**Kaminski Laboratory - Material and Methods**

Description of the test articles used, study material evaluations (diet, drinking water, cage and bedding leachates), general study design, animal treatments, and animal allocations to the 2 year toxicology study conducted at NCTR and the grantee studies can be found in Heindel et al. (2015).

**2.1 Sample Collection**

In the present study, thymus glands on postnatal day (PND) 21 and spleens on PND 21, PND 90, 6 month, and 1 year were collected from animals dosed continuously with vehicle (0.3% aqueous carboxymethylcellulose (CMC)), BPA (2.5, 25, 250, 2,500 or 25,000 μg/kg bw/day), or reference estrogen ethinyl estradiol (EE2) (0.05 or 0.5 μg/kg bw/day). The individual body and organ weights were recorded at necropsy and the samples were transferred from the FDA's National Center for Toxicological Research (NCTR) to Michigan State University in 1640 RPMI medium (Gibco Invitrogen, Carlsbad, CA) supplemented with 5% bovine calf serum (BCS; HyClone, Logan, UT) and penicillin (100 U/ml)/streptomycin (100 g/ml; Gibco Invitrogen) on ice overnight and processed the following day.

**2.2 Cell culture and activation**

Splenocytes and thymocytes were isolated by mechanical disruption and made into single cell suspensions in RPMI 1640 medium supplemented with 10% BCS and penicillin/streptomycin. Red blood cells were lysed using Zap-oglobin prior to counting splenocytes on Beckman Coulter Counter following manufacturer's instructions with a size threshold of 4.0 μm. Splenocytes were then cultured at 1 x $10^6$ cells/well in 96 well plates or 5 x $10^6$ cell/well in 48 well plates. Cells were treated with 15 μg/ml lipopolysaccharide (LPS, *S. typhosa*, Sigma Aldrich, St. Louis, MO), 15 μg/ml pokeweed mitogen (PWM, Sigma Aldrich), or 1 μg/ml anti-CD3 (clone 145-2C11, Biolegend, San Diego, CA) plus 10 μg/ml anti-CD28

(clone 37.51, Biolegend). For anti-CD3/CD28 treatment, culture plates were coated overnight with 1 μg/ml anti-CD3 at 4 °C, washed twice with 1640 RPMI, and then seeded with cells and 10 μg/ml anti-CD28. Cells were culture at 37 °C with 5% $CO_2$ for up to 72 hours.

Post-activation, cells were harvested by centrifugation at 500 x g for 5 minutes. Supernatants were collected for IgM ELISA, and cells were washed in Hank's Balanced Salt Solution and stained for flow cytometry.

**2.3 ELISA**

IgM responses by activated splenocytes was characterized by ELISA. 96-well ELISA plates (Immulon 4 HBX strips, Thermo Scientific, Milford, MA) were coated with 1 μg/ml purified mouse anti-rat IgM antibody (clone G53-238, BD Biosciences, San Jose, CA) in 0.1 M sodium bicarbonate buffer (pH 9.6) at 4°C overnight. Plates were washed with phosphate-buffered saline (PBS) containing 0.05 % tween-20 and incubated with 3% bovine serum albumin (BSA)-PBS at room temperature for 1-2 hours. Plates were washed again as described and samples incubated at 37 °C for 1-1.5 hours. Plates were washed three times and incubated with 1 μg/ml of biotin mouse anti-rat IgM secondary antibody (clone G53-238, BD Biosciences) for 1.5 hours. Plates were washed following incubation and developed with 1 mg/ml ABTS buffer (Riche, Branford, CT). Samples were read using BioTek Synergy HT plate reader (BioTek, Winooski, VT) at 405 nm every minutes for 1 hour on kinetic mode.

2.4 Proliferation Assay

Splenocytes were activated with 15 μg/ml LPS, PWM, or 1 μg/ml anti-CD3 and 10 μg/ml anti-CD28 in 96-well plates. Cells were pulsed with [3H]-thymidine (1 μCi/well) 48 hours after activation. Cells were harvested on filter paper 72 hours after activation. The incorporation of [3H]-thymidine in the chromosomal DNA during cell proliferation were quantified using Ultima Gold liquid scintillation cocktail (PerkinElmer, Waltham, MA) and Tri-Carb 2100 TR scintillation counter (PerkinElmer).

**2.5 Flow Cytometry**

Cells were washed using 1X Hank's Balanced Salt Solution (HBSS, pH 7.4, Invitrogen) and stained using LIVE/DEAD Fixable Near-IR Dead Cell Stain (Gibco Invitrogen) to assess cell viability, according to the manufacturer's instructions. Cell surface Fc receptors were blocked with purified mouse anti-rat CD32 (BD Biosciences, San Jose, CA). Cells were incubated in FACS buffer (1× HBSS containing 1% BSA and 0.1% sodium azide) and stained for surface proteins using the following antibodies from BD Biosciences or Biolegend: AF647-CD3 (clone 1F4), PE/Cy7-CD4 (clone W3/25), PerCP-CD8 (clone OX-8), FITC-CD11b/c (clone OX-42), FITC-CD25 (clone OX-39), PE-CD80 (clone 3H5), APC-CD86 (clone 24F), FITC-CD161a (clone 10/78), PE-CD172a (clone OX-41), FITC-IgM (clone G53-238), PerCP-MHCII (clone OX-6), BV450 CD11b (clone WT.5), FITC CD11c (clone 10B631), AffiniPure Rabbit Anti-Rat FITC IgG + IgM (Jackson Immuno Research, West Grove, PA), and PE CD161α (clone 10/78). Cells were incubated with the antibodies for 30 min, washed three times with FACS buffer, and fixed by incubating with Cytofix (BD Biosciences) for 10 min. To measure intracellular IgM, cells were washed and incubated with 1x Perm/Wash solution (BD Biosciences) for 30 minutes, then incubated with mouse anti-rat IgM for 30 minutes. Cells were washed and resuspended in FACS buffer. In all cases, flow cytometric analyses were performed on a FACS Canto II cell analyzer (BD Biosciences) and data were analyzed using FlowJo v8.8.6 (Tree Star, Ashland, OR) software.

**2.6 Statistical Analysis**

Statistical analyses were performed using GraphPad Prism version 4.0a (GraphPad Software, La Jolla, CA). Samples were excluded from analysis when the integrity of samples was judged unacceptable in cases where the cell viability upon tissue delivery was less than one standard deviation of the mean. To determine statistically significant changes between the treatment groups and vehicle control within male and female rats, a two-way ANOVA with Dunnett's posttest was used. The mean ± SE is displayed in all

bar graphs. At the 6 month PND time point, treatments were compared to the vehicle-overlap group, due to insufficient number of vehicle control animals.

During the chronic CLARITY-BPA study, the potential for unintentional exposure of vehicle control rats to BPA, termed “overlap” was acknowledged and evaluated (Heindel et al. 2015). It was hypothesized that the unintentional exposure resulted from housing of CLARITY-BPA control rats in the same room as those dosed with 250,000 μg BPA/kg bw/day. These vehicle control and potentially BPA-exposed rats were identified and tracked throughout the study. To eliminate any possibility of confounders and for the purpose of complete transparency, all vehicle control group rats potentially exposed to BPA were excluded from the data analysis, except for the vehicle group at 6 months. The 6-month rats were not excluded because all of vehicle treated female rats at 6 months of age were identified as potential overlap. Therefore, only at the 6-month time point were the vehicle overlap rats included in our analysis but were analyzed as a separate group and identified as "vehicle-overlap" (VH-Ov). At the 6-month time point, treatments in female rates were compared to the vehicle-overlap group, due to the insufficient number of vehicle control animals.

**References:**

Heindel JJ, Newbold RR, Bucher JR, Camacho L, Delclos KB, Lewis SM, Vanlandingham M, Churchwell MI, Twaddle NC, McLellen M, Chidambaram M, Bryant M, Woodling K, Gamboa da Costa G, Ferguson SA, Flaws J, Howard PC, Walker NJ, Zoeller RT, Fostel J, Favaro C, Schug TT. NIEHS/FDA CLARITY-BPA research program update. Reprod Toxicol. 2015 Dec; 58:33-44.