Gestational and lactational exposure to low-dose bisphenol A increases Th17 cells in mice offspring

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Running Title

BPA promotes Th17 cell development

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Abstract

Increasing evidence demonstrates that perinatal exposure to Bisphenol A (BPA) can cause immune disorders throughout the life span. However, the biological basis for these immune disorders is poorly understood and the effects of exposure to BPA on Th17 development are unknown. The present study sought to characterize alterations of Th17 cells in childhood and adulthood following gestational and lactational exposure to environmentally relevant low-dose of BPA and the underlying mechanisms. Pregnant dams were exposed to BPA (10, 100 or 1000 nM) via drinking water from gestational day (GD) 0 to postnatal day (PND) 21. At PNDs 21 and 42, offspring mice were anesthetized, blood was obtained for cytokine assay and spleens were collected for Th17 cell frequency and ROR γ t mRNA expression analysis. Perinatal exposure to low-dose BPA resulted in a dose-dependent and gender-specific persistent rise in Th17 cells accompanied by an increase of ROR γ t mRNA expression in the offsprings. The contents of major Th17 cell-derived cytokines (IL-17 and IL-21) and those essential for Th17 cell differentiation (IL-6 and IL-23) were also increased compared to those in controls. These changes were more pronounced in female than in male offsprings. However, perinatal exposure to low-dose BPA had little effect on serum TGF- β , another key regulator for Th17 cell development. Our results suggest that gestational and lactational exposure to a low-dose of BPA can affect Th17 cell development via an action on its transcription factor and the regulatory cytokines. These findings provide novel insight into sustained immune disorders by BPA exposure during development.

Key words Bisphenol A; cytokine; maternal exposure; ROR γ t; Th17 cells;

1. Introduction

Bisphenol A (BPA) is extensively used as a monomer component in the manufacture of polycarbonate plastics and epoxy resins that have been found in numerous human consumer products. BPA is readily released from these products into food and water, and hence contributes to human exposure through diet or drinking water (Hengstler et al., 2011; Michałowicz, 2014). Bio-monitoring studies have reported that BPA can be detected in over 90% of blood, urine and other tissue samples around the world (Vandenberg et al., 2010). Studies have shown that the developing immune system is highly sensitive to BPA exposure. In humans, prenatal and postnatal environmental BPA exposure is associated with various autoimmune diseases and inflammation during childhood and adulthood, including allergy, asthma and obesity (Spanier et al., 2012; Trasande et al., 2012; Mikołajewska et al., 2015). In animals, early-life exposure to BPA may produce considerable adverse effects to the immune system, such as cytokine secretion changes, immune-associated gene expression, T helper (Th)1/Th2 cell shifts, decrease in regulatory T cell (Treg) number, immune response abnormality, and allergic inflammation in offspring mice during the life course (Yoshino et al., 2004; Yan et al., 2008; Midoro-Horiuti et al., 2010; Bauer et al., 2012). However, the molecular mechanisms for immune disorders of BPA following perinatal exposure remain to be elucidated (Rogers et al., 2013), in particular the autoimmune-specific pathways.

Th17 cell is a subset of CD4⁺ effector T -cells, complements the function of the Th1 and Th2 cell lineages (Harrington et al., 2005; Damsker et al., 2011) and contributes specifically to autoimmune processes. Th17 cells characteristically express the transcription factor, retinoic acid-related orphan receptor γ t (ROR γ t), and mainly produce pro-inflammatory interleukin-17A (IL-17) and IL-21.

Importantly Th17 cells are involved in the pathogenesis of various autoimmune and inflammatory diseases, which were previously recognized as Th1 cell-mediated diseases (Park et al., 2005; Wilson et al., 2007; Miossec et al., 2009; Lee et al., 2012). To date, whether gestational and lactational exposure to a low-dose of BPA affects the development of Th17 cells in the offspring has not been examined.

In the present study, we investigated whether Th17 cell development in mouse offspring would be altered following gestational and lactational exposure to a low-dose of BPA by analyzing the frequency of Th17 cells in the spleens of the offspring. The mode of action and the underlying mechanisms were further explored by examining ROR γ t mRNA expression, and serum levels of key

cytokines that are either secreted mainly from Th17 cells or essential for Th17 cell differentiation. Specifically, we evaluated whether these effects have a gender difference, and whether developmental exposures to BPA affect the immune functions in later life, which are of important public health implications

2. Materials and methods

2.1. BPA and Vehicle

BPA (purity > 99%) was purchased from Sinopharm Chemical Reagent Co.(China) and BPA dosing solutions were prepared in the vehicle, 0.1% DMSO (Sigma-Aldrich, St. Louis, MO) in purified water. Prewashed glass bottles were used. The concentrations of BPA in water were monitored using high performance liquid chromatography coupled with mass spectrometry (HPLC-MS); the levels of BPA in the purified water were below the average analytical blanks.

2.2. Animals and treatments

Eight-week old ICR mice were purchased from Animal Center of Anhui Province and housed in prewashed polycarbonate cages. Throughout the study, animal rooms were maintained at $23 \pm 2^{\circ}\text{C}$ temperature and $50 \pm 5\%$ relative humidity with a 12-hr light/dark cycle. Standard chow and water were available *ad libitum*. After one week's acclimation, mice were mated and gestation day (GD) 0 was determined when vaginal plug was presented. Pregnant dams were housed individually and randomly assigned to five dose groups: blank control (purified water, n=10), vehicle control (0.1% DMSO, n=10), 10 nM BPA(n=10), 100 nM BPA(n=10) and 1000 nM BPA(n=11). Dams were exposed to BPA through drinking water from GD0 until weaning [postnatal day (PND) 21]. In order to maintain concentrations of BPA in water, drinking water was replaced every two days and consumption of water for each dam was recorded by scaled bottle. The health of dams was monitored daily and body weight of dams during gestation was recorded every three days. On PND0, the numbers of pups alive and dead, gender ratio, and live litters' weight were recorded. Litter body weight of pups was recorded weekly. Pups were weaned on PND21 and assigned to the same gender groups with free access to food and purified water without BPA. At PND21 and PND42, blood and spleen from one male and one female offspring mouse from each litter were sampled randomly. The animals were treated humanely and all experiments were done with approval of the Animal Care and Use Committee at Anhui Medical University.

2.3. Flow cytometry (FCM) analysis for Th17 cell frequency in spleen

Splenocytes were isolated and suspended in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Tianhang Biological Technology Co. China). Splenocytes suspensions with a density of 1×10^5 cells/mL were planted into 24-well culture plate and stimulated with 50 μ g/L phorbol 12-myristate 13-acetate (PMA, eBioscience, San Diego, CA), 500 μ g/ml ionomycin (eBioscience) and 200 μ M monensin (eBioscience) for 4 hours at 37°C under 5% CO₂ environment. Surface staining was conducted with phycoerythrin (PE) - conjugated antimouse-CD4 monoclonal antibody (Biolegend) for 1/2 hour at room temperature. After being fixed and permeabilized, intracellular staining was performed using fluorescein isothiocyanate (FITC)-conjugated antimouse-IL-17 (Biolegend). Finally the cells were sorted by FCM with a FACSScan (BD Bioscience) and data were analyzed using CellQuest software.

2.4. Real-time quantitative polymerase chain reaction (RT-qPCR) analysis for ROR γ t mRNA expression in spleens

Total RNAs were extracted from spleen tissues by RNeasy Mini Kit (Qiagen), and then reversely transcribed into cDNA using Reverse Transcription Kit PrimeScript[®] RT Master Mix (Takara). cDNA analysis was performed on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) using Real time-PCR kit SYBR[®] Premix Ex TaqTM II (Takara). Relative quantification of mRNA was calculated using the standard $2^{-\Delta\Delta Ct}$ method with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as internal standard. The primers for ROR γ t (Takara) were: 5'-CCGCTGAGAGGGCTTCAC-3'(sense) and 5'-TGCAGGAGTAGGCCACATTACA-3' (anti-sense); those for GAPDH (Takara) were: 5'-AGCAATGCCTCCTGCACCACCAAC-3'(sense) and 5'-CCGGAGGGGCCATCCACAGTCT-3'(anti-sense). The PCR conditions were as follows: 5 minutes at 94°C for pre-degeneration, followed by 35 cycles of 20 seconds at 94°C for denaturing, then 20 seconds at 60°C and 20 seconds at 72°C for annealing, and finally 10 seconds at 87°C for extension.

2.5. Enzyme-linked immunosorbent assay (ELISA) quantification of cytokine in serum

Contents of IL-6, IL-23, IL-17, IL-21 and transforming growth factor- β (TGF- β) in serum were quantified by ELISA Kits from Rapidbio Co (RapidBio Laboratory, USA), according to the manufacturer's instructions.

2.6. Statistical analysis

Data are expressed in graphs as mean $\pm$ SEM unless otherwise stated. Statistical analysis and graphic presentation of the results were performed using SPSS software 17.0 (IBM Corporation, New York). Comparison among the group means was made using two-way analysis of variance (ANOVA) with gender and maternal exposure dose as factors followed by *post hoc* test [Dunnett t (2-sided)]. $P < 0.05$ was considered statistically significant.

3. Results

3.1. BPA exposure levels

Daily water consumption of dams was (5.77 ± 0.98) ml/day during pregnancy and (9.08 ± 0.46) ml/day during lactation. The maximum value was 10.0 mL; thus, it was estimated that a dam exposed to 1000 nM BPA in the drinking water had a BPA maximal intake of 47.5 $\mu\text{g/kg/day}$. This level is still lower than the current reference dose of 50 $\mu\text{g/kg/day}$, as established by the US Environmental Protection Agency (EPA) and the European Food Safety Authority (EFSA) and thus is relevant to environmental exposure. There was no significant difference in daily maternal water consumption between the BPA-exposed group and the control group at each time point during pregnancy and lactation (data not shown).

3.2. Maternal low-dose BPA exposure did not affect birth outcomes and weight of pups before weaning, but increased weight of male pups after weaning

There was no significant difference in the body weight of dams between BPA-exposure group and the control group at each time point during gestation (Fig. 1). Perinatal exposure to low-dose BPA did not significantly influence birth weight, litter size, survival and gender ratio of the offspring mice (Table. 1). Gestational and lactational exposure to low-dose BPA had no significant effect on the average weights of pups before weaning, but increased the average weight of male pups after weaning. Significant change in the average weight of male pups occurred on PND35 at 1000 nM BPA (Fig. 2A). No significant change was observed in the spleen coefficient from either female or male offspring mice following maternal BPA exposure on PND21 and PND42 (Fig. 2B).

3.3. Gestational and lactational exposure to low-dose BPA increased Th17 cells in the spleen in offspring mice

Th17 cells are importantly involved in immune-mediated diseases. We first investigated the effects of gestational and lactational low-dose BPA exposure on Th17 cell frequency in the spleen of offspring

mice on PND21 and PND42. As shown in Figure 3, Th17 cell frequency in the spleen of offspring mice was increased on PND21 and remained high on PND42 (Figure 3A -C). This suggests that gestational and lactational low-dose BPA exposure can stimulate Th17 differentiation and the effect remains after stopping the exposure in later life. Th17 cell frequency was increased in a dose-dependent manner ($P=0.006$). The mean value of increase in Th17 cell frequency was greater in female than that in male mice, although the difference was not statistically significant. However, in female mice, BPA generated an effect at 100nM BPA while in male mice the effect was observed at 1000nM BPA, showing a more sensitive effect in female mice. There was a significant synergistic interaction between the effects of gender and maternal exposure doses on Th17 cell frequency ($P = 0.014$), consistent with the notion that female mice are more sensitive to BPA effect.

3.4. Mechanisms of action – effect of BPA on ROR γ t expression in the spleen of offspring mice

To examine whether expression of ROR γ t, a characteristic transcription factor of Th17 cells, would underlie a higher frequency of Th17 cells in the offspring mice after gestational and lactational exposure to low-dose BPA, we further examined ROR γ t mRNA expression in spleens of the offspring mice using real-time qRT-PCR on PND21 and PND42. As shown in Figure 4, gestational and lactational exposure to low-dose BPA resulted in a dose-dependent up-regulation of ROR γ t mRNA expression ($P = 0.017$). Changes in ROR γ t mRNA expression followed similar patterns of dose and time-dependence to the changes in Th17 in response to gestational and lactational exposure to low dose BPA and correlated with Th17 changes ($r=0.591$, $p<0.01$, $n=204$). Female mice were more sensitive to BPA effect as seen with a lower effective concentration at PND21 (Figure 4). There was a significant synergistic interaction between the effects of gender and maternal exposure doses on ROR γ t mRNA expression ($P = 0.021$), with female gender resulting in a greater effect. These data suggest that changes in ROR γ t mRNA expression contribute significantly to Th17 up-regulation and also gender difference in BPA action.

3.5. Mechanisms of action – effect of BPA on serum IL-17 and IL-21 in offspring mice

We explored whether gestational and lactational exposure to low-dose BPA had any effects on serum IL-17 and IL-21, which are mainly produced by Th17 cells, in the offspring mice on PND21 and PND42, respectively. As shown in Figure 5, IL-17 and IL-21 were elevated in BPA exposure groups compared with those in control groups, and were associated with the changes in Th17 cell frequency in the spleens (IL-17 and Th17, $r=0.629$; $p<0.01$, $n=204$; IL-21 and Th17, $r=0.572$, $p<0.01$, $n=204$).

The levels of IL-17 and IL-21 were increased on PND21 and on PND42 (Figure 5), with similar time dependence as seen in Th17 cells in Figure 3. Figure 5A shows that serum IL-17 was increased in a dose-dependent manner ($P = 0.002$); the effect was more prominent in female than in male mice with statistical significance ($P = 0.046$). The interaction between the effects of gender and maternal exposure dose on serum IL-17 was not significant. As shown in Figure 5B, serum IL-21 also increased in a dose-dependent ($P = 0.004$) and gender-specific manner ($P=0.032$); the effect was more prominent in female than in male mice. There was no significant interaction between the effects of gender and maternal exposure dose on serum IL-21. These data suggest that altered IL-17 and IL-21 levels are associated with and contribute to Th-17 development following gestational and lactational exposure to low-dose BPA.

3.6. Mechanisms of action – effect of BPA on serum IL-6, IL-23, and TGF- β in offspring mice

To examine the effects of gestational and lactational exposure to low-dose BPA on additional regulatory cytokines, we quantified IL-6, IL-23 and TGF- β in serum from the offspring mice by ELISA on PND21 and PND42. As shown in Figure 6A and 6B, the levels of IL-6 and IL-23 were increased on PND21 and PND42 in both male and female offspring mice, with similar time-dependence as seen with Th17 cells. IL-6 was increased in a dose-dependent ($P = 0.002$) and gender-specific manner ($P = 0.046$), with the effects more pronounced in female than in male mice. IL-23 was increased in a dose-dependent manner ($P = 0.003$), and the mean values in female mice were higher than those in male mice, despite not reaching a statistical significance. There were no significant interactions between the effects of gender and maternal exposure doses on the production of IL-6 and IL-23. Maternal exposure to low-dose BPA did not cause significant changes in TGF- β regardless of doses and gender differences (Figure 6C). These data suggest that altered IL-6 and IL-23 levels contribute to the up-regulation of Th17 differentiation in BPA-mediated response while TGF- β is little involved.

4. Discussion

A growing body of evidence suggests that gestational and lactational exposure to environmentally relevant doses of BPA causes adverse effects on immune function in offspring mice, but the molecular mechanisms are poorly understood. The present study has demonstrated that gestational and lactational exposure to low-dose BPA leads to a significant increase in Th17 cells in offspring mice

from infant to adulthood in a dose-dependent and gender-specific manner. To our knowledge, this is the first experimental evidence for a specific effect on Th17 cells by maternal exposure to environmentally relevant levels of BPA. This finding has important public health implications.

Gestational and lactational exposure to environmentally relevant levels of BPA is attracting considerable attention worldwide. Studies have suggested that maternal exposure to a dose below the current reference dose of 50µg/kg/day, as established by EPA and EFSA, can have deleterious effects on offspring mice (Kundakovic et al., 2013; Medwid et al., 2016). Oral exposure to BPA seems to be the most important route and the most appropriate way of extrapolation from animal data to human conditions (Sieli et al., 2011). Therefore, we selected an order of magnitude lower level of BPA (10, 100, 1000 nM) for maternal exposure via drinking water.

Several studies support that exposure to low-dose BPA leads to gender dimorphism and persistent disruption of maturation (Jasarevic et al., 2011; Beronius et al., 2013; DeBenedictis et al., 2016). In the present study, gestational and lactational exposure to low-dose BPA was found to induce persistent and more prominent changes in female offspring mice in terms of Th17 cell frequency, IL-17, IL-21, and ROR γ t mRNA expression. These gender-specific effects are consistent with previous studies showing that perinatal BPA exposure induces inflammation, alterations in energy expenditure, body composition and hormone levels, as well as behavioral changes (Bauer et al., 2012; Anderson et al., 2013; Kundakovic et al., 2013). Differential tissue distribution of BPA in male and female offspring mice may play a critical role in gender dimorphic phenotypes (Doerge et al., 2011). These persistent disruptive effects by BPA may be associated with accumulation in the fetal and infant tissues through placenta and milk transfer (Balakrishnan et al., 2010). However, the molecular mechanisms underlying BPA-induced disruption of gender dimorphic and persistent phenotypes are not well understood. Recently, several studies have suggested that epigenetic mechanisms may contribute to these consequences (Kundakovic et al., 2013). A major step forward from the present study is the identification of Th17 cells as the target by low level exposure of BPA which may explain immune reaction and inflammation as a result of environmental exposure to BPA.

To further characterize Th17-associated effect by BPA and explore the underlying mechanisms, the present study discovered that the increase in Th17 cells was accompanied by serum IL-17 and IL-21 rises and these variables were correlated with each other. IL-17 is a main effector cytokine for Th17 cells and can induce the expressions of several genes that encode pro-inflammatory cytokines and

chemokines, thus promoting the development of inflammation and autoimmune disorders (Park et al., 2005; Miossec et al., 2009; Lee et al., 2012). IL-21 is a member of IL-2 family cytokines and is produced mainly by Th17 cells. IL-21 can positively regulate Th17 cells and stimulates non-immune cells to produce inflammatory mediators, thus facilitating autoimmune diseases (Wang et al., 2011). It has been shown that IL-21 can substitute for IL-6 to promote Th17 cell differentiation (Korn et al., 2007; Nurieva et al., 2007). Thus up-regulation of Th17 cells and increase of these cytokines may explain, at least partially, autoimmune and pro-inflammatory reactions in infants and adults caused by perinatal exposure to BPA. The increase of these cytokines, especially IL-21, may also contribute to Th17 development via an autocrine action.

In seeking for the transcriptional mechanism of BPA-induced Th17 increase, we examined the role of ROR γ t. The ROR γ t has been identified as a key transcription factor, which promotes differentiation of Th17 cell lineage, induces and stabilizes IL-17 expression (Ivanov et al., 2006; Xiao et al., 2014). Indeed, we found that mRNA expression of ROR γ t in offspring mice was enhanced by gestational and lactational BPA exposure and the pattern of changes in ROR γ t expression was similar to those observed for Th17 cells, IL-17 and IL-21, supporting that up-regulation of ROR γ t expression plays a critical role in the development of Th17 cells and production of IL-17 and IL-21.

Full differentiation of Th17 cells is also regulated by various additional cytokines. TGF- β along with IL-6 is required for the initial commitment of naive T cells to Th17 cells in mice (Bettelli et al., 2006; Littman and Rudensky, 2010). Unlike TGF- β and IL-6, IL-23 is found to be critical for maintaining proliferation and survival of Th17 cells by binding to the IL-23 receptor (IL-23R) in the presence of IL-6 or IL-21 (Wilson et al., 2007; Volpe et al., 2008; Gaffen et al., 2014). Examination of these cytokines showed that gestational and lactational exposure to low-dose BPA also led to an elevation of IL-6 and IL-23 that was concordant with the increase in Th17 cells. Contrary to the common belief, TGF- β was not markedly altered by gestational and lactational BPA exposure. Thus TGF- β is not the main site of action for BPA. Here, Th17 cells in offspring mice were still increased by gestational and lactational BPA exposure without an alteration of TGF- β production. We speculate that changes in levels of TGF- β are undetectable, but basic TGF- β levels may be sufficient for the initiation of Th17 differentiation in the presence of IL-6 and IL-21 induced by gestational and lactational BPA exposure. Based on our results, we conclude that the elevation of IL-6 and IL-21 induced by maternal exposure

to BPA may initiate commitment of naive CD4⁺T cells into Th17 cells, and IL-23 subsequently further boosts the development of Th17 cells (Figure 7).

Our findings suggest that gestational and lactational exposure to low-dose BPA results in changes of Th17 cells development, which may contribute to its developmental immunotoxicity. Whether exposure to BPA generates similar effect in utero or during early infancy remains to be examined. These experimental findings warrant in vivo experiments to assess Th17 cells in response to experimental antigen stimulation and Th17-mediated pathology, as well as further epidemiological studies to assess the effects of BPA burden in mothers on the risk of developing childhood and adult immune-mediated diseases in the offspring. Our study has further characterized the mode of action of BPA on Th17 in relation to its effector cytokines and also identified altered ROR γ t expression and several Th17 regulatory cytokines as the underlying mechanism for Th17 proliferation in response to low level exposure to BPA. However, the molecular mechanisms how BPA induces T cell cytokines and transcription factor, and how these factors interact with each other remain to be clarified.

5. Conclusion

In summary, our results have demonstrated for the first time that gestational and lactational exposure to low-dose BPA leads to a sustained, gender-specific, and dose-dependent increase in Th17 cells in the offspring mice mediated by specific alteration to their transcription factor and regulatory cytokines. This may represent a novel mechanism by which perinatal exposure to BPA promotes autoimmunity and inflammation in childhood and adulthood.

Abbreviations: BPA, bisphenol A; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FCM, flow cytometry; GD, gestational day; IL-17, interleukin-17A; PND, postnatal day; ROR γ t, retinoic acid-related orphan receptor γ t; RT-qPCR, real-time quantitative polymerase chain reaction; TGF- β , transforming growth factor- β ; Th17, T helper 17 cell; Treg, regulatory T cell

Conflict of interest statement

The authors declare that there are no actual or potential conflicts of interest.

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Legends

Figure 1. Effects of exposure to low-dose BPA during gestation on body weight of dams. Pregnant dams were exposed to BPA (10, 100 or 1000 nM) via drinking water from GD 0 to PND21. Body weight was recorded every three days. Data are expressed as mean $\pm$ SEM (n=10 except 11 for 1000nM BPA group).

Figure 2. Effects of maternal exposure to BPA during gestation and lactation on the weight of pups and spleen coefficient in female and male offsprings. Litter body weight of pups was recorded weekly, average body weight of pups (A) was then calculated. At PND21 and PND42, offsprings were sacrificed, spleen weight was recorded and spleen coefficient (B) was then calculated. Data are expressed as mean $\pm$ SEM (n=10 except 11 for 1000nM BPA group). *p < 0.05 compared with blank control group for the same gender.

Figure 3. Effects of maternal exposure to BPA during gestation and lactation on Th17 cell frequency in the spleen in offspring mice. At PND21 and PND42, offsprings were sacrificed, spleens were collected, and Th17 cell frequency in spleen was analyzed in males and females by FCM. (A) and (B) are representative flow cytometry plots for CD4 and IL-17 staining of T17 cell frequency in spleen on PND21 and PND42, respectively. (C) Quantification of the frequency of Th17 cells in males and females on PND21 and PND42. Data are expressed as mean $\pm$ SEM (n=10 except 11 for 1000nM BPA group). *p < 0.05 compared with blank control group for the same gender, **p < 0.01 compared with blank control group for the same gender.

Figure 4. Effects of maternal BPA exposure during gestation and lactation on ROR γ t mRNA expression in the spleen in offspring mice. At PND21 and PND42, offsprings were sacrificed, spleens were collected, and ROR γ t mRNA expression in the spleen was detected in males and females using RT-qPCR. Data are expressed as mean $\pm$ SEM (n=10 except 11 for 1000nM BPA group). *p < 0.05 compared with blank control group for the same gender, **p < 0.01 compared with blank control group for the same gender.

Figure 5. Effects of maternal BPA exposure during gestation and lactation on serum IL-17 and IL-21 in offspring mice. Contents of IL-17 (A) and IL-21 (B) in serum from the offspring mice were measured in males and females by ELISA on PND21 and PND42. The results are expressed as mean $\pm$ SEM (n=10 except 11 for 1000nM BPA group). *p < 0.05 compared with blank control group for the same gender, **p < 0.01 compared with blank control group for the same gender.

Figure 6. Effects of maternal BPA exposure during gestation and lactation on serum IL-6, IL-23 and TGF- β in the offspring mice. Contents of IL-6 (A), IL-23(B) and TGF- β (C) in serum of the offspring mice were measured in males and females by ELISA on PND21 and PND42. The results are expressed as mean $\pm$ SEM (n=10 except 11 for 1000nM BPA group). *p < 0.05 compared with blank control group for the same gender, **p < 0.01 compared with blank control group for the same gender.

Figure 7. Schematic picture describing the experimental set-up and the main findings. Dams were exposed to BPA via drinking water from GD0 until weaning. At PND21 and PND42, frequency of Th17 cells and expression of ROR γ t mRNA in the spleens of the offspring were analyzed, serum levels of key cytokines that are either secreted mainly by Th17 cells or are essential for Th17 cell differentiation in the offspring were quantified. Results show that gestational and lactational exposure to a low-dose of BPA can promote Th17 cell development and increase in IL-17 and IL-21 secretion by up-regulating its transcription factor ROR γ t mRNA and the regulatory cytokines (IL-6 , IL-21 and IL-23).

Table 1 Effects of maternal exposure to various doses of BPA during gestation on birth
outcomes ($\bar{x} \pm s$ mean $\pm$ SEM)

Group	n	litter size at birth	Average birth weight(g)	Gender ratio (Male/Female)	Survival ratio at PND21(%)
Blank Control	10	12.8 $\pm$ 1.9	1.67 $\pm$ 0.09	1.12 $\pm$ 0.25	93.26 $\pm$ 3.89
Vehicle Control	10	12.6 $\pm$ 1.6	1.68 $\pm$ 0.10	1.07 $\pm$ 0.28	90.08 $\pm$ 5.71
10nM BPA	10	12.9 $\pm$ 3.6	1.65 $\pm$ 0.11	1.13 $\pm$ 0.25	89.92 $\pm$ 8.06
100nM BPA	10	13.0 $\pm$ 2.7	1.64 $\pm$ 0.08	1.16 $\pm$ 0.23	90.36 $\pm$ 5.91
1000nM BPA	11	13.1 $\pm$ 3.4	1.69 $\pm$ 0.09	1.21 $\pm$ 0.23	89.48 $\pm$ 6.48
<i>F</i> Value		0.049	0.547	0.449	0.653
<i>P</i> Value		0.995	0.702	0.773	0.628

Fig. 1

Fig. 2

Fig.3

Fig.4

Fig.5

Fig.6

C

Fig.7