (Figs. 2E and 2D). In contrast, the intracellular protein levels of IgH, IgJ, and Igκ, as determined by flow cytometry, were not suppressed by TCDD treatment in human B cells (Figs. 3A–D). To verify the flow cytometry results for human B cells, Western blotting was performed. Western blotting revealed that TCDD treatment (10 nM), significantly increased intracellular IgM compared with the respective VH control group in human B cells (Figs. 3E and 3F). To further confirm the observation that AHR activation did not impair IgM production by human primary B cells, the effect of TCDD was assessed using a different activation stimulus, pokeweed mitogen (PWM). As observed with CD40L, PWM-activated human primary B cells showed no decrease in intracellular IgM protein levels at any of the TCDD concentrations tested (Figure 3G). The difference between human and mouse B cells in the intracellular protein levels of IgH, IgJ, and Igκ indicated divergent mechanisms responsible for impairment of the IgM response between the two species after AHR activation.

AHR Activation Suppressed the mRNA Levels of IgH, IgJ, and Igκ in Mouse but Not Human Primary B Cells

Previous studies have demonstrated that AHR activation decreased the mRNA expression levels of *IgH*, *Igκ*, and *IgJ* chains in mouse B cells after LPS activation (Schneider *et al.*, 2008). In this study, we also observed a concentration-dependent decrease of the mRNA levels of *IgH*, *IgJ*, and *Igκ* in CD40L-activated mouse B cells when treated with TCDD (Figs. 4A–C). In contrast, the mRNA levels of *IgH*, *Igκ*, and *IgJ* were not altered in CD40L-activated human B cells with AHR activation at TCDD concentrations as high as 10 nM (Figs. 4D–F).

AHR Activation by TCDD Caused the Intracellular Accumulation of Assembled IgM Pentamer

The secreted IgM pentamer consists of 5 IgM monomers that are joined together by a single J chain (Kirk *et al.*, 2010). Due to the observation by Western blotting, that intracellular IgM was accumulating in human B cell treated with TCDD (Figure 3F), studies were conducted to explore the possibility that AHR activation interfered with the assembly of the IgM pentamer. Native-PAGE assays were used to assess the amount of intracellular IgM pentamer in activated human B cells. TCDD treatment increased the intracellular levels of IgM pentamers, trimers and dimers compared with the VH control (Figure 5). Longer exposure of the same Native-PAGE film showed noticeably more intracellular IgM pentamer in the TCDD treatment groups compared with VH group controls (Supplementary Data 1).

TCDD-Mediated Activation of AHR Decreased the Secreted IgG in Human Primary B Cells

IgG is secreted by B cells after undergoing class switching and is the mature form of immunoglobulin secreted by B cell (Tangye *et al.*, 2002). Activation by cytokines has also shown to promote class switch in human B cells (Malisan *et al.*, 1996). Therefore, further studies were conducted to determine if impairment of immunoglobulin secretion was limited to IgM by AHR activation. ELISA were conducted to quantify supernatant IgG levels from human B cell cultures. These studies showed that the levels of supernatant IgG in culture medium decreased as TCDD concentrations increased, with a significant decrease at 10-nM TCDD (Figure 6).

DISCUSSION

Suppression of antibody responses after AHR activation by high-affinity ligands, most notably by TCDD, has been observed in many animal species (Sulentic and Kaminski, 2011; Travis and Hattemer-Frey, 1987). Investigations in well-established mouse models have been especially useful for studies aimed at elucidation of the molecular mechanism for impairment of humoral immunity in response to AHR activation (North *et al.*, 2010). Murine studies have shown that suppression of the IgM response by AHR ligands is closely linked to a decrease in mRNA levels for the immunoglobulin chains (IgH, IgJ, and Igκ chains) (Schneider *et al.*, 2008). Previous studies have also demonstrated that AHR activation impairs IgM responses by human B cells to a variety of stimuli including: PWM; co-stimulation by CD40L with cytokines; and toxic shock syndrome toxin

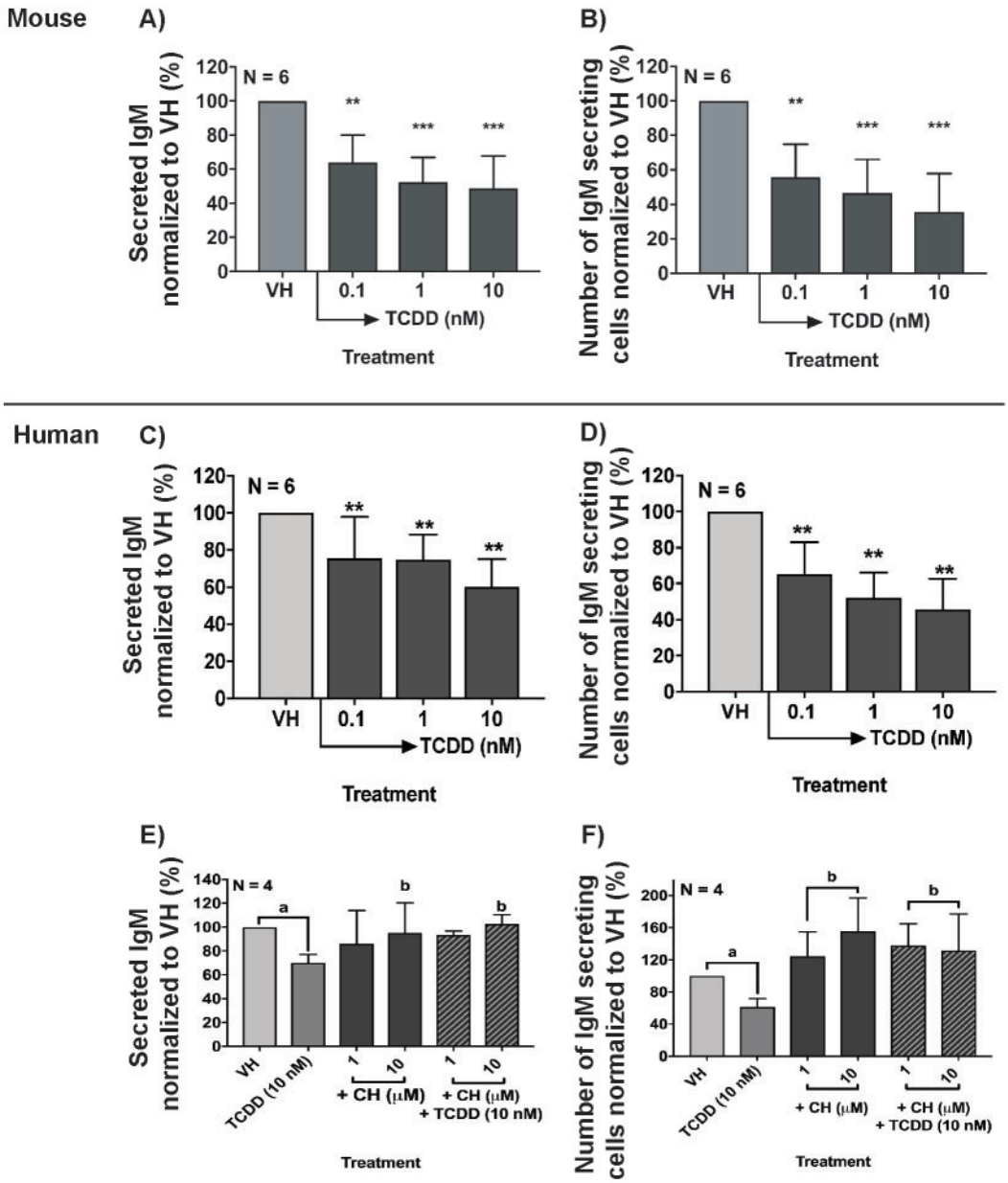

Figure 1. AHR-mediated suppression of the IgM response in mouse and human primary B cells. Mouse and human primary B cells were treated with VH (0.02% DMSO) or TCDD (0.1, 1, and 10 nM) and, subsequently activated by co-culture with CD40 ligand expressing L cells plus cytokines (IL-2, IL-6, and IL-10) for 6 days (mouse) or 7 days (human). A, Mouse primary B cells were collected to quantify the concentration of secreted IgM by ELISA on day 6. The IgM concentration in supernatants from mouse B cells ranged from 16 000 to 25 000 ng/ml in the VH control group. B, Mouse primary B cells were collected to quantify the number of IgM-producing cells by ELISPOT on day 6. The number of IgM-secreting mouse B cells ranged from 12 000 to 20 000 per 10^6 cells in the VH control group. C, Supernatant from human primary B cell cultures were collected and quantified for IgM by ELISA on day 7. The IgM concentration in the supernatants ranged from 12 000 to 27 000 ng/ml in VH control group. D, Human primary B cells were collected to quantify the number of IgM-secreting cells by ELISPOT on day 7. The number of human IgM-secreting B cells ranged from 10 000 to 20 000 per 10^6 cells in the in the VH control group. "N" indicates the number of human donors or mice use in the study. Significant differences from VH control are indicated by ** $p < .01$, *** $p < .001$ by 1-way ANOVA following by Fisher's post hoc test. Naive human primary B cells were treated with AHR antagonist, CH-223191 (CH) for 30 min. After treatment with antagonist, B cells were then treated with VH (0.02% DMSO) or TCDD (10 nM) and activated as described in the "Materials and Methods" section. E, Concentration of human secreted IgM in culture supernatants was quantified by ELISA on day 7. F, The number of human IgM-secreting B cells was quantified by ELISPOT on day 7. a indicates a significant difference at $p < .05$ compared with the respective VH groups as determined by 1-way ANOVA followed by Fisher's LSD post hoc test. b indicates a significant difference at $p < .05$ compared with the respective TCDD (10 nM) groups by 1-way ANOVA as determined by Fisher's LSD post hoc test. Results are the normalized percentage to the VH group.

superantigen (Kovalova *et al.*, 2016a; Lu *et al.*, 2011; Phadnis-Moghe *et al.*, 2015; Wood and Holsapple, 1993). It has been broadly assumed that the molecular mechanisms responsible for immune suppression by AHR ligands, including suppression of the IgM antibody response, would be similar across animal species. Here we report that the molecular mechanism responsible for TCDD-mediated suppression of the IgM response in human and mouse primary B cells differs significantly.

In the current side-by-side studies comparing primary mouse and human B cells, we observed a decrease of immunoglobulin proteins (IgH, IgJ, and Igκ) following AHR activation in

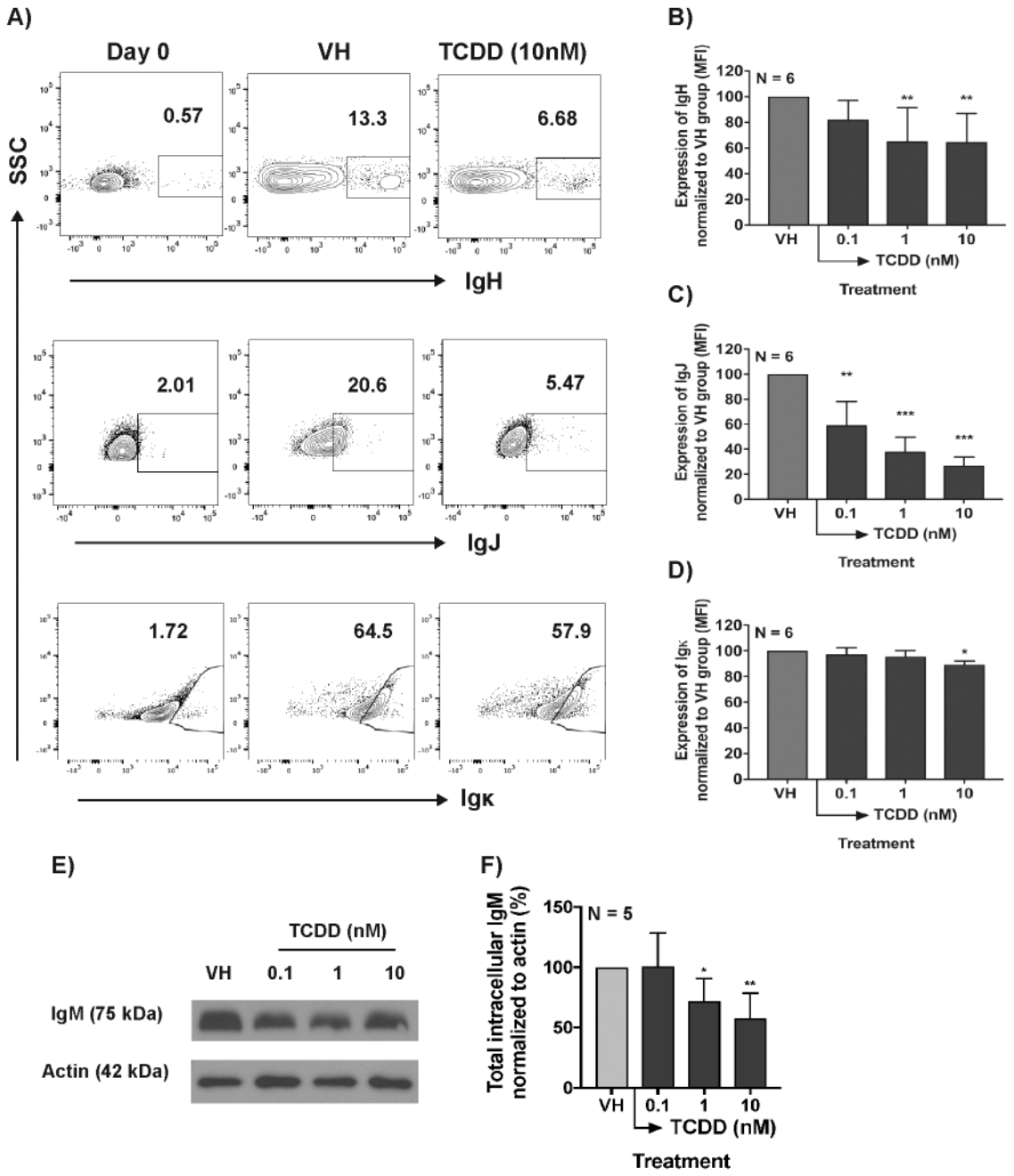

Figure 2. AHR activation by TCDD suppressed IgH, IgJ, and Igκ chains in mouse primary B cells. Mouse primary B cells were treated with VH (0.02% DMSO) or TCDD (0.1, 1, and 10 nM) and then activated by co-culture with CD40L expressing L cells plus cytokines (IL-2, IL-6, and IL-10) for 6 days. A, Flow cytometry dot plots of intracellular IgH, IgJ, and Igκ chains with VH or TCDD treatment in mouse primary B cells. B, Level of intracellular IgH protein. C, Level of intracellular IgJ protein. D, Level of intracellular Igκ protein. E, Western blotting for IgM in whole cell lysates. F, Level of total IgM protein normalized to actin protein quantified by Western blotting. Results are the normalized percentage to the VH group. "N" indicates the number of mice used in the study. Significant differences from VH control are indicated by * $p < .05$, ** $p < .01$, and *** $p < .001$ as determined by a 1-way ANOVA followed by Fisher's LSD post hoc test.

mouse primary B cells, as determined by intracellular staining and Western Blotting. This finding is consistent with prior reports of decreased mRNA levels for IgH, IgJ, and Igκ, resulting from an impairment of the B cell to plasma cell developmental program through changes in the regulation of paired box 5 (Pax5) and B lymphocyte-induced maturation protein-1 (BLIMP-1) (North *et al.*, 2009, 2010; Sulentic *et al.*, 2004). In contrast, AHR activation in human primary B cells does not alter the transcription, translation, or assembly of IgM (Figs. 3 and 4), but rather the ability of B cell to secrete assembled IgM, which is also consistent with an absence in altered Pax5 or BLIMP-1 regulation in human B cells (Lu *et al.*, 2011). Collectively, the studies presented in this manuscript indicate a divergence in the mechanism by which AHR activation suppresses the IgM response in human and mouse primary B cells.

The accumulation of IgM pentamers with TCDD treatment further suggests that TCDD-mediated activation of AHR did not impair the synthesis and assembly of IgM pentamers in human B cells but rather IgM secretion (Figure 5). Impairment in IgM secretion is further supported by accumulation of IgM dimers and

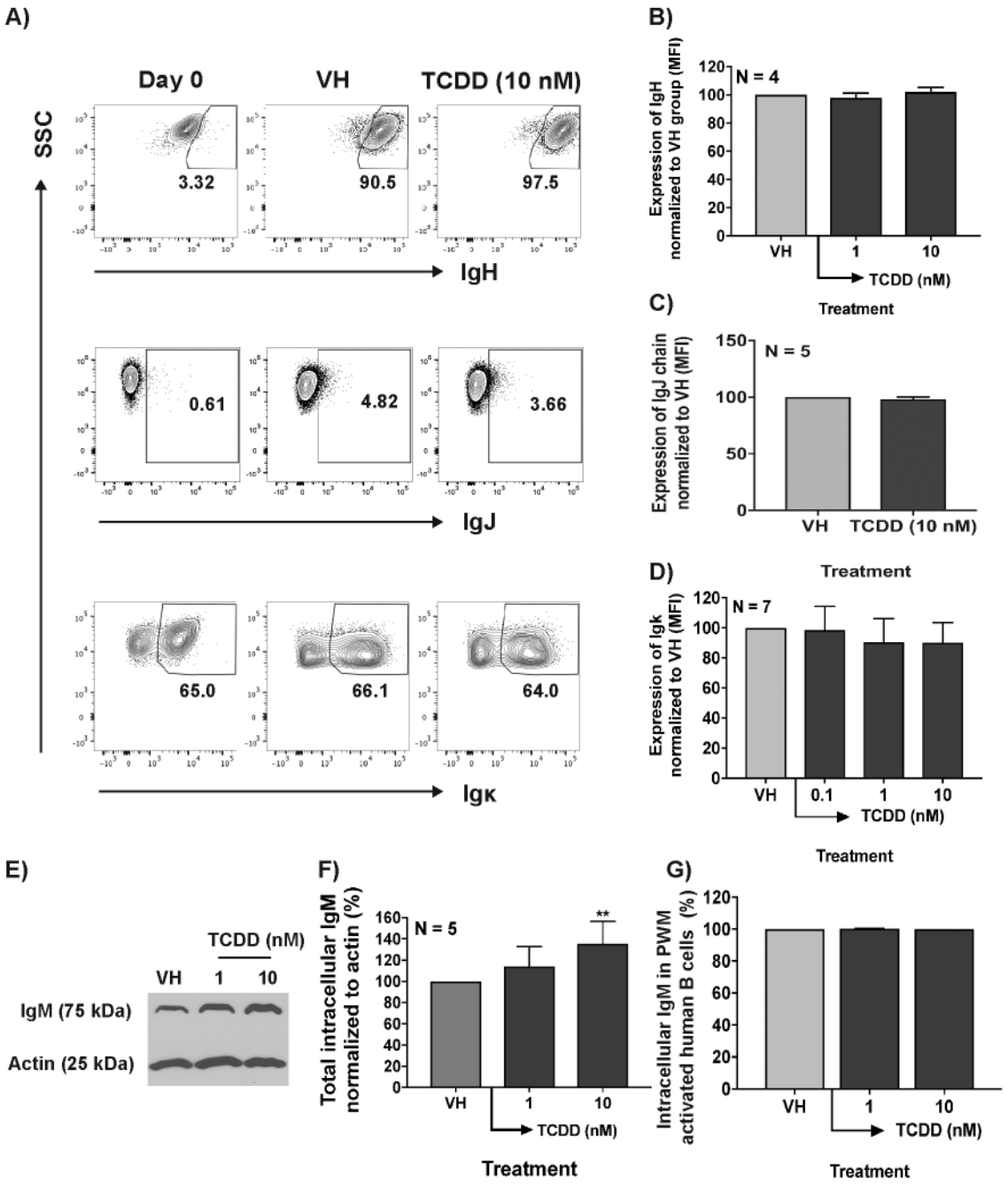

Figure 3. AHR activation did not alter the intracellular IgH, IgJ, and Igκ chains in human primary B cells. Human primary B cells were treated with VH (0.02% DMSO) or TCDD and activated by co-culture with CD40L expressing L cells plus cytokines (IL-2, IL-6, and IL-10) for 7 days. A, Flow cytometry dot plots of intracellular IgH, IgJ, and Igκ in human B cells. B, Level of intracellular IgH protein. C, Level of intracellular IgJ protein. D, Level of intracellular Igκ protein. E, Western blotting for IgM in whole cell lysates. F, Level of total IgM protein normalized to actin protein quantified by Western blotting. G, Human B cells were treated with VH (0.02% DMSO), or TCDD (1 and 10 nM) and activated with PWM for 5 days. On day 5, human B cells were collected and quantified for intracellular IgH protein by flow cytometry. Results are the normalized percentage for each donor's VH control response. "N" indicates the number of human donors. Significant differences from VH control are indicated by ** $p < .01$ as determined by a 1-way ANOVA followed by Fisher's LSD post hoc test.

trimers in TCDD-treated human B cells (Figure 5). The assembly of IgM dimers to trimers and pentamers occurs between the ER and Golgi (Anelli *et al.*, 2007), further suggesting that IgM protein trafficking is likely being affected after Golgi-associated processes are completed.

Recent transcriptomic analysis investigating the effect of AHR activation in PWM-activated mouse and human primary B cells revealed remarkably few common differentially expressed genes across the two species (Kovalova *et al.*, 2016b), which provided the first insights suggesting a divergence in mechanism by which AHR activation influences human and mouse B cell function. Although the current investigation indicates that AHR activation impairs immunoglobulin secretion, it is presently unclear which specific processes within the immunoglobulin secretory pathway are affected. Studies by other laboratories have shown that the upregulation of critical secretory genes and the enlargement of the ER and Golgi are critical in the secretion of immunoglobulins by human B cells

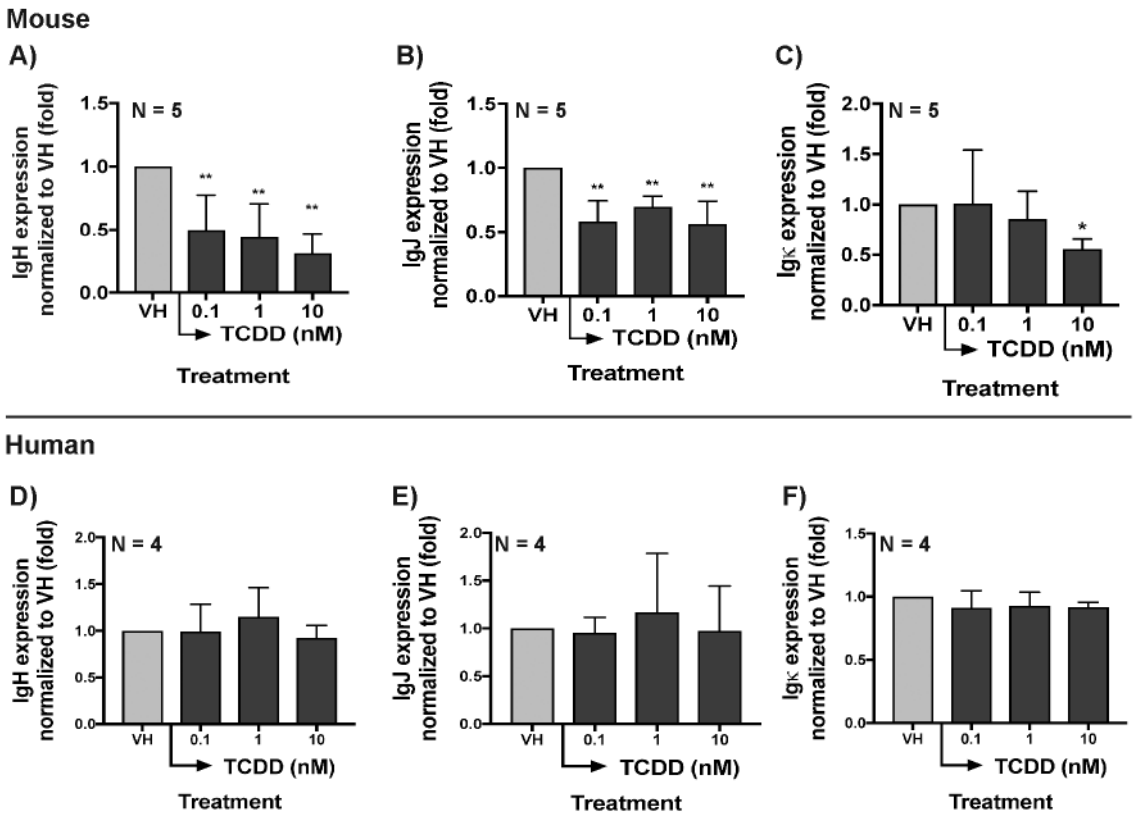

Figure 4. AHR activation suppressed the mRNA levels of *IgH*, *IgJ*, and *Igκ* in mouse but not human primary B cells. Mouse and human primary B cells were treated with VH (0.02% DMSO), or TCDD (0.1, 1, and 10 nM) and activated by co-culture with CD40L expressing L cells plus cytokines (IL-2, IL-6, and IL-10) for 6 days (mouse) or 7 days (human). A, Mouse B cells treated with VH or TCDD were collected on day 6 and quantified for mRNA levels of *IgH*; (B) mRNA levels of *IgJ*; or (C) mRNA levels of *Igκ*. D, Human B cells were quantified for the mRNA levels of *IgH*; (E) mRNA levels of *IgJ*; or (F) mRNA expression level of *Igκ*. Results are presented as the normalized fold change for each treatment to VH control for both human donors and mice. "N" indicates the number of mice or donors used in the study. Significant differences from VH control are indicated by * $p < .05$ and ** $p < .01$ as determined by a 1-way ANOVA followed by Fisher's LSD post hoc test.

(Anelli and van Anken, 2013). B cells then undergo structural modification in preparation to become an antibody-secreting cell (Anelli and van Anken, 2013). In the current studies, we have not only observed a decrease in secreted IgM but also IgG antibodies by human B cells with AHR activation suggesting the effects are not limited to the IgM isotype (Figure 6). Future studies focused on the B cell secretory processes will be required to determine the specific molecular targets responsible for AHR-mediated impairment of immunoglobulin secretion.

One interesting phenomenon that we have observed when studying TCDD-mediated suppression of IgM responses in human B cells is that even when high concentrations of TCDD are employed (>10 nM), the magnitude of suppression rarely exceeds 50% of the vehicle control response, regardless of whether it is quantified by ELISPOT or ELISA. The ELISPOT results, which enumerate the number of IgM-secreting cells, show that there is a population of B cells that is refractory to suppression by TCDD. One explanation for the lack of suppression in a subset of B cells, although unlikely, is that TCDD does not distribute homogenously to all of the B cells in culture, thus resulting in AHR activation in only a fraction of the cultured B cells. A second explanation is that an insufficient number of AHRs are activated in some of the cells; hence impairment of IgM secretory processes only occurs in a subset of B cells. Although plausible, it is difficult to test until the molecular targets, responsible for impaired IgM secretion have been elucidated. This hypothesis is worthy of further study. A third possibility, and what we believe may be the most plausible explanation, is that only a specific subpopulation of human B cells is, in fact, sensitive to suppression by high-affinity AHR ligands such as TCDD. This would explain why close to a 100% suppression of the IgM response cannot be attained unless cytotoxic concentrations of an AHR ligands are used to treat B cells. Moreover, approximately 1 in 7, donors are what we have previously termed "non-responders," are defined by a lack of sensitivity to IgM suppression by TCDD (Sulentic and Kaminski, 2011). It is tempting to speculate whether these nonresponding donors have a very low number of the putative subset of B cells that is responsive to TCDD, and future studies are planned to address this phenomenon. In addition to "classical" B cells, the best characterized B cell subset has been termed innate-like. These innate-like B cells are the major source of natural IgM antibodies and they play a critical role in early immune responses (Majolini *et al.*, 1998). Innate-like B cells can produce a large amount of circulating IgM following Toll-like receptors or CD40L activation to provide critical early defense against infections (Choi *et al.*, 2012; Martins and Calame, 2008). This newly generated circulating IgM plays an important role in neutralizing pathogens and enhancing complement activation to protect the host prior to the generation of an adaptive immune response (Baumgarth *et al.*, 2000).

A second interesting phenomenon that we and others have described in B cells is that AHR activation must occur within the first 24 h post B cell activation to induce suppression of the IgM response (Kovalova *et al.*, 2016a). The temporal relationship between AHR activation and the activation of the B cell suggests

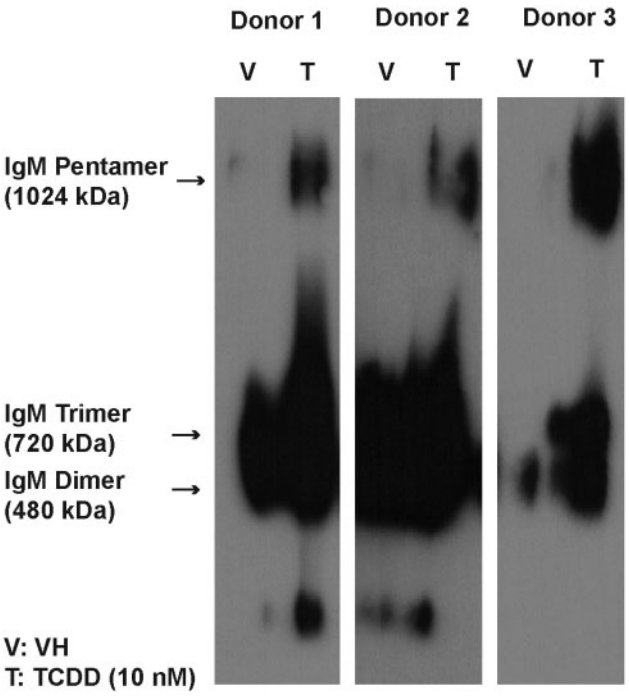

Figure 5. AHR activation by TCDD caused intracellular accumulation of the assembled IgM pentamer. Human primary B cells were treated with VH (0.02% DMSO) or TCDD (10 nM) and activated by co-culture with CD40L expressing L cells plus cytokines (IL-2, IL-6, and IL-10). Cells were collected on day 7 and whole cell lysates prepared for identification of IgM isoforms using Native-PAGE assay. The top band represented the 1024 kD IgM pentamer. The bottom large dark band indicated the combination of IgM trimers (720 kDa) and dimers (480 kDa). The analysis was performed on B cells isolated from 3 separate human donors with all 3 of the donor's B cells exhibiting suppression of the IgM response by TCDD treatment as quantified by ELISA and ELISPOT.

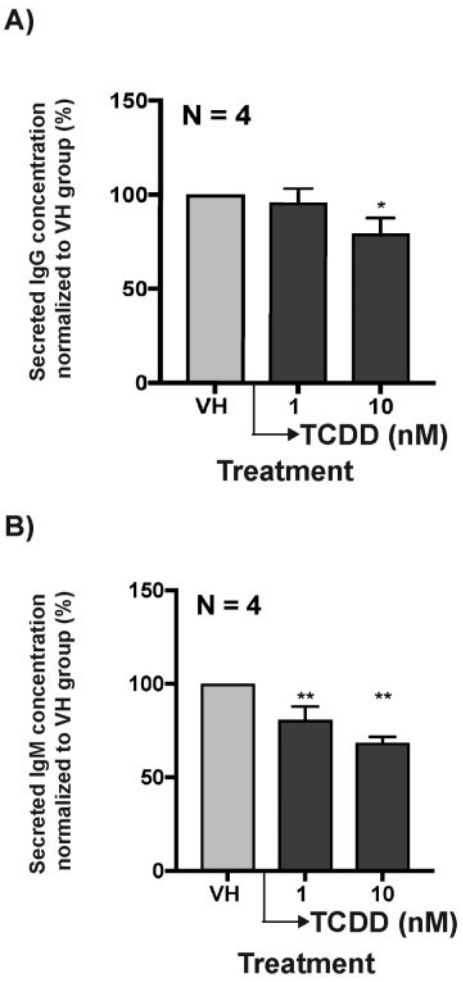

Figure 6. TCDD-mediated activation of AHR decreased the secreted IgG in human primary B cells. Human primary B cell were treated with VH (0.02% DMSO) or TCDD (1 and 10 nM) and activated by co-culture with CD40L expressing L cells plus cytokines (IL-2, IL-6, and IL-10). On day 7, the culture medium was collected and quantified for (A) IgG; and (B) IgM by ELISA. Results are normalized to the individual VH group for each donor. The level of IgG ranged from 9000 to 10 000 ng/mL and the level of IgM ranged from 15 000 to 25 000 ng/mL in the VH treated control B cells. "N" indicates the number of donors. Significant differences from VH control are indicated by * $p < .05$, ** $p < .01$ as determined by a 1-way ANOVA following by Fisher's LSD post hoc test.

that AHR activation may induce diminished assembly or function of the necessary secretory machinery required for immunoglobulin release from B cells. Putative targets include ERp44, ERGIC53, ER-associated proteins (Anelli *et al.*, 2007) and secretory signal cascades, all of which have the potential of decreasing immunoglobulin secretion, which is supported by the observation in the Native-PAGE (Figure 5). Likewise, activation of AHR may alter immunoglobulin trafficking within human B cells, therefore leading to diminution of antibody transport and secretion.

Our current studies also demonstrate that the AHR is directly involved with impaired IgM secretion by TCDD-treated human B cells as pretreatment of B cells with an AHR antagonist restored IgM secretion. Interestingly, we have also observed increased IgM secretion in B cells treated only with AHR antagonist in the absence of treatment with an AHR ligand. These data suggest AHR plays a role in the regulation of immunoglobulin secretion through its activation by one or more endogenous ligands. A similar phenomenon has also been observed in AHR-null rats, which exhibited an increase in secreted IgM compared with wild type rats (Phadnis-Moghe *et al.*, 2016). Moreover, since TCDD-mediated AHR activation also results in decreased secreted IgG, this finding suggests that there is a generalized effect on the secretory processes in human primary B cells.

Collectively, these studies are the first to report divergent mechanisms governing impaired B cell effector function by AHR activation in mouse and human primary B cells. In mouse B cells, AHR activation impairs transcription of immunoglobulin IgH, IgJ, and Igκ chains and is therefore the likely cause for the decrease of synthesis and production of IgM. In contrast, in human B cells AHR activation has no effect on transcription, translation or IgM assembly but appears to impair IgM secretion. Although the findings presented in this study are the first to show key differences in AHR-mediated suppression of IgM responses between human and mouse primary B cells, further investigation is required to elucidate the mechanisms underlying these differences.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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