# Sex-dependent Effects of Bisphenol A on Type 1 Diabetes Development in Non-obese Diabetic (NOD) Mice

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## Abstract

Type 1 diabetes (T1D) is an autoimmune disease caused by immune-mediated pancreatic β-cell destruction. The endocrine disrupting chemical bisphenol A (BPA) has widespread human exposure and can modulate immune function and the gut microbiome (GMB), which may contribute to the increasing T1D incidence worldwide. It was hypothesized that BPA had sex-dependent effects on T1D by modulating immune homeostasis and GMB. Adult female and male non-obese diabetic (NOD) mice were orally administered BPA at environmentally relevant doses (30 or 300 μg/kg). Antibiotic-treated adult NOD females were exposed to 0 or 30 μg/kg BPA. BPA accelerated T1D development in females, but delayed males from T1D. Consistently, females had a shift towards pro-inflammation (e.g. increased macrophages and *Bacteroidetes*), while males had increases in anti-inflammatory immune factors and a decrease in both anti- and pro-inflammatory GMB. Although bacteria altered during sub-acute BPA exposure differed from bacteria altered from chronic BPA exposure in both sexes, the GMB profile was consistently pro-inflammatory in females, while males had a general decrease of both anti- and pro-inflammatory gut microbes. However, treatment of females with the antibiotic vancomycin failed to prevent BPA-induced glucose intolerance, suggesting changes in Gram-positive bacteria were not a primary mechanism. In conclusion, BPA exposure was found to have sex dimorphic effects on T1D with detrimental effects in females, and immunomodulation was identified as the primary mechanism.

## Keywords

Bisphenol A; type 1 diabetes; NOD mice; immunomodulation; microbiome; vancomycin

## Introduction

Bisphenol A (BPA), an endocrine disrupting chemical (EDC) used widely in a variety of polycarbonate plastics and epoxy resins (e.g., water bottles and the lining of food cans), has been identified in more than 90% of human urine samples (Lang et al. 2008). Worldwide, there has been a parallel increase in type 1 diabetes (T1D) and EDC exposure (Bergman et al. 2013; Pitkäniemi et al. 2004). While there are still uncertainties about the involvement of BPA in T1D risk, animal studies have shown that BPA can increase T1D and/or autoimmune responses in non-obese diabetic (NOD) female mice (Bodin et al. 2014; Bodin et al. 2013) and streptozotocin-treated C57BL/6 male mice (Cetkovic-Cvrlje et al. 2017). Furthermore, human studies demonstrate a correlation of increased diabetes with increased BPA exposure (Lang et al. 2008).

T1D, once afflicting mainly juveniles, is increasing in incidence among US adults with a 40% increased risk of death in women compared to men with T1D (Chiang et al. 2014; Huxley et al. 2015). Various studies have established the association between T1D and gut microbiome (GMB) (Brown et al. 2011; de Goffau et al. 2014). Immune function and development are influenced by GMB and the composition and localization of GMB are modulated by immunity (Belkaid and Hand 2014). Studies have shown that BPA can alter GMB and cause dysbiosis (Lai et al. 2016; Liu et al. 2016). However, whether alteration of GMB by BPA can increase disease risk for T1D has yet to be studied.

Differences in hormone levels in different sexes affect T1D susceptibility (Markle et al. 2013). Sex-biased changes from BPA exposure have been reported for human glucose homeostasis (Beydoun et al. 2014). NOD mice, a spontaneous T1D model that has similarities to human T1D, have also been known for their sex-dependent dysregulation of immune system and T1D development (Young et al. 2009). BPA has sex-related effects on sex hormone levels (Scinicariello and Buser 2016), and various hormones can regulate insulin secretion and metabolic function in T1D patients (Fu et al. 2013). The overall hypothesis of this study was that BPA would differentially affect T1D susceptibility depending on sex through alteration of immune responses in NOD mice in which changes in immune cell populations and cytokine/chemokine expressions lead to the destruction of pancreatic β-cells (Jörns et al. 2014). We have evaluated the effects of BPA on T1D development and the roles of the immune system and GMB using NOD mice exposed during adulthood. This is the first study to determine the role of BPA's immunomodulatory mechanisms on T1D based on sex.

## Materials and Methods

### Animal Husbandry

Specific pathogen free (SPF) NOD mice were initially obtained from Taconic Biosciences (Hudson, NY). NOD mice were housed in polysulfone cages with irradiated laboratory animal bedding and Bed-r'Nest for enrichment (The Andersons Inc., Maumee, Ohio). Recent studies have reported that a negligible amount of BPA leaches from new or used polysulfone cages maintained at room temperature (Johnson et al. 2016). The animal room

was maintained at 22–25°C with relative humidity 50±20 and 12-h light/dark cycle (7:00AM lights on). The mice had access to water and food *ad libitum*. Only mice that were receiving the same treatment were housed together to prevent coprophagy between groups. All procedures were conducted under an approved animal protocol by the University of Georgia Institutional Animal Care and Use Committee (IACUC). Animals were treated humanely with regard for alleviating suffering.

### Bisphenol A Exposure

**Exposure of NOD mice to BPA.—**Mice (8–12 wks old; 10/group/sex) were randomized into vehicle (VH) or BPA groups, and initial body weight (BW) and blood glucose levels (BGLs) were not significantly different before treatment started. All mice fed with PicoLab diet (LabDiet, St. Louis, MO) containing ~ 237–655 ppm isoflavone (Huang et al. 2017) were dosed daily with VH, 30 or 300 μg BPA/kg BW by gavage. BPA was dissolved in 100% ethanol and added to corn oil at a final concentration of 0.05% ethanol. VH mice received the same volume of corn oil with 0.05% ethanol. The doses used in this study have previously been shown to alter the immune system (Yoshino et al. 2003); 30 μg/kg is within estimated human exposure levels, and 300 μg/kg is also relevant to human exposure based on median human blood levels (Calafat et al. 2005; Taylor et al. 2011). Females and males were euthanized at different time points (after 155 and 363 days of treatment, respectively) because female NOD mice developed T1D faster.

**Exposure of NOD females to BPA in combination with antibiotics.—**To determine the role of GMB alteration from BPA exposure, female NOD mice (10–12 wks old; 6/group) were randomized into VH or BPA groups according to BW and BGLs. They were dosed with either 0 or 30 μg BPA/kg BW as described above. Water bottles containing 0.5 g/L vancomycin were provided starting one week before dosing to ensure bacteria depletion and continuing throughout the experiment. Water bottles were changed once a week. This antibiotic and amount were chosen based on previous studies that determined their effects on GMB in female NOD mice (Hansen et al. 2012).

### Measurement of BW, BGL And Diabetic Incidence

BW and non-fasting BGLs were measured every 1–2 weeks. BGLs were measured from a small sample of venous blood (tail nick) using Accu-Chek Diabetes monitoring kit (Roche Diagnostics, Indianapolis, IN) or Contour Blood Glucose Meter (Ascensia Diabetes Care, Parsippany, NJ). Mice were considered diabetic if 2 consecutive BGL measurements were 250 mg/dL. Mice were humanely euthanized using $CO_2$ asphyxiation once a BGL 600 mg/dL was detected twice, or at the end of the study.

### Tolerance Tests And Insulin Measurement

For the glucose tolerance test (GTT), mice were fasted overnight for 15 h and then injected with glucose (2 g/kg BW; i.p.; (Susiarjo et al. 2015)). For the insulin tolerance test (ITT), mice were injected with 1.5 IU/kg BW insulin (i.p.; (Cui et al. 2015)). Baseline BW and BGLs, and BGLs after injection at 15, 30, 60 and 120 minutes were measured. Glucose and insulin were obtained from Sigma. For insulin measurement, the Ultra Sensitive Mouse

Insulin ELISA Kit (Crystal Chem, Downers Grove, IL) was performed following manufacturer's protocol.

## Organ Collection, Flow Cytometry And Histopathology

At euthanization, pancreas, spleen, kidneys with adrenals, liver and thymus were removed and cleaned of connective tissue before weighing. Histopathology is included in the supplementary methods. For flow cytometry, the spleens were mashed in 3 mL PBS solution on ice. Leukocyte populations were characterized by flow cytometric analysis with different combinations of fluorochrome-labeled antibodies (diluted 1:80–1:100; BD PharMingen, San Diego, CA) including CD4-CD8-CD25 (PE-PerCP-FITC), CD40L-B220 (PE-FITC) and Mac3-Gr1 (PE-FITC) in females. For males, we used CD4-CD8-CD25 (PE-PerCP-FITC), CD40-LB220 (PE-FITC) and F4/80-Gr1 (PE-FITC). CD4-CD8-CD25-Mac3-CD45R (V450-APCH7-APCA-FITC-PE), CD40L-B220 (PE-FITC), CD5-CD24 (PE-FITC) and CD44-CD40 (PE-FITC) were used in antibiotic treated females. Isotype matched irrelevant antibodies were used as controls. Following addition of the antibodies, the cells were incubated at 4°C in the dark for 30 min. The cells were washed and enumeration performed on a Becton Dickinson LSRII Flow Cytometer (BD Biosciences, San Jose, CA) in which log fluorescence intensity was read and a forward scatter threshold high enough to eliminate red blood cells. Ten thousand cells were counted for each sample. Analysis was done using FlowJo software (FlowJo LLC).

## Cytokine/chemokine Measurement

Milliplex MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore, Billerica, MA) was used for measuring cytokines and chemokines from sera collected at euthanasia according to manufacturer protocols along with the quality controls provided in the kit. Bio-Plex MAGPIX™ Multiplex Reader with Bio-Plex Manager™ MP Software (Luminex, Austin, TX) was used to run the plates, and concentration was measured as pg/ml.

## Antibody Measurement

ELISA kits (Bethyl Laboratories, Montgomery, TX) were used to measure IgG subclasses ($IgG_1$, $IgG_{2a}$, $IgG_{2b}$) and IgM levels in sera obtained at euthanasia as previously described (Huang et al. 2017). $IgG_{2a}$ ELISA kits have been shown to be a measure of $IgG_{2c}$ in NOD mice (Martin et al. 1998). $IgG_{2a}$ and $IgG_{2c}$ are generally assumed identical in function (Collins 2016), so we have referred to $IgG_{2a}$ results as $IgG_{2a}/IgG_{2c}$. Plates were read using the Synergy 4 Hybrid Microplate Reader (BioTek, Winooski, VT) either at a wavelength of 405 nm or at 450 nm following adding 100 μL/well stop solution (2N sulfuric acid).

## GMB, Bioinformatics And Metabolomics And Bioinformatics Analysis

Feces were collected from individual mice immediately after defecating and kept at −20°C until use. DNA was extracted using QIAamp DNA stool mini kits (Qiagen, Valencia, CA) following manufacturer protocols. For library preparation, DNA was normalized and the V3–V4 region of 16S rRNA was targeted using locus-specific primers for the first round of PCR. Illumina-specific iTru_R1_5'_fusion and iTru_R2_5'_fusion Read 1 and Read 2

sequencing primers (forward: 16S_341_F, and reverse: 16S_785_R) were used with 20 internal tags (8 forward fusion primers and 12 reverse fusion primers) ranging from 5 nucleotides (NT) to 8 NTs long (Glenn et al. 2016). For antibiotic treated females, the forward primer S-D-Bact-0564-a-S-15 and the reverse primer S-D-Bact-0785-a-A-21 were used. The PCR mix was from Kapa Biosystems, Inc. (Boston, MA). Next, the PCR amplicon aliquot was purified and quantified using Speedbeads and Qubit, respectively, or for the antibiotic treated females was purified with AMPure beads (Amplicon et al. 2013). The second round PCR was run using Illumina i5 and i7 primers, and sequencing was done on Illumina Miseq (Illumina Inc., San Diego, US). Bioinformatics analysis was performed as previously described (Lefever et al. 2016). Linear Discriminant Analysis (LDA) Effect Size (LEfSe) analysis was used to identify significantly different taxa following the conditions on the website: http://huttenhower.sph.harvard.edu/galaxy and increasing the LDA score to > 3 (Segata et al. 2011). Metabolomics analysis can be found in the supplementary methods.

### Statistical Analysis

Likelihood ratio and Logrank test were performed to analyze the rate of T1D development and total T1D incidence over time, respectively. Correlational analysis was conducted using Spearman's correlation test. Two-way ANOVA and Tukey's test were used for sex × treatment interactions. For all other data sets, one-way ANOVA and Dunnett's test (VH as the reference group) were used for comparisons when the equal variance assumption was met; otherwise, Wilcoxon test was performed to compare the means. A group was considered statistically significant if $p < 0.05$. JMP Pro 12 (SAS Inc., Cary, NC) and GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA) were used for statistical analysis and data visualization.

## Results

### Effect of BPA Exposure on BGLs and T1D Incidence.

First, we determined BPA's effect on BGLs and T1D outcome in NOD mice. Due to NOD males having a delayed T1D development compared to females, males were tested longer for T1D effects. BPA induced early onset of T1D in females with a significant change observed on day 76 after the initial dosing in the low dose group, although Logrank test analysis indicated total T1D incidence over time was not significantly altered (Fig. 1a). Consistently, increased BGLs in the time-course study were observed with a significant increase in the low dose group on day 76 in females; a similar increase, albeit not statistically significant (e.g. P = 0.07 at time 15 min for low dose), was produced in the day 119 (4 mo.) GTT, but no significant difference on day 84 serum insulin concentration was observed (Fig. S1a–c). Additionally, histopathology of the pancreas in females showed no difference in insulitis score (Table S1). Low dose non-diabetic female mice also had increased absolute spleen weight, and the high dose non-diabetic females had increased absolute kidney weight (Table S2).

In contrast, BPA treated males at the high dose had a significant delay in T1D with a significant decrease in incidence compared to the control on day 174 (5 mo.), but not total diabetes incidence over time (Fig. 1e). No statistically significant effects on the BGLs in the

time-course study or in GTTs were found, but the day 361 ITT showed the BGLs at 120 min and the area under the curve after insulin injection (Fig. S1d–h) in the low dose group was significantly increased. Additionally, both the absolute and % weights of spleen were increased by the low dose BPA in non-diabetic males (Table S3).

### Effect of BPA on Immune Cells.

To determine the mechanisms of T1D modulation by BPA, splenic immune cells were analyzed. BPA exposed females had increased %Gr-1$^-$Mac-3$^+$ (macrophages) in both low and high dose groups, and %CD8$^+$CD25$^+$ cells, %CD4$^+$CD8$^+$ cells and %CD25$^+$ cells in the low dose group (Fig. 1b–c). In contrast, %CD4$^+$CD8$^-$ cells and B220 mean fluorescence intensity (MFI; expression levels of B220 on cells) of B220$^+$CD40L$^+$ and B220$^+$CD40L$^-$ cells (Fig. 1c–d) were decreased by BPA. However, no changes were found for neutrophils (Gr-1$^+$Mac-3$^-$), %B cells or CD40L MFI in females (Fig. 1b and S2a–b). BGLs from days 63–119 were found to significantly correlate with immune endpoints, and BGLs from day 134 correlated with absolute kidney weight (Fig. 2a).

Unlike the females, immune cells in the spleen of males had significantly decreased neutrophils (Gr-1$^+$F4/80$^-$) in the high dose group, while macrophages were not significantly changed (Fig. 1f). As for splenic T cell populations, the low dose BPA decreased %CD25$^+$ cells, whereas the high dose decreased %CD4$^+$CD8$^+$ cells. The %CD4$^+$CD25$^+$ cells were also decreased by the low dose, but this did not reach the level of statistical significance (p=0.053; Fig. 1g). The low dose group had a decrease in activated B cells (%B220$^+$CD40L$^+$), while in the high dose group, B220 MFI of naïve B cells (B220$^+$CD40L$^-$) was increased (Fig. S2c and 1h). Furthermore, both BPA treatment groups in males had increased CD40L MFI of B220$^+$CD40L$^+$ (Fig. S2d). BGLs on days 68 and 109 correlated with neutrophils and CD40L MFI of B220$^+$CD40L$^+$, respectively (Fig. 2b).

### Effects of BPA on Cytokine/Chemokine and Antibody Levels.

Cytokine, chemokine and antibody levels in sera were measured to establish if the alterations in immune cells resulted in pro-inflammatory or anti-inflammatory changes. In general, the cytokines/chemokines were increased in females and decreased in males by BPA showing a sex-dependent alteration (Fig. 2c). In the high dose female group, significant increases included IL-2, IL-9, IL-13, granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-$\gamma$, macrophage inflammatory protein (MIP)-1$\alpha$ and MIP-1$\beta$; however, decreased IL-7 was observed as compared to the VH control (Table S4). In the low dose female group, significant increases included IL-2, IL-9 and IP-10. In males, the high dose BPA significantly decreased pro-inflammatory cytokines IL-1$\beta$ and IL-5, and the low dose BPA also decreased IL-1$\beta$ compared to the VH control (Table S4). As for antibody changes, high dose females had decreased IgG$_1$ and IgG$_{2a}$/IgG$_{2c}$, while males had decreased IgG$_1$ and IgM levels in the low and high dose groups, respectively (Fig. 2d–e).

### BPA Alteration of GMB.

Next, BPA alteration of GMB composition was determined as a possible mechanism of immunomodulation and T1D development. Sub-acute (16 days) BPA exposure significantly altered the alpha diversity between all groups and the weighted and unweighted beta

diversity in the high dose groups when compared to the VH (Fig. 3a–c). At the phylum level, both BPA doses increased Bacteroidetes, and the high dose increased Cyanobacteria and TM7. In contrast, Proteobacteria was decreased from the low dose, and Firmicutes and Tenericutes were decreased from the high dose (Fig. 3d; Fig. S3a). The Bacteroidetes to firmicutes (B:F) ratio was increased in the high dose from sub-acute exposure (Fig. 3e). Bacteria significantly altered at the class level from both BPA doses including increased *Bacteroidia* and decreased *Betaproteobacteria*. At the genus level, *Bacteroidales* (Other), *Odoribacter* and unclassified bacteria from *Rikenellacaea* were increased, and *Bacteroides*, *Clostridiales* (Other), *Ruminococcaceae* (Other), *Sutterella* and *Turicibacter* were decreased (Fig. S3b–c). Additionally, these bacteria correlated with significant phenotypic endpoints (e.g. macrophages) and with day 63 BGLs (Fig. S4).

After chronic BPA exposure (90 days), the B:F ratio was no longer significant (Fig. 3e), and no significant differences were found at the phylum level (Fig. S5a). However, the low dose NOD females had significantly increased *Bacteroidetes* (listed as *Bacteroidetes*:Other) at the class and genus levels and unclassified bacteria *Ruminococcaceae* at the genus level when compared to the VH control (Fig. 3f; Fig. S5b). At the genus level in the high dose, unclassified bacteria from *Rikenellacaea* was increased, while unclassified bacteria from *Bacteroidales* and *Lactobacillus* were decreased when compared to VH control (Fig. 3f). No significant difference was seen in alpha or beta diversity in BPA exposed females (Fig. S5c–e).

As for males after sub-acute exposure (27 days), bacteria classified as other at the phylum level was significantly increased from the low dose, while Tenericutes was significantly decreased from both BPA doses (Fig. 3g; Fig. S6a). At the class level, *Bacilli* was decreased from the low dose, and *Mollicutes* was decreased by both BPA doses (Fig. 3h). At the genus level, the high dose increased *Odoribacter* and unclassified *Campylobacterales* and decreased *Anaeroplasma* and *Camplybacter*, while *Anaeroplasma*, unclassified *RF32* and *Lactobacillus* were decreased from the low dose (Fig. S6b). The weighted beta diversity was significantly different between the low dose and VH, but no significant effect was found for the alpha diversity or unweighted beta diversity (Fig. S6c–e).

In males after chronic BPA treatment (122 days), bacteria at the phylum level was not significantly altered, but the B:F ratio was significantly increased (Fig. 3i; Fig. S7a). The various bacteria significantly altered at the class level in the high dose group included increases in *Bacteroidia* and decreases in *Erysipelotrichi*, *Verrucomicrobiae*, *YS2* and *Bacilli* compared to the VH group (Fig. S7b). At the genus level, the high dose BPA increased *Bacteroides* and decreased *Akkermansia*, *Ruminococcus*, *Betaproteobacteria* listed as other, unclassified bacteria from *YS2* and unclassified bacteria from *Erysipelotrichaceae* (from the class *Bacilli*) compared to VH control. Additionally, both doses significantly reduced abundances of unclassified bacteria from *Lachnospiraceae* and *Erysipelotrichaceae* (from the class *Erysipelotrichi*) when compared to the VH (Fig. S7c). No significant difference was found in alpha or weighted beta diversity, but unweighted beta diversity was significantly different between the low dose and VH (Fig. S7d–f). Several bacteria were found to correlate with changes in spleen weight, cytokines and immune cells, and with different timepoints of BGLs (Fig. S8).

### Analysis of Urine Metabolic Profiles.

BPA's effect on the metabolites in urine at 4 mo. after initial dose was next determined. Our results showed that BPA did not significantly alter the global urinary metabolomics profile compared to control in either females or males (Fig. S9).

### Effects of BPA in Antibiotic Treated Females.

Our data in Figure 3 suggested that BPA increased unclassified *Ruminococcaceae*. To further examine the role of GMB in T1D exacerbation from BPA exposure, female mice were exposed to BPA in combination with vancomycin since vancomycin decreases microbes from Bacteroidetes and Firmicutes, including unclassified *Ruminococcaceae* (Hansen et al. 2012). No statistically significant difference was observed in T1D incidence and little effect on the time course of BGLs; however, the 3 mo. GTT and ITT (day 100 and 103, respectively) showed increased BGLs (Fig. 4a–b and Fig. S10a–b). Additionally, %macrophages (Mac3$^+$CD45R$^-$), %CD5$^-$CD24$^+$, %CD40$^+$CD44$^+$ and %CD40$^-$CD44$^+$ splenic immune cells were increased, while no changes were observed in %T cell populations (Fig. 4c–d and Fig. S10c–d). Macrophages also positively correlated with BGLs on days 96 (Spearman $\rho = 0.63$; $P = 0.03$) and 106 (Spearman $\rho = 0.61$; $P = 0.04$). BPA exposure in antibiotic treated mice increased several bacteria (e.g. Actinobacteria) compared to the antibiotic treated VH control at the phylum, class and genus levels that were not altered in females without antibiotics (Fig. 4e–f and Fig. S11a–b). No significant difference was observed in alpha or beta diversity (Fig. S11c–e).

## Discussion

Currently, there are no epidemiological studies correlating BPA exposure with T1D and only a few animal studies have been published concerning BPA's impact on T1D risk (Xu et al. 2016). This is the first study to determine the role of BPA alteration of GMB in T1D pathogenesis and examine immunity after chronic BPA exposure. Although total T1D incidence was not increased as further evidenced from no significant difference in the insulitis score at the end of study, exposure to BPA in female NOD mice accelerated T1D development with a greater effect in the low dose group. It was possible that insulitis was higher in this group during early BPA exposure (i.e. 76 days). Similarly, the only other study to determine T1D alteration after BPA exposure during adulthood in NOD mice did not find a significant difference in total T1D incidence, but saw increased insulitis at 8 weeks of treatment from their low dose (about 150 μg/kg; (Bodin et al. 2013)). This previous study and our results showing accelerated T1D development and increased BGLs at day 76 indicate that low dose BPA exposure during adulthood has detrimental effects to accelerate T1D development. While it is still uncertain why low dose BPA exposure can have a greater effect than higher doses, this non-monotonic dose effect of BPA has been previously described in different tissues including pancreas, and possibly from low dose activation of ER-extranuclear signaling pathways (Nadal et al. 2017).

Changes seen in immune cells (e.g. an increase in macrophages) corroborated the finding of accelerated T1D incidence from BPA exposed females. We also found decreased CD4$^+$CD8$^-$ cells, which might be the Th2 phenotype that has been previously indicated to

decrease in female NOD mice with an increased amount of inflammation (Young et al. 2009). $CD8^+CD25^+$ cells decreased $CD4^+CD8^-$ cell proliferation and might be a mechanism for the decreased $CD4^+CD8^-$ levels found (Bisikirska et al. 2005). Additionally, autoreactive cells that contributed to autoimmune diseases including $CD4^+CD8^+$ T cells and B cells with low B220 expression (Abo et al. 2012) were increased by BPA. Activated B cells could increase inflammation and activate autoreactive T cells to accelerate T1D development (Leeth et al. 2016). We also found that BPA altered B cell antibody production. BPA-exposed females had decreases in $IgG_1$, a Th2 supporting antibody, and $IgG_{2a}/IgG_{2c}$, which delayed T1D in NOD mice. These decreases could also promote a pro-inflammatory response by increasing Th1 cytokines including IFN-γ (Kaplan et al. 2001; Todd et al. 1998).

This immunomodulation towards a pro-inflammatory state in females was further confirmed by the increase of several pro-inflammatory cytokines/chemokines that could exacerbate T1D, including IFN-γ (Barthson et al. 2011), GM-CSF (Alnek et al. 2015), IP-10 (Devaraj et al. 2011), MIP-1α (Cardozo et al. 2003), MIP-1β (Codella et al. 2015) and IL-9 (Ryba-Stanislawowska et al. 2016). We found many of these changes in the T cell populations (e.g. $CD4^+CD8^+$ cells) significantly correlated with BGLs on or before the day T1D was significantly accelerated in the low dose group. On the other hand, many of the cytokines/chemokine and B220 MFI of $B220^+CD40L^+$ correlated with BGLs after the significant effect in T1D from BPA exposure was observed. This suggests BPA's alteration of T cell populations were important for initiating the acceleration of T1D and should be further investigated in future experiments. BPA's immunomodulation towards pro-inflammation was further suggested as having a primary role in accelerating T1D by the increased macrophages in both BPA exposed females with and without antibiotics.

Sub-acute BPA exposure in female mice resulted in an overall pro-inflammatory GMB profile. The increased Bacteroidetes from sub-acute BPA exposure characterizes preclinical T1D and is involved in the progression of β-cell autoimmunity, which usually coincides with a decrease in Firmicutes in T1D patients compared to controls (Knip and Siljander 2016). Consistent with our results, Bacteroidetes and Cyanobacteria have been found increased, while Firmicutes, Tenericutes and Proteobacteria were decreased in T1D patients compared to controls and in NOD mice compared to NOD mice on an anti-diabetogenic diet (Brown et al. 2011; Hansen et al. 2014). Conversely, TM7, which was increased from high dose BPA, was previously found increased in NOD mice receiving an anti-diabetogenic diet (Hansen et al. 2014). Chronically BPA exposed female's GMB continued to be pro-inflammatory (e.g. decreased T1D protecting *Bacteroidales* and *Lactobacillus*; see Supplemental Material, Supplementary Discussion; (Krych et al. 2015)). However, BGLs did not significantly correlate with GMB except a few bacteria altered from sub-acute exposure on day 63. Since females receiving antibiotics still had increased BGLs from BPA exposure in the tolerance tests with a different set of bacteria altered from BPA exposure, the later GMB changes were unlikely to be the primary contributor of the accelerated T1D development. The increased B:F ratio, BGL correlations and clear separation in diversity between the control and high dose BPA treated females after sub-acute exposure suggests that early changes in GMB drove the changes in the immune system and led to an accelerated T1D.

As for the males, BPA exposure protected them from T1D development as demonstrated by a decreased T1D incidence in mice treated with the high dose of BPA. This was also seen from immune cell changes including decreased neutrophils (Guo et al. 2014) along with increased B cells with high B220 expression indicating less autoreactivity (Abo et al. 2012). Males given the high BPA dose also had decreased IgM, which indicated that there were less activated B cells. BPA modulation towards anti-inflammation in high dose males was further confirmed from the overall decrease of cytokines/chemokines. Specifically, the pro-inflammatory IL-1β, which exacerbates T1D (Tuller et al. 2013), was decreased by BPA. On the other hand, while IL-1β was also decreased in low dose males, no effect was observed in T1D incidence; long term low dose BPA treatment resulted in insulin resistance and decreased Th2 supporting $IgG_1$.

The GMB profile after sub-acute BPA exposure in males showed decreased Tenericutes, which was similar to what was found in females, and was inconsistent with the delayed T1D in males. However, at the class level, decreased *Bacilli* was consistent with our T1D results (de Goffau et al. 2014). Currently, there is a lack of information for the effect of bacteria labeled as other and *Mollicutes* on T1D. The male GMB changes following chronic BPA exposure were a mix of protective (e.g. decreased *Lachnospiraceae*; (Krych et al. 2015)) and exacerbating effects (e.g. decreased *Betaproteobacteria*; (Guo et al. 2016)). These results, together with the increased B:F ratio in chronically high dose exposed males, metabolomics and female results indicated that the later alterations in the GMB were not driving the changes in T1D incidence. Additionally, other studies that have examined the GMB changes share little commonalities with this study and each other on what bacteria BPA alters (Javurek et al. 2016; Lai et al. 2016; Malaisé et al. 2017). This could be from differences in timepoints used or other study parameters, and further suggests BPA impacts on later GMB may be from secondary effects.

BPA doses in this study are relevant for human exposure since the current US EPA reference dose is 50 μg/kg/day. Other studies in mice have found 250 μg/kg and 400 μg/kg doses of BPA provide environmentally relevant levels of total and unconjugated serum BPA that are within the range reported in humans (Rubin et al. 2017; Taylor et al. 2011). However, future studies using a wider range of BPA doses, fecal transfer and/or a phytoestrogen-free diet would help elucidate the causal relationship of GMB, immunity and T1D modulation from BPA exposure. More time points examining the effects of BPA on the GMB, fecal metabolites, cytokine/chemokine and antibody levels will benefit further studies in examining the immune and GMB changes leading to T1D pathogenesis. Additional studies also are needed to determine the cause of these sex-related differences (see Supplemental Material, Supplementary Discussion).

## Conclusions

In summary, this study has shown sex plays an important role in BPA altering T1D risk. BPA accelerated T1D development in adult NOD females, but delayed male mice from T1D development. The alteration of immune homeostasis was found to be the most likely mechanism of altering T1D risk. BPA's promotion of autoimmunity related to T1D should be further examined with more mechanistic experiments, so biomarkers of risk factors for

early T1D development from BPA exposure can be determined. Thus, the potential health consequences from BPA and other EDC exposure on T1D should further be evaluated in adult females of different species and mouse strains.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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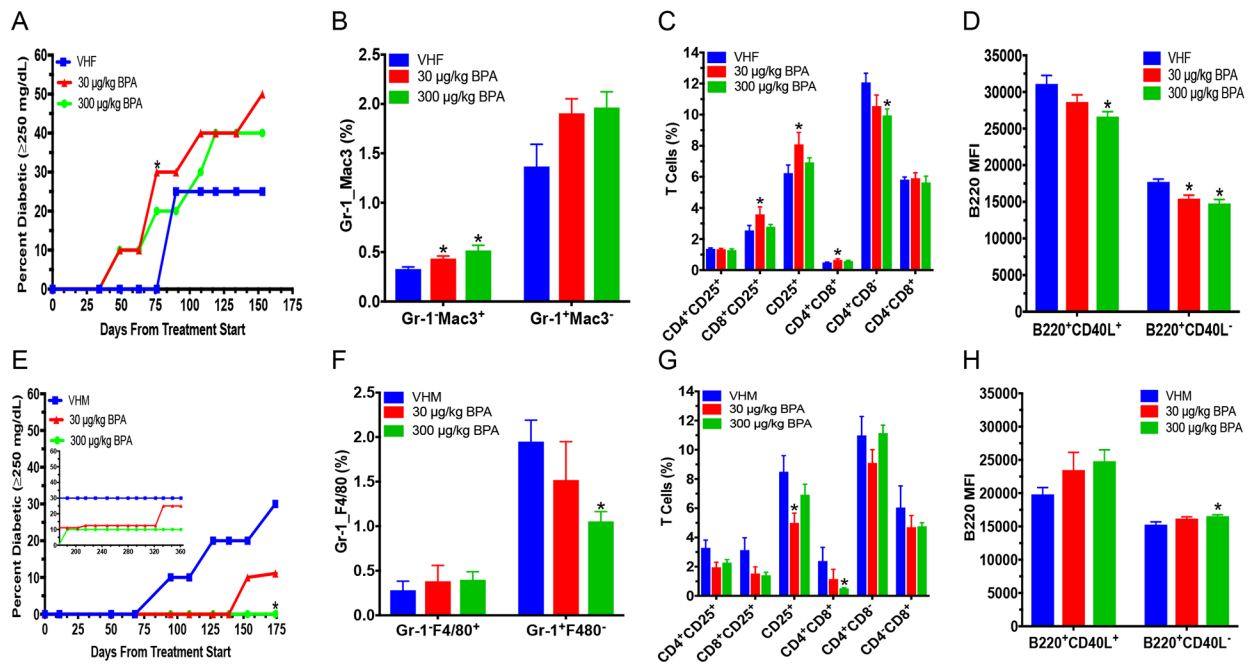
**Figure 1.**
Diabetic incidence and flow cytometric analysis of spleen cells from adult NOD mice. T1D incidence in female (A) and (E) male mice. Blood glucose 250 mg/dL was considered diabetic. (B) % macrophages (Gr-1⁻Mac3⁺ or F4/80⁻Mac3⁺) and neutrophils (Gr-1⁺Mac3⁻ or F4/80⁺Mac3⁻) in adult females (B) or males (F), respectively. % T cell populations in females (C) and males (G). B220 mean fluorescence intensity (MFI) in females (D) and males (H). The values are presented as mean ± SEM. *, $p < 0.05$ using Dunnett's test or Wilcoxon based on whether equal variance assumption was met as compared to the respective vehicle (VH) control group. N = 6–10. VHF, vehicle females; and VHM, vehicle males
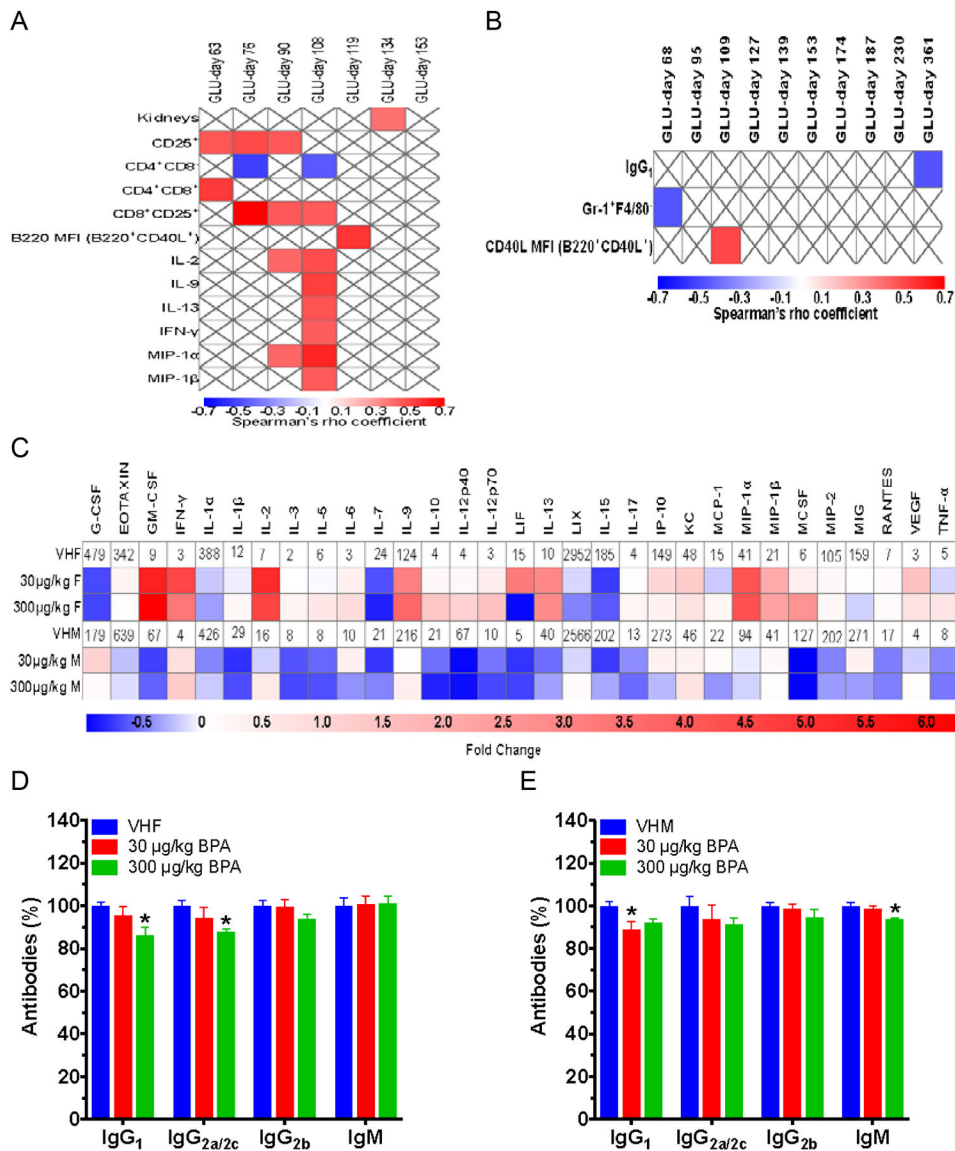
**Figure 2.**
Effect of BPA exposure on serum cytokine/chemokine and antibody levels, and their correlations with BGLs. Correlation between significant phenotypic endpoints and different timepoints of BGLs in females (A) and males (B) with red showing significantly positive and blue significantly negative correlation using Spearman correlation test ($p < 0.05$). Blank boxes with an X were not significantly different. (C) Heat map of serum cytokine/chemokine changes at time of euthanization for adult BPA exposed female (VHF) and male (VHM) NOD mice. Values of VHF and VHM are shown as mean pg/mL. (D) $IgG_1$, $IgG_{2a/2c}$, $IgG_{2b}$ and IgM in NOD females, which were measured at dilutions of 1:2,000, 1:50, 1:25 and 1:500, respectively, following titration by serial dilution. (E) $IgG_1$, $IgG_{2a/2c}$, $IgG_{2b}$ and IgM in NOD males, which were measured at dilutions of 1:1000, 1:50, 1:25 and 1:500, respectively, following titration by serial dilution. The values are presented as mean ± SEM.

\*, $p < 0.05$ using Dunnett's test or Wilcoxon based on whether equal variance assumption was met as compared to the respective vehicle (VH) control group. N = 6–9
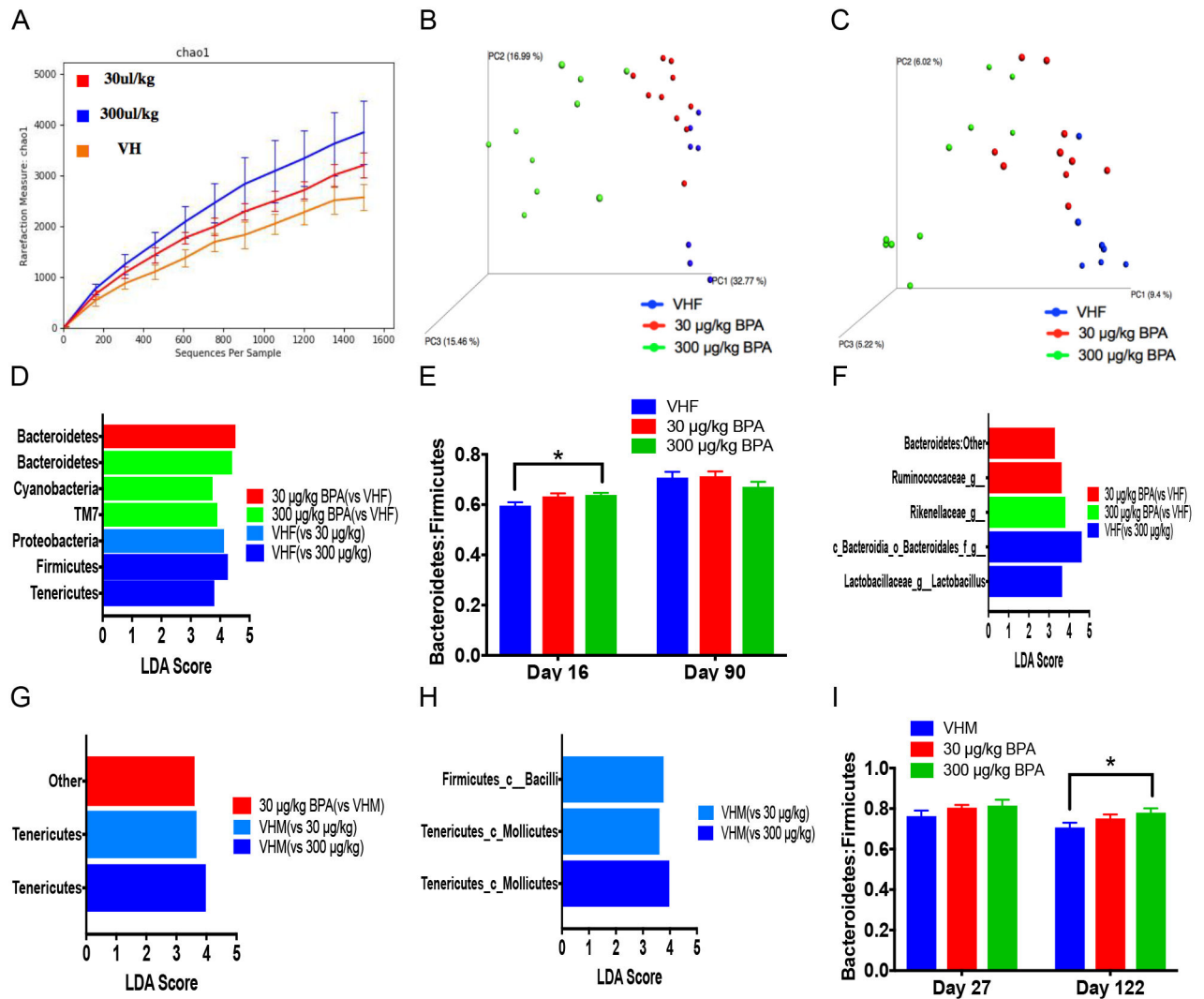
**Figure 3.**

Gut microbiome composition based on 16S rRNA sequencing in female (A-F) and male (G-I) mice after sub-acute and chronic exposure. The alpha diversity (chao1; A), weighted (B) and unweighted (C) UniFrac beta diversity from sub-acute exposure in females. (D) LEfse results of gut microbiome shown at the phylum level from female sub-acute exposure. (E) Female bacteroidetes to firmicutes (B:F) ratio alteration after sub-acute (16 days) and chronic exposure (90 days). (F) LEfse results for the genus level from female chronic exposure. LEfse results of gut microbiome shown at the phylum (G) and class (H) levels from male sub-acute exposure. (I) B:F ratio alteration after sub-acute (27 days) and chronic exposure (122 days) in males. N = 6–10. VHF, vehicle females; VHM, vehicle males
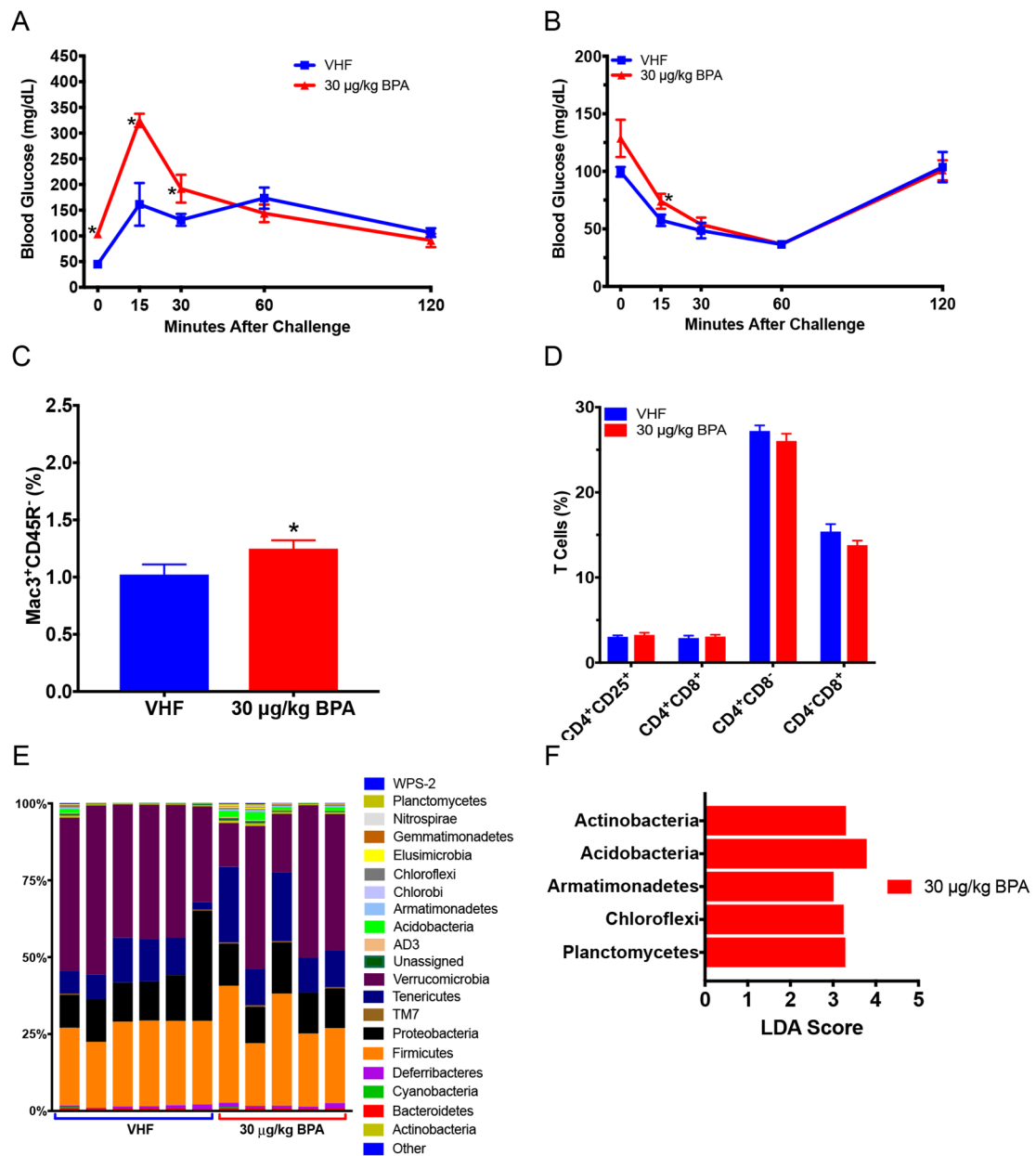
**Figure 4.**

Glucose tolerance test (GTT), insulin tolerance test (ITT), splenocyte differentials and microbiota alterations in BPA exposed female NOD mice treated with antibiotics. GTT (A) and ITT (B) of non-diabetic adult females were performed at 3 mo. after initial dose. % macrophages (C; Mac3$^+$CD45R$^-$) and % T cells (D) were measured using flow cytometric analysis. Phylum level taxonomy of gut microbiome excluding bacteria less than 0.05% (E) and LEfse results (F) are shown. The values are presented as mean ± SEM. *, $p < 0.05$ using Dunnett's test or Wilcoxon based on whether equal variance assumption was met as compared to the respective vehicle (VH) control group. N = 4–6. VHF, vehicle females