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Bisphenol A (BPA) stimulates the interferon signaling and activates the inflammasome activity in myeloid cells

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Abstract

Environmental factors contribute to the development of autoimmune diseases, including systemic lupus erythematosus (SLE), which exhibits a strong female bias (female-to-male ratio 9:1). However, the molecular mechanisms remain largely unknown. Because a feedforward loop between the female sex hormone estrogen (E2) and type I interferon (IFN- α/β)-signaling induces the expression of certain p200-family proteins (such as murine p202 and human IFI16) that regulate innate immune responses and modify lupus susceptibility, we investigated whether treatment of myeloid cells with bisphenol A (BPA), an environmental estrogen, could regulate the p200-family proteins and activate innate immune responses. We found that treatment of murine bone marrow-derived cells (BMCs) and human peripheral blood mononuclear cells with BPA induced the expression of ER α and IFN- β , activated the IFN-signaling, and stimulated the expression of the p202 and IFI16 proteins. Further, the treatment increased levels of the NLRP3 inflammasome and stimulated its activity. Accordingly, BPA-treatment of BMCs from non lupus-prone C57BL/6 and the lupus-prone (NZB $\times$ NZW)F₁ mice activated the type I IFN-signaling, induced the expression of p202, and activated an inflammasome activity. Our study demonstrates that BPA-induced signaling in the murine and human myeloid cells stimulates the type I IFN-signaling that results in an induction of the p202 and IFI16 innate immune sensors for the cytosolic DNA and activates an inflammasome activity. These observations provide novel molecular insights into the role of environmental BPA exposures in potentiating the development of certain autoimmune diseases such as SLE.

Keywords

Bisphenol A; Estrogen; IFN- β ; p202; Inflammasome; Lupus Susceptibility

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Conflicts of interest

Authors declare no conflict of interest.

1. Introduction

Multiple factors such as genetic background and environmental exposures contribute to the development and progression of autoimmune diseases such as systemic lupus erythematosus (SLE) (Pisetsky, 2008; Muñoz et al., 2010; Tsokos, 2011). The development of SLE in patients (and certain mouse models) exhibit a strong female gender bias (female-to-male ratio 9:1) and the female sex hormone estrogen (E2) through activation of the estrogen receptor- α (ER α), but not ER β , contributes to the female gender bias (Roubinian et al., 1976; Rubtsov et al., 2010; Hill et al., 2010). Further, dysregulation of innate and adaptive immune responses is central to the development of pathogenic autoantibodies in the development of autoimmune diseases such as SLE (Pisetsky, 2008; Muñoz et al., 2010; Tsokos, 2011). Additionally, increased serum levels of type I interferon (IFN- α) and increased expression of the IFN-inducible genes (the "IFN-signature") in most SLE patients and certain mouse models are associated with the severity of the disease (Baechler et al., 2004; Banchereau and Pascual, 2006; Lu et al., 2007).

BPA, an environmental estrogen and an endocrine disruptor, is widely used in the manufacture of polycarbonate plastics and epoxy resins found in food containers (Rao and Richardson, 1999; Melnick et al., 2002; vom Saal, 2007; Cooper et al., 2008). Human exposure to the BPA occurs through a variety of sources by consumption of contaminated food and drinking waters. In North American waters, effluent levels of BPA are estimated to be up to 12 nM (Rogers et al., 2013). However, in European waters, the levels are estimated to be 3-times higher (up to 40 nM) (Rogers et al., 2013). BPA-induced signaling in cells involves estrogen receptors (ERs)-dependent and independent mechanisms (Rao and Richardson, 1999; vom Saal, 2007; Cooper et al., 2008; Rogers et al., 2013). ERs-dependent signaling modulates the transcription of ER-responsive genes (Melnick et al., 2002; vom Saal, 2007; Rogers et al., 2013). The ER α -responsive immunoregulatory genes encode for the activation-induced cytidine deaminase (AID) (Mai et al., 2010), IL-17 (Khan et al., 2010), IRF5 (Shen et al., 2010), and p202 (Panchanathan et al., 2009) proteins. Increased expression of these estrogen-inducible proteins in immune cells is associated with the development of SLE (Panchanathan et al., 2009; Mai et al., 2010; Khan et al., 2010; Shen et al., 2010). Notably, BPA-induced signaling in cells interferes with endocrine signaling that regulates a number of immune cell types and their immune functions (Rao and Richardson, 1999; Cooper et al., 2008; Rogers et al., 2013; Kharrazian, 2014). Accordingly, the BPA exposure (2.5 μ g BPA/kg body weight) of lupus-prone intact (NZB $\times$ NZW)F₁ female mice is reported to reduce the IFN- γ and IgG2a levels and also increase the symptom-free (development of proteinuria) period in mice (Sawai et al., 2003). In contrast, in another study, the BPA (300–350 mg BPA/kg body weight) exposure of ovariectomized (NZB $\times$ NZW)F₁ female mice (through silastic tube implant) enhanced the autoantibody (IgG) production by B1 cells (Yurino et al., 2004).

Sensing of the endogenous "danger" signals that are released by the dying cells by "danger" sensing cytosolic receptors activates a complex of cytoplasmic proteins that is termed inflammasome in innate immune cells (such as monocytes and macrophages) (Martinon et al., 2002). These receptors include members of the NOD-like receptor (NLR) family proteins such as NLRP3 and certain members of IFN-inducible p200-family proteins (such

as murine p202, Aim2 and human AIM2 and IFI16) (Martinon et al., 2002; Shaw et al., 2011; Schattgen and Fitzgerald, 2011; Choubey, 2012). Upon activation, the NLRP3 recruits the inflammasome adaptor protein ASC and pro-caspase-1 to form NLRP3 inflammasome (Martinon et al., 2002). Similarly, upon sensing the cytosolic DNA, Aim2/AIM2 protein assembles the Aim2/AIM2 inflammasome (Schattgen and Fitzgerald, 2011; Choubey, 2012). Activation of the inflammasome promotes proteolytic cleavage of the immature pro-IL-1 β and pro-IL-18 to the mature IL-1 β and IL-18 (Martinon et al., 2002; Shaw et al., 2011). Additionally, activation of inflammasome induces inflammatory cell death (termed pyroptosis) (Miao et al., 2011). In contrast to the Aim2 protein, upon sensing cytosolic DNA, the p202 and IFI16 proteins activate the STING-TBK1-IRF3 axis to stimulate the expression of the IFN- β (Unterholzner et al., 2010; Panchanathan et al., 2011; Brunette et al., 2012; Choubey, 2012). Binding of IFN- β to the cell surface IFN-receptor activate the JAK-STAT signaling pathway that stimulates the expression of the IFN-inducible proteins such as the p200-family proteins (Choubey, 2012; Stark and Darnell, 2012). This response is referred as the type I IFN response (Taniguchi and Takaoka, 2001; Hall and Rosen, 2010; González-Navajas et al., 2012; Ivashkiv and Donlin, 2014). Further, increased expression of p202 in certain strains of female mice is associated with increased production of type I IFNs (IFN- α/β), increased expression of Unc93B1 and B cell activating factor (BAFF), development of autoantibodies to DNA and nuclear antigens, and lupus nephritis (Choubey, 2012; Panchanathan et al., 2013; Panchanathan and Choubey, 2013). Similarly, increased expression of the *IFI16* gene in patients with certain autoimmune diseases has been noted (Mondini et al., 2007; Choubey et al., 2008). Therefore, increased expression of both p202 and IFI16 proteins and their ability to sense the cytosolic DNA (a "danger" signal) under certain pathogenic conditions and induce the production of type I IFN are predicated to enhance the susceptibility to develop SLE (Choubey, 2012).

We identified a feedforward loop between the female sex hormone estrogen (E2)-induced signaling through the ER α and type I interferon (IFN)-signaling in inducing the expression of ER α and the IFN-inducible lupus susceptibility modifier genes, including the *Ifi202* (encodes for the p202) (Panchanathan et al., 2010). Further, an increased expression of p202 protein in myeloid cells (and other cell types) activated the type I IFN-signaling (Panchanathan et al., 2011; Brunette et al., 2012). Because interactions between the environmental factors and lupus susceptibility modifier genes remain largely unknown, we investigated whether BPA, an environmental endocrine disruptor, treatment of innate immune cells could activate innate immune responses through ERs. Here we report that treatment of human and murine innate immune cells with BPA at environmentally relevant concentrations increased levels of ER α and activated innate immune responses that included the production of IFN- β , activation of the IFN-signaling and the inflammasome activity. Our observations for the first time implicate environmental BPA exposures in influencing the development and progression of SLE through the activation of the innate immune responses.

2. Materials and methods

2.1. Mice, immune cells, and treatments

The Animal Care and Use Committee (IACUC) of University of Cincinnati approved the experimental procedures that involved mice. Female C57BL/6J (B6) and [(NZB × NZW)F₁] mice between 6–8-wk of age were purchased from The Jackson Laboratory (Bar Harbor, Maine). All mice were housed in specific pathogen-free Laboratory of Animal and Medical Services (LAMS) facilities of the University of Cincinnati.

Bone marrow-derived cells (BMCs) were prepared from the age-matched female mice and characterized as described previously (Panchanathan and Choubey, 2013). Freshly isolated bone marrow cells (cells isolated from two or more age-matched female mice were pooled) were suspended in RPMI-1640 cell culture medium that was supplemented with 10% fetal bovine serum (FBS) and antibiotics (Invitrogen, Carlsbad, CA). From pooled bone marrow cells, CD11b⁺ cells were purified using magnetic MicroBeads (purchased from Miltenyi Biotech, Auburn, CA) through the positive selection (Panchanathan and Choubey, 2013). ~90–95% pure cells (the purity of cells determined by flow-cytometry and the expression of cell-type-specific genes by quantitative real-time PCR) were either used immediately for experiments or maintained in culture up to 24 h in the medium that was supplemented with granulocyte macrophage colony stimulating factor (GM-CSF; 10 ng/ml).

Human monocytic THP-1 cell line (established from a male infant) was originally purchased from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in suspension culture as described (Veeranki et al., 2011). For differentiation of cells in culture, the cell cultures were either treated with 1 μM PMA for 6 h or 100 nM PMA for two days. PMA-differentiated THP-1 cells were either left untreated or treated with BPA (10–100 nM) for the indicated time. Cells were then primed with LPS (100 ng/ml, Sigma) for 3–4 h and then treated with the NLRP3 inflammasome activator nigericin (10 μg/ml) for 45 min to an hour as described (Veeranki et al., 2011). Cells lysates were prepared and analyzed for the levels of activated caspase-1 and the mature IL-1β as described (Veeranki et al., 2011). Human purified (CD14⁺) monocytes from a healthy male donor were purchased from ReachBio (Seattle, WA) and cultured as described (Veeranki et al., 2011).

Murine macrophage J774.A1 cell line was purchased from the ATCC and cells were maintained in culture as suggested by the supplier. Sub-confluent cultures of cells were either treated with E2 or BPA in phenol-free medium as described (Panchanathan and Choubey, 2013).

Stock solutions (100 μM, 10 μM or 1 μM) of 17β-estradiol (E2) or BPA (cat # 239658-50G; >99% pure from Sigma-Aldrich, St. Louis, MO, USA) were prepared in absolute alcohol (vehicle; from Sigma-Aldrich). For treatment of cells, stock solutions were diluted 1:1000 fold (the final concentration of alcohol 0.1%) and control cells were treated with equal volume of the vehicle. Cells were cultured in the phenol red-free DMEM medium (Invitrogen, Carlsbad, CA). To minimize the endogenous levels of steroid hormones in the culture medium, the medium was supplemented with 10% charcoal-stripped fetal bovine serum (Invitrogen) (Panchanathan and Choubey, 2013). Cells in culture were either treated

with E2 or BPA at the indicated concentrations for the indicated time. As a control, cells were treated with equal volume of ethanol (vehicle).

2.2. Isolation of RNA, RT-PCR, and real-time Taq-Man PCR

TRIzol (Invitrogen) RNA isolation procedure was used to isolate total RNA from control and BPA-treated cells. To remove any contaminating genomic DNA in RNA preparations, RNA preparation was digested with DNase I. The digested RNA preparation containing 0.5–2 µg of RNA was used for reverse transcriptase (RT) reaction (Panchanathan and Choubey, 2013). For PCR, the following primer pair was used to amplify the murine *Esr1* cDNA: forward primer: 5'-AATTCTGACAATCGACGC CAG -3'; backward primer: 5'-GTGCTTCAACATTCTCCCTCCTC -3'; *Ifi202*: forward primer: 5'-GGTCATCTACCAACT CAGAAT -3'; backward primer: 5'-CTCTAGGATG CCACTGCTGTTG -3'; *Aim2*: forward primer: 5'-ACAGTGGCCACGGAGA -3'; backward primer: 5'-AGGTGAC TTCACTCCACA -3'; and *Ifnb*: forward primer: 5'-CTGCGTTCCTGCT GTGCTTCTCCA -3'; backward primer: 5'-TTCTCCGTCATCTCCATAGGGATC-3'. Levels of actin mRNA were used as an internal control. To estimate a fold change (FC) in levels of an mRNA following a treatment, the intensity of the actin RT-PCR DNA band (an internal control) on the agarose gel and the RT-PCR DNA band of a gene of interest were measured by the Molecular Imager Gel Doc XR⁺ System (Bio-Rad, Hercules, CA) with Image Lab Software. Next, the ratio was calculated using the DNA band intensity value for the gene of interest and actin DNA band. This ratio in control cells was indicated as 1 and the FC for treated samples was calculated by calculating ratio between the value from treated samples (calculated as in the case of control sample) and the control value 1.

Gene-specific TaqMan PCR assays for murine *Esr1* (Mm00433149_m1), *Ifnb* (Mm00439552_s1), *Ifi202* (Mm03048198_m1), *Aim2* (Mm01295719_m1) and the endogenous control β -actin genes (4352933) were purchased from the Applied Biosystems (Foster City, CA). In brief, the quantitative real-time PCR reactions (in triplicates for each RNA sample) were performed using all reagents and plates from Applied Biosystems (Foster City, CA, USA) as suggested by the supplier using the 7300 Real Time PCR System. The PCR cycling program consisted of denaturing at 95°C for 10 min and 40 cycles at 95°C for 15 seconds, and annealing and elongation at 60°C for 1 min. The PCR signal in samples was normalized using the housekeeping gene as suggested by the supplier and the initial data analyses were performed using RQ Manager software supplied along with the PCR machine by Applied Biosystems. Quantification was performed using the comparative C_T method (or C_T) according to Livak and Schmittgen (2001). Further, statistical analyses were done using the GraphPad Prism software 5 (GraphPad Software, Inc., La Jolla, CA). A Student's *t*-test and one-way analysis of variance (ANOVA) were used for comparisons among the experimental values. When indicated, data are presented here as mean $\pm$ SE from three independent experiments and a *p*-value <0.05 was considered statistically significant.

2.3. Immunoblotting

Cells were lysed in a modified radio-immune precipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1%

SDS). The buffer was supplemented with protease inhibitor (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (Sigma-Aldrich) as described (Veeranki et al., 2011). The cell lysates were incubated on ice for 30 min. After the incubations, the lysates were sonicated briefly, centrifuged at 14,000 rpm for 5 min at 4°C, and the supernatants were collected. Cell lysates containing approximately equal amounts of total proteins were subjected to immunoblotting using the indicated antibodies. Antibodies to murine ER α (sc-542), IFI16 (sc-8023), ASC (sc-22514), IL-18 (sc-7984), and IL-1 β (sc-7884) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to IRF5 (# 4950), STAT1 (# 9172), p-STAT1 (Tyr-701) (# 9167), actin (# 5125) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies to NLRP3 (HPA012878) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies to the p202 (IMG-6670A) were purchased from Imgenex (San Diego, CA). Polyclonal antibodies to the murine Aim2 protein have been described (Panchanathan et al., 2011).

When indicated, actin protein was used as an internal control (because levels of actin protein did not change after BPA treatment of cell types that were used). To calculate a fold change (FC) in levels of a protein of interest following a treatment, enhanced chemiluminescence (ECL) signals of actin protein (an internal standard) and the protein of interest were measured by the Molecular Imager Gel Doc XR⁺ System (Bio-Rad, Hercules, CA) with Image Lab Software. To estimate a relative FC in levels of a protein of interest after a treatment, the protein band ECL signal for the protein of interest was divided by the actin protein band ECL signal (which was normalized based on equal protein amounts per lane). This ratio between the protein band ECL signal and the actin protein band ECL signal in control cells was indicated as 1. Further, FC for treated samples was estimated by calculating ratio between the value from treated samples (calculated as in the case of control sample) and the control value 1.

2.4. Reporter assays

Sub-confluent cultures of the J774.A1 cells in a six-well plate were transfected with the reporter plasmids, either the ERE-luc (a reporter plasmid in which the expression of the firefly luciferase reporter gene is regulated by estrogen-responsive element) or the 202-luc (a reporter plasmid in which the expression of the firefly luciferase reporter gene is regulated by ~0.8 Kb 5'-regulatory region of the *Ifi202* gene; plasmid DNA: 1.8 μ g) and pRL-TK (0.2 μ g) using the FuGENE 6 (Roche Applied Science, Indianapolis, IN) transfection reagent as suggested by the supplier. Cells were harvested between 40 and 45 h after transfections, and the firefly and *Renilla* dual luciferase activities were determined (Panchanathan et al., 2011).

2.5. Statistical methods and analyses

Statistical analyses were done using the GraphPad Prism software 5 (GraphPad Software, Inc., La Jolla, CA). Further, a Student's *t*-test and one-way analysis of variance (ANOVA) were used for comparisons among the experimental values. When indicated, data are presented as mean $\pm$ SEM from at least three experiments. A *p*-value <0.05 was considered statistically significant.

3. Results

3.1. Treatment of murine bone marrow cells with BPA increases levels of ER α and IFN- β mRNAs in sex-dependent manner

Treatment of murine cells with the female sex hormone estrogen activated a type I IFN-signaling and induced the expression of certain IFN-inducible genes, including the *Ifi202* (Panchanathan et al., 2010). Additionally, the activation of type I IFN-signaling in cells increased levels of the ER α and stimulated the expression of estrogen-inducible genes in sex-dependent manner (Panchanathan et al., 2010). Therefore, to explore whether treatment of murine cells with BPA, an environmental estrogen that can signal through the ER α (Gould et al., 1998), could regulate the expression of ER α (encoded by the *Esr1* gene) and activate a type I IFN response, we treated CD11b⁺ bone marrow cells (BMCs) from C57BL/6 (B6) female mice with increasing concentrations (0, 1, 10, or 100 nM; we chose these concentrations because drinking waters are reported to contain 12–40 nM concentration of BPA; see Rogers et al., 2013) of BPA. As shown in Fig. 1A–C, treatment of cells under our experimental conditions with BPA at 10 nM, as compared with 1 nM, concentration significantly increased levels of mRNAs encoding for the ER α , IFN- β , and IFN-inducible p202 proteins. Intriguingly, higher (100 nM) concentration of BPA did not result in further increases in the levels of these mRNAs. Instead, we noted some decreases in the mRNA levels (Fig. 1A–C). Notably, no significant increase in the *Aim2* mRNA levels was evident at any of the BPA concentration used (data not shown). Because basal and induced (induced by IFN- α) of *Esr1* mRNA in BMCs (and other immune cell types) were higher in cells from female mice as compared with age-matched males (Panchanathan et al., 2010), we also compared levels of the *Esr1*, *Ifnb*, and *Ifi202* mRNAs in BMCs isolated from B6 males and age-matched females without and after BPA-treatment. As shown in Fig. 1D, consistent with our previous observations (Panchanathan et al., 2010), the basal levels of *Esr1* mRNA were higher in females than males and BPA-treatment (10 nM) of cells increased the levels further. Further, as expected from our previous observations (Panchanathan et al., 2010), basal levels of *Ifnb* and *Ifi202* mRNAs were relatively higher in female cells than males and BPA-treatment increased the levels further. Together, these observations indicated that BPA-treatment of CD11b⁺ BMCs significantly increased levels of mRNAs encoding for ER α , IFN- β , and IFN-inducible p202 proteins in a concentration and sex-dependent manner.

3.2. Treatment of murine BMCs with BPA activates innate immune responses

We also sought to determine whether treatment of murine BMCs with BPA could activate innate immune responses. Therefore, we incubated CD11b⁺ BMCs from C57BL/6 females with either E2 (10 nM; a positive control) or BPA (10 nM) for 14 h. We chose BPA concentration 10 nM based on our experiments described above (Fig. 1), which revealed that this concentration of BPA significantly increased levels of ER α and IFN- β mRNAs. As shown in Fig. 2A, the treatment with E2 increased steady-state levels of mRNAs encoding for the ER α , p202, and IFN- β as compared to untreated cells (compare lane 2 with 1). Levels of the *Aim2* mRNA did not change appreciably (compare lane 2 with 1). Consistent with our above observations (Fig. 1), the BPA-treatment of cells increased steady-state levels of mRNAs encoding for ER α , p202, and IFN- β (compare lane 3 with 1). Again, levels of *Aim2*

mRNA did not change measurably. The increase in mRNA levels encoding for ER α (Fig. 2B), IFN- β (Fig. 2C), and p202 (Fig. 2D) was found to be significant in quantitative real-time PCR. Consistent with the above observations (Fig. 2A), no significant change was noted in mRNA levels encoding for the Aim2 protein (data not shown).

Because treatment of BMCs with E2 or BPA significantly increased levels of ER α mRNA (Fig. 2), we investigated whether pre-treatment of cells with MPP (10 μ M), an antagonist of ER α -signaling (Zhou et al., 2009), or PHTTP (10 μ M), an antagonist of ER β -signaling (Santollo et al., 2010), could inhibit BPA-mediated induction of ER α or IFN- β . As shown in Fig. 3A, the treatment with MPP significantly reduced levels of the mRNAs encoding for ER α and IFN- β . In contrast, the treatment with PHTTP did not decrease levels of these mRNAs, thus suggesting that BPA-induced signaling in bone marrow-derived CD11b⁺ cells depends upon the ER α , but not ER β , expression. Consistent with the above observations, we found that treatment of cells with BPA increased levels of the ER α (~2.5-fold; Fig. 3D), activated the STAT1 transcription factor (as determined by activating phosphorylation of STAT1 on Tyr701 residue) and increased the levels of p-STAT1 (~2.4-fold, Fig. 3D), increased steady-state levels of STAT1 (~2.5-fold, Fig. 3D) and p202 (~2.6-fold, Fig. 3B, compare lane 2 with 1) proteins. Levels of the Aim2 protein did not change appreciably. Because the activation of the type I IFN-signaling stimulates the activity of certain inflammasomes (Jones et al., 2010; Choubey, 2012), we also tested whether the treatment could activate an inflammasome activity. As shown in Fig. 3C, BPA-treatment of CD11b⁺ BMCs increased levels of the NLRP3 (about 4 to 5-fold, see Fig. 3C and D), activated caspase 1 (p20 and p10), and increased the levels of the mature IL-1 β (p17), thus, indicating that the treatment activated an inflammasome activity. Together, our observations revealed that BPA-treatment of murine bone marrow-derived CD11b⁺ cells that were isolated from C57BL/6 females activated the innate immune responses that included an activation of the type I IFN-signaling and an inflammasome activity.

3.3. Treatment of murine macrophage cell line with BPA activates the estrogen and type I IFN-induced signaling and induces the expression of the p202

Next, we treated murine macrophage cell line J774.A1 with either estrogen (10 nM, a positive control) or with BPA (10 nM) for 14 h. Consistent with our above observations (Figs. 1 and 2), the treatment of cells with E2 increased steady-state levels of mRNAs encoding for the ER α , p202, and IFN- β as compared with untreated cells (Fig. 4A, compare lane 2 with 1). Levels of the Aim2 mRNA did not change appreciably (compare lane 2 with 1). Further, BPA-treatment of cells also increased steady-state levels of mRNAs encoding for the ER α , p202, and IFN- β (compare lane 3 with 1). Again, levels of the Aim2 mRNA did not change measurably. Accordingly, treatment of cells with BPA increased levels of ER α , activated the IFN-activatable STAT1 transcription factor (as determined by activating phosphorylation of STAT1 on Tyr701 residue), and increased steady-state levels of STAT1 and p202 proteins (Fig. 4B and C; compare lanes 2 and 3 with lane 1). Levels of Aim2 protein did not change appreciably. Consistent with the above observations, estrogen or BPA treatment of J774.A1 cells significantly stimulated the activity of estrogen inducible reporters such as the ERE-luc (Fig. 4D) and 202-luc (Fig. 4E). Together, these observations

revealed that treatment of J774.A1 cells with BPA activated estrogen and type I IFN-induced signaling that induced the expression of lupus susceptibility enhancer p202.

3.4. BPA-treatment of BMCs from non lupus-prone as well as lupus-prone strains of mice activates innate immune responses

In two studies (Sawai et al., 2003; Yurino et al., 2004), treatment of lupus-prone [NZB × NZW)F₁] female mice with BPA differentially modulated the adaptive immune responses. However, these two studies did not investigate whether BPA-treatment also modulated innate immune responses. Therefore, we investigated whether BPA-treatment of bone marrow-derived CD11b⁺ cells from the [NZB × NZW)F₁] strain of female mice regulates the innate immune responses. As shown in Fig. 5, constitutive levels of ER α , NLRP3, activated STAT1 (PY-STAT1), STAT1, activated caspase-1 (p20), and the mature IL-1 β (p17) were measurably higher in cells from the [NZB × NZW)F₁] females than the age-matched C57BL/6 females used as controls (compare lane 4 with lane 1) in two experiments. Further, BPA-treatment of cells appreciably increased levels of the ER α , p202, PY-STAT1, STAT1, activated caspase-1 (p20), and the mature IL-1 β (p17) in B6 cells more than the cells from [NZB × NZW)F₁] female mice. These observations suggested that BPA-induced signaling in CD11b⁺ BMCs from non lupus-prone (C57BL/6) and the lupus-prone [NZB × NZW)F₁] female mice differentially activated the innate immune responses.

3.5. Treatment of human innate immune cells with BPA activates the innate immune responses

Our above observations that treatment of murine immune cells with BPA activated innate immune response (Figs. 3–5) prompted us to explore whether BPA-treatment can activate similar responses in the human cells. For this purpose, we treated human monocytic cell line THP-1 (because THP-1 cell line is a well-characterized cell model system to study activation of innate immune responses; see Martinon et al., 2002) with increasing concentrations (0–100 nM) of BPA. As shown in Fig. 6A, the treatment increased levels of the estrogen-inducible transcription factor IRF5 (Shen et al., 2010), activated the STAT1 transcription factor (PY-STAT1) and increased the levels of p-STAT1 (2.5 to 4-fold, Fig. 6C), increased levels of STAT1 (2.5 to 3-fold, Fig. 6C), IFI16 (2.2 to 2.6-fold, Fig. 6C), and pro-caspase-1 in a dose-dependent manner (compare lanes 2 and 3 with lane 1). Notably, levels of the AIM2 protein did not change appreciably. Additionally, the treatment increased levels of the mature IL-18 (Fig. 6B). Consistent with these observations, treatment of human peripheral blood mononuclear cells (CD14⁺) with BPA (10 nM) for 16 h also appreciably increased the levels of the NLRP3, IFI16, STAT1, and procaspase-1 (p45) proteins (Fig. 6D). Further, we noted an appreciable increase in the levels of AIM2 protein in mononuclear cells and the increase was consistent with an activation of the IFN response and corresponding increases in the levels of the IFN-inducible IFI16 proteins (compare lane 2 with 1). Notably, the treatment decreased the levels of the pro-IL-1 β , suggestive of its proteolytic cleavage by an activated inflammasome and secretion of the mature form (p17) from cells (although we were unable to detect the secreted levels of the mature IL-1 β ; see Martinon et al., 2002). Together, these observations revealed that BPA-induced signaling in human immune cells can activate the innate immune responses.

3.6. BPA-treatment potentiates activation of NLRP3 inflammasome

Our observations that treatment of murine and human innate immune cells with BPA increased levels of NLRP3 and activated an inflammasome activity (as determined by increases in levels of activated caspase 1 and/or the cellular levels of the mature IL-1 β) prompted us to investigate whether the treatment could activate the NLRP3 inflammasome activity. As shown in Fig. 7A, treatment of the murine bone marrow-derived CD11b⁺ cells, which were primed with LPS (100 ng/ml for 3 h), with nigericin, an activator of the NLRP3 inflammasome (Gross et al., 2009), increased levels of the activated caspase 1 (p10) about 3.5-fold (Fig. 7B) and decreased levels of the pro-IL-1 β (p31) (compare lane 2 with lane 1). Notably, the treatment increased the cellular levels of the mature IL-1 β (p17) about 2.6-fold (compare lane 2 with lane 1; Fig. 7B). Of note, treatment of cells with BPA (10 nM for 14 h) prior to the priming of cells with LPS appreciably increased constitutive levels of NLRP3, activated caspase 1 (p10) about 3.8-fold (Fig. 7B), and the cellular levels of the mature IL-1 β (p17) about 2.1-fold (Fig. 7B). Interestingly, treatment of BPA-treated cells with nigericin further increased levels of the activated caspase 1 (p10) about 4.4-fold (compare lane 4 with lane 3 or 2; Fig. 7B). Further, the treatment measurably decreased cellular levels of IL-1 β (p17) (compare lane 4 with lane 3; Fig. 7B), suggesting its secretion from cells (Martinon et al., 2002). These observations revealed that treatment of murine bone marrow-derived CD11b⁺ cells with BPA increased levels of NLRP3 and potentiated activation of the NLRP3 inflammasome activity.

We also investigated whether treatment of PMA-treated and LPS primed human monocytic THP-1 cells (a cell model to study inflammasome activation; Martinon et al., 2002) with BPA potentiates activation of the NLRP3 inflammasome. As shown in Fig. 7C, BPA treatment of primed cells increased levels of ER α , the inflammasome adaptor ASC, activated caspase 1 (p10), and IL-1 β (p17) (compare lane 3 with 1). Consistent with our above observations (Fig. 7A and B), treatment of BPA-treated cells with nigericin decreased the cellular levels of caspase1 (p10) and IL-1 β (p17) (compare lane 4 with 3), thus suggesting their secretion into the medium. Together, our observations indicated that BPA treatment of murine and human myeloid cells increased the constitutive activity of the inflammasome and potentiated the activity of the NLRP3 inflammasome.

4. Discussion

The molecular mechanisms through which environmental factors influence the development and progression of certain autoimmune diseases remain largely unknown. Additionally, it remains unknown how environmental factors influence the sex/gender bias in the development of certain autoimmune diseases, including SLE. Therefore, our observations that treatment of murine and human myeloid cells with BPA at 10 nM concentration that is often detected in drinking waters (Rogers et al., 2013) increased levels of ER α and stimulated the expression of the IFN- β (a type I IFN), activated the type I IFN-signaling that resulted in an induction of the p202 and IFI16 innate immune sensors for the cytosolic DNA, and activated an inflammasome activity may provide novel molecular insights into the potential role of environmental BPA exposures in potentiating the development of certain autoimmune diseases that exhibit a female sex/gender bias (Fig. 8).

Expression of p202 protein is up-regulated by estrogen through the ER α in a variety of immune cell types, including the myeloid cells (Panchanathan et al., 2010; Panchanathan et al., 2013; Panchanathan and Choubey, 2013). Further, mouse strain and sex-dependent basal and estrogen and the ER α -induced levels of the p202 protein in immune cells up-regulate the expression of certain innate immune regulators such as Toll-like receptors (such as the TLR7 and TLR9) and their transporter Unc93b1 (Panchanathan and Choubey, 2013). Additionally, p202 protein up-regulates the expression of B cell activating factor (BAFF), an important regulator of the B cell functions and an enhancer of lupus disease in mouse models and SLE patients (Panchanathan and Choubey, 2013). Therefore, our observations that BPA-induced signaling in murine innate immune cells that were isolated from the female mice increased p202 protein levels through activation of the ER α -mediated transcriptional activity (Fig. 4) support the idea that environmental exposures to BPA may contribute to the female sex bias in the development of murine lupus in part through up-regulation of the p202 protein.

Activation of the ER α -signaling regulates the development of dendritic cells (DCs) (Mao et al., 2005; Kovats 2012). These antigen-presenting cells play a crucial bridging role between the innate and adaptive immune responses (Kovats, 2012). In the presence of an inflammatory environment, increased levels of GM-CSF that is produced by innate immune cells and T cells potentiate the development of new DCs from monocytes. These inflammatory DCs play an important role in programming T cell-mediated responses during the development of autoimmunity. Notably, estrogen through ER α , but not ER β , promotes the GM-CSF-mediated inflammatory pathway of DC differentiation (Mao et al., 2005; Kovats 2012). Therefore, our observations that treatment of innate immune cells with BPA activated the inflammasome activity (Fig. 7) raise the possibility that BPA-induced increased production of IL-1 β by innate immune cells promotes the differentiation of inflammatory DCs through increasing the production of GM-CSF. Therefore, further work is in progress to test this possibility.

While *in vitro* treatment of BMCs from non lupus-prone C57BL/6 (B6) and lupus-prone (NZB/W)F1 mice with BPA resulted in activation of innate immune responses (Fig. 5), it remains unknown whether a chronic exposure of B6 mice with BPA results in a break in tolerance, thus resulting in the development of autoimmunity. Further, it is known that B6 mice do not spontaneously develop autoimmunity and a genetic deficiency in the *Trex1* gene (encoding for the three prime repair exonuclease 1) in the B6 mice results in autoimmunity through activation of an IFN response (Gall et al., 2012). Considering the above interesting observations and our observations in this manuscript, it is conceivable that a chronic exposure of B6 female mice to BPA could break tolerance in this non autoimmune strain of mice. Therefore, further work will needed to test this hypothesis.

Treatment of mice with BPA causes pyometra, an inflammatory disease of the uterus that is accompanied by an increase in accumulation of macrophages in uterus (Kendzierski et al., 2012). Notably, the development of the disease is shown to be mouse strain-dependent- C57BL/6, but not CD1, females develop the disease (Kendzierski et al., 2012). Although, it remains unclear how BPA-induced responses in mice differ with the genetic background, we found that innate immune cells from both non lupus-prone C57BL/6 as well as lupus prone

[(NZb × NZW)F₁] strains of female mice activated the innate immune responses following treatment with BPA (Fig. 5). Further work will be needed to determine whether mouse strain-specific factors influence the strength and duration of BPA-induced innate immune responses in other strains of mice.

Studies involving mice have indicated that prenatal or adulthood exposure to various doses of BPA influences the development of adaptive immune responses that involve T (Yoshino et al., 2003; Yoshino et al., 2004; Yan et al., 2008) and B cells (Sawai et al., 2003; Yurino et al., 2004). Further, treatment of mice with BPA differentially regulated the development of autoimmunity in the [(NZB × NZW)F₁] mice (Sawai et al., 2003; Yurino et al., 2004). Therefore, it is conceivable that different doses of BPA (2.5 µg BPA *versus* 300–350 mg/kg body weight) and different routes of exposure (feeding in cereal versus through a silastic tube implant) could account for the BPA-mediated differential regulation of the adaptive immune responses. Notably, these previous studies (Sawai et al., 2003; Yoshino et al., 2003; Yurino et al., 2004; Yoshino et al., 2004; Yan et al., 2008) did not investigate whether BPA exposure of mice differentially activated the innate immune responses. Therefore, our observations described here are novel and do not contradict the previous observations (Sawai et al., 2003; Yurino et al., 2004). Because dysregulation of innate as well as adaptive immune responses is central to the development of autoimmune diseases (Pisetsky, 2008; Muñoz et al., 2010; Tsokos, 2011), our observations will serve as basis to further investigate the role of BPA-induced innate immune responses in the development of autoimmunity.

Treatment of human T cell leukemia line, Jurkat, with BPA is reported to increase the expression of ERα (Cipelli et al., 2014). Similarly, BPA exposure in rats increases the expression of ERα in splenic cells derived from females, but not males (Cao et al., 2013). Consistent with these observations, we also found that treatment of murine and human myeloid cells with BPA also increased levels of the ERα (Figs. 3 and 5). Further, the increase was associated with activation of an ER-responsive reporter gene (Fig. 4) and an induction of estrogen and IFN-inducible *Irf202* gene (Figs. 1 and 2). Although these observations support the idea that BPA-induced signaling in innate immune cells increases levels of the ERα and stimulate the expression of estrogen and ERα-inducible genes, our observations do not rule out the possibility that the ER-independent signaling contributes to the regulation of p202 levels. Because estrogen and ERα-induced signaling can stimulate the expression of the IFN-inducible genes (including the *Esr1* gene) that encodes for the ERα (Panchanathan et al., 2010), our observations make it likely that BPA-mediated activation of ERα-mediated transcription in innate immune cells activates a feedforward loop between the ERα and IFN-signaling through up-regulation of the p202 protein (Fig. 8).

A deficiency of the *Esr1* gene, but not *Esr2* (encodes for the ERβ) in lupus-prone mice reduces the development of lupus-associated glomerulonephritis and significantly increases the survival of mice (Bynoté et al., 2008; Svenson et al., 2008). Further, the deficiency reduces the development of pathogenic anti-histone/DNA auto-antibodies (Bynoté et al., 2008). Because BPA can signal through ERα (Gould et al., 1998), our observations that BPA treatment of innate immune cells increased levels of ERα and stimulated the transcriptional activity of the ER-responsive 202-luc reporter (Fig. 4) are consistent with the idea that activation of the ERα by environmental BPA contribute to the female sex/gender

bias in the development of autoimmunity through up-regulation of estrogen-inducible p202 and BAFF.

In summary, our observations demonstrate that BPA-induced signaling in murine and human myeloid cells activates innate immune responses that include stimulation of type I IFN-signaling, an antiviral response, and activation of the inflammasome activity, a proinflammatory response. Dysregulated activation of both types of these innate immune responses is associated with the development of autoimmune and inflammatory diseases in patients and mouse models (Pisetsky, 2008; Muñoz et al., 2010; Tsokos, 2011). Therefore, our observations will serve as basis to further investigate the role of environmental BPA exposures in the regulation of innate immune responses and their potential role in the development of certain autoimmune diseases that exhibit the female sex/gender bias.

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Highlights

- Exposures to bisphenol A (BPA), which signals through activation of ER α , are linked to the development of autoimmunity.
- BPA treatment of myeloid cells increased levels of ER α and activated interferon (IFN) signaling.
- BPA treatment of myeloid cells increased levels of NLRP3 and activated the inflammasome activity
- We identified novel potential links between BPA exposures and autoimmunity

Fig. 1.

Treatment of murine bone marrow cells (BMCs) with BPA increases levels of ER α and IFN- β mRNAs in a dose-dependent manner. (A–C) CD11b⁺ BMCs from C57BL/6 female mice were treated with vehicle alone or with the indicated concentration of BPA in a phenol red-free medium as described in methods for 14 h. At the end of the treatment, cells were harvested and total RNA was prepared. The RNA in triplicates was analyzed by quantitative real-time PCR using the TaqMan assay specific to the *Esr1* (A), *Ifnb* (B), or *Ifi202* (C) gene. The ratio of the test gene to actin mRNA was calculated in units (one relative unit being the ratio of the test gene to actin mRNA). Levels of mRNA in control cells are indicated as 1.0 unit. Standard errors are represented with bars. * Significantly different ($p < 0.05$; *** $p < 0.001$) as compared with vehicle treated control group (NS, not significant). These assays were repeated at least 2–3 times with similar results. (D) CD11b⁺ BMCs from

C57BL/6 male (lanes 1 and 2) or age-matched female (lanes 3 and 4) mice were either treated with vehicle (lanes 1 and 3) or 10 nM BPA (lanes 2 and 4) for 14 h in a phenol red-free medium as described in methods. At the end of the treatment, total RNA was isolated and the RNA samples were analyzed by semi-quantitative RT-PCR using a pair of primer specific to the indicated gene. Approximate fold change (FC) in the indicated mRNAs levels was calculated as described in methods.

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Fig. 2.

Estrogen or BPA-treatment of murine bone marrow cells increases levels of ER α and IFN- β mRNAs. (A) CD11b⁺ BMCs from C57BL/6 female mice were either treated with vehicle, E2 (10 nM) or BPA (10 nM) in a phenol red-free medium as described in methods for 14 h. At the end of the treatment, total RNA was prepared from cells. The RNA samples were analyzed by semi-quantitative RT-PCR using a pair of primer specific to the indicated gene. (B–E) RNA samples described in the panel (A) were also subjected to quantitative real-time PCR (in triplicates) using the TaqMan assay specific to the *Esr1* (B), *Ifnb* (C), *Ifi202* (D), or

Aim2 (E) gene. The ratio of the test gene to actin mRNA was calculated in units (one relative unit being the ratio of the test gene to actin mRNA). Levels of mRNA in control cells are indicated as 1.0 unit. The SEM are indicated by bars. * Indicates significantly different ($p < 0.05$; *** $p < 0.001$) as compared with vehicle treated control group (NS, not significant).

Fig. 3.

BPA-treatment of murine bone marrow cells activates the innate immune responses. (A) Purified CD11b⁺ BMCs from C57BL/6 females were left untreated (lane 1) or pretreated with the vehicle (DMSO, lane 2), MPP (10 μ M, lane 3) or PHTTP (10 μ M, lane 4) for an hour. Subsequently, cells were either left untreated (lane 1) or treated with BPA (10 nM, lanes 2–4). 14 h after the treatments, total RNA was prepared from cells and was analyzed by RT-PCR using primers specific to the indicated genes. Two experiments gave similar results. Fold change (FC) in levels of the indicated mRNAs in response to BPA treatment of cells as compared with vehicle treated cells was estimated as described in methods. (B and C). Purified CD11b⁺ BMCs from C57BL/6 female mice were either left untreated (lanes 1)

or treated with BPA (10 nM; lane 2) for 14 h under the culture conditions described in Methods. After the treatment, total cell lysates were prepared and lysates containing equal amounts of proteins were analyzed by immunoblotting using antibodies specific to the indicated proteins. Fold changes (FC) in levels of the indicated proteins in response to BPA treatment of cells (as compared with vehicle treated cells) were estimated as described in methods section. (D) Fold changes in the levels of the indicated proteins in BPA-treated CD11b⁺ cells as compared with vehicle-treated cells shown in the panels B and C were estimated in three independent experiments as described in Materials and method section. The SEM values are shown. The value in the vehicle-treated cells is indicated as 1. Significance levels assessed by paired one-tailed *t*-test are **p*<0.05 and ***p*<0.01.

Fig. 4.

BPA-treatment of murine macrophage cell line activates type I IFN-signaling and induces the expression of p202. (A) Sub-confluent cultures of J774.A1 cells were either left untreated (lane 1, control), treated with estrogen (10 nM, lane 2) or BPA (10 nM, lane 3) for 14 h under the culture conditions described in Methods. Total RNA was prepared from cells and was analyzed by semi-quantitative RT-PCR using a pair of primers that were specific to the indicated genes. Two experiments gave similar results. Fold change (FC) in levels of the IFN- β mRNA in response to BPA treatment of cells (as compared with vehicle treated cells) was estimated as described in methods. (B) Sub-confluent cultures of J774.A1 cells were either left untreated (control, lane 1) or treated with the indicated concentration of BPA under the culture conditions described in Methods. Total cell lysates were prepared and lysates containing equal amounts of proteins were analyzed by immunoblotting using antibodies specific to the indicated proteins. (C) Fold changes in the levels of ER α , p-STAT1, and p202 proteins in BPA-treated J774.A1 cells as compared with vehicle-treated cells (panel B) were estimated in three independent experiments as described in Materials and method section. The SEM values are shown for the ER α (left panel), p-STAT1 (middle panel), and p202 (right panel) proteins. The value in the vehicle-treated cells is indicated as 1. Significance levels assessed by the paired one-tailed *t*-test are * $p < 0.05$. (D and E) Sub-confluent cultures of J774.A1 cells were either transfected with the ERE-luc-reporter (1.8 μ g) or 202-luc-reporter plus pRL-TK plasmid (0.2 μ g). 24 h after transfections, the transfected cells were treated with vehicle, estrogen (10 nM) or BPA (10 nM) for 14 h. After the treatments, cells were lysed and cell lysates were analyzed for dual luciferase activity as described in methods. The ratio between the firefly luciferase and *Renilla* luciferase activity in the control cells is indicated as 1. The SEM are represented with bars. * Indicates significantly different ($p < 0.05$) as compared with vehicle treated control group.

Fig. 5.

BPA-treatment of BMCs from non lupus-prone as well as lupus-prone strains of female mice activates innate immune responses. Purified CD11b⁺ BMCs (cells pooled from 3–5 age-matched females) were isolated from non lupus-prone C57BL/6 or age-matched lupus-prone [(NZB × NZW)F₁] female mice as described in Methods. The purified CD11b⁺ cells were either left untreated (lanes 1 and 4) or treated with the indicated concentrations of BPA for 14 h under the culture conditions described in Methods. After the treatments, total cell lysates were prepared and lysates containing equal amounts of proteins were analyzed by

immunoblotting for the indicated proteins. Fold changes (FC) in levels of the indicated proteins in response to BPA treatment of cells (as compared with vehicle treated cells) in two experiments were estimated as described in methods section. An upward arrow indicates an increase in either constitutive and/or BPA-treatment induced levels of the indicated protein. Two repeats of the same experiment gave similar results.

Fig. 6.

BPA-treatment of human myeloid cells activates innate immune responses. (A and B) Human monocytic THP-1 cells were cultured under the conditions described in Methods. Cells were either left untreated (lane 1) or treated with the indicated concentrations of BPA (lanes 2 and 3) for 16 h. After the treatment, cells were lysed and lysates containing equal amounts of proteins were analyzed by immunoblotting for levels of the indicated proteins. Similar results were obtained in two experiments. (C) Fold changes in the levels of the indicated proteins in BPA-treated CD11b⁺ cells as compared with vehicle-treated cells shown in the panels A were estimated in three independent experiments as described in Materials and method section. The SEM values are shown. The value in the vehicle-treated cells is indicated as 1. Significance levels assessed by the paired one-tailed *t*-test are **p*<0.05 and ***p*<0.01. (D) Human peripheral CD14⁺ cells from a healthy male donor were cultured as described in Methods. Cells were either left untreated (lane 1) or treated with 10 nM BPA for 14 h. After the treatment, cell lysates were analyzed by immunoblotting using antibodies that were specific to the indicated proteins. An upward arrow indicates an increase in levels of the indicated protein due to BPA treatment. Two experiments resulted in similar results and a representative experiment is shown.

C **Fig. 7.**

Activation of the NLRP3 inflammasome activity by BPA-induced signaling. (A) Bone marrow-derived purified CD11b⁺ cells from C57BL/6 female mice were either left untreated (lanes 1 and 2) or treated with BPA (10 nM) for 14 h under the culture conditions described in Methods. After the treatment, cells were primed with LPS (100 ng/ml) for 3 h. The "primed" cells were either left untreated (lanes 1 and 3) or treated with nigericin (15 μ g/ml) for 45 min. After the treatment, cell were lysed and total cell lysates containing equal amounts of proteins were analyzed by immunoblotting using antibodies specific to the

indicated proteins. (B) Fold changes in the levels of cellular levels of activated caspase-1 (p10) and the mature IL-1 β (p17) proteins in BPA-treated cells, as compared with vehicle-treated cells, that are shown in the panel A were estimated in three independent experiments as described in methods section. The SEM values are shown. The value in the vehicle-treated cells is indicated as 1. Significance levels assessed by the paired one-tailed *t*-test are **p*<0.05 and ***p*<0.01. NS, not significant. (C) Human monocytic THP-1 cells were treated with PMA (100 nM) for two days and the PMA-treated cells were either left untreated (lanes 1 and 2) or treated with BPA (10 nM) for 14 h under the culture conditions described in Methods. After the treatment, cells were primed with LPS (100 ng/ml) for 3 h. The "primed" cells were either left untreated (lanes 1 and 3) or treated with nigericin (15 μ g/ml) for 45 min. After the treatment, cell were lysed and total cell lysates containing equal amounts of proteins were analyzed by immunoblotting using antibodies specific to the indicated proteins. Fold changes (FC) in levels of the indicated proteins in response to BPA treatment of cells (as compared with vehicle treated cells) were estimated as described in methods section. An upward arrow indicates an increase in either constitutive and/or BPA-treatment induced levels of the indicated protein. Two repeats of the experiment gave similar results and results from a representative experiment are shown.

Fig. 8.

Proposed model for the potential role of BPA exposures in activation of the innate immune responses. Dysregulation of these responses plays a role in the development of autoimmunity.