Transmaternal Bisphenol A Exposure Accelerates Diabetes Type 1 Development in NOD Mice

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Diabetes mellitus type 1 is an autoimmune disease with a genetic predisposition that is triggered by environmental factors during early life. Epidemiological studies show that bisphenol A (BPA), an endocrine disruptor, has been detected in about 90% of all analyzed human urine samples. In this study, BPA was found to increase the severity of insulitis in the pancreatic islets of NOD mice offspring after transmaternal exposure through the dams’ drinking water (0, 0.1, 1, and 10 mg/l). Both the severity of insulitis in the pancreatic islets at 11 weeks of age and the diabetes prevalence at 20 weeks were significantly increased for female offspring in the highest exposure group compared to the control group. Increased numbers of apoptotic cells, a reduction in tissue resident macrophages and an increase in regulatory T cells were observed in islets prior to insulitis development in transmaternally exposed offspring. The detectable apoptotic cells were identified as mostly glucagon producing alpha-cells but also tissue resident macrophages and beta-cells. In the local (pancreatic) lymph node neither regulatory T cell nor NKT cell populations were affected by maternal BPA exposure. Maternal BPA exposure may have induced systemic immune changes in offspring, as evidenced by alterations in LPS- and ConA-induced cytokine secretion in splenocytes. In conclusion, transmaternal BPA exposure, in utero and through lactation, accelerated the spontaneous diabetes development in NOD mice. This acceleration appeared to be related to early life modulatory effects on the immune system, resulting in adverse effects later in life.

Key Words: bisphenol A; insulitis; diabetes mellitus type 1; NOD mice; developmental toxicity; immunotoxicity.

Most cases of diabetes mellitus type 1 (T1DM) develops in young people up to 18 years of age, and is seen to increase in many European countries (Patterson et al., 2009). T1DM is an autoimmune disease where pancreatic beta-cells are destroyed by autoreactive immune cells, resulting in insulin deficiency. The onset of the disease could be triggered by several environmental factors, such as intestinal viruses, vitamin D deficiency or foetal disturbances, acting together with a genetic predisposition, although there is a need for evidence to confirm the influence of these factors (Stene and Gale 2013). Also, some environmental pollutants like PCB, particulate air pollution, ozone, and sulphate have been reported to be associated with T1DM (Hathout et al., 2006; Langer et al., 2002; Longnecker et al., 2001), but experimental data supporting the causality is limited. Interestingly, there has also been a parallel occurrence of increased T1DM prevalence (Maahs et al., 2010) and an increased exposure burden of endocrine disruptors like Bisphenol A (BPA) (Global Industry Analysts 2013).

BPA is used in polycarbonate plastic for food and beverage containers and in epoxy resins in the lining of metal cans. Leaking of BPA into food and beverages is the major source of human BPA exposure. BPA is only biologically active in its conjugated form. However, the majority of BPA is glucuronidated in the liver and excreted into the urine within 24 h after exposure. Conjugated BPA has been detected in 90% of all analyzed human urine samples, indicating a widespread human exposure.

Epidemiological studies report associations between high urinary levels of BPA and obesity, diabetes type 2, heart disease, altered thyroid hormones, and allergic asthma (Carwile and Michaels 2011; Clayton et al., 2011; Donohue et al., 2013; Meeker and Ferguson 2011; Melzer et al., 2010; Shankar and Teppala 2011; Silver et al., 2011; Vaidya and Kulkarni 2012). Transmaternal BPA exposure has been reported to modulate the immune system by promoting asthma and allergy development in experimental mouse models (Midoro-Horiuti et al., 2010), as well as function as a thyroxine hormone receptor antagonist and transiently increase the serum thyroxine level in rat offspring (Zoeller et al., 2005). In addition, BPA appears to have a modulatory effect on macrophage activity, important for
viral infection defense and physiological clearance of apoptotic cells (Byun et al., 2005; Hong et al., 2004; Pyo et al., 2007; Yamashita et al., 2005). Interestingly, in vitro studies of rat primary pancreatic islets have suggested a direct effect of BPA on the insulin producing beta-cells including impaired mitochondrial function and altered morphology at concentrations as low as 100 nM (Song et al., 2012). Recently, we reported that long term BPA exposure starting at 4 weeks of age accelerated the spontaneous development of diabetes type 1 in NOD mice (Bodin et al., 2013). Insulitis can be seen as early as at 2 weeks of age in NOD mice (Diana et al., 2013) when the developing immune system is particularly vulnerable to environmental influences. We therefore hypothesized that a BPA-effect on the diabetes development would be further accelerated if the BPA exposure was initiated in early life through in utero and lactational exposures. In humans, BPA levels in amniotic fluid in the second and third trimesters as well as in the placenta are higher than levels in maternal blood and cord blood at birth, suggesting a high transmaternal burden of exposure (Edlow et al., 2012; Schönfelder et al., 2002).

The non obese diabetic NOD mouse, where 70%–80% of the female mice spontaneously develop diabetes type 1, is a model for human T1DM (Eizirik et al., 2009; Jansen et al., 1994; Lo et al., 2011; Melanitou et al., 2004). Specific features in this mouse model is the decrease in numbers or function of tissue resident macrophages, regulatory T-cells, NKT-cells and/or dendritic cells, which in turn will result in decreased clearance of apoptotic cells and increase the severity of the insulin development (D’Alise et al., 2011; Ehlers et al., 2012; Kadri et al., 2012; Lo et al., 2011; O’Brien et al., 1997, 2002, 2006; Subramanian et al., 2012; Tang et al., 2008; Yu et al., 2000).

In the present study, we investigated the influence of transmaternal BPA exposure on the diabetes onset and insulitis grade in NOD mice. BPA exposure via the maternal drinking water resulted in a transmaternal BPA exposure of the fetus during gestation and lactation that stopped at weaning. We hypothesized that BPA may interfere with the induction of beta-cell apoptosis and/or regulatory T cell and macrophage populations in the pancreatic islets and therefore we also determined the pancreatic number of these cells, as well as T-cell populations (T-helper cells, regulatory T cells, and NKT-cells) in the pancreatic lymph node. Systemic effects of transmaternal BPA exposure, like alterations in serum autoantibodies, cytokines, thyroxine levels, and splenocyte cytokine secretion and proliferation, were also determined. Our data contribute to the evidence suggesting a causal role for early life exposure to endocrine disruptors in the growing incidence of immune diseases.

MATERIAL AND METHODS

Mice and exposure conditions. One hundred twenty female (randomized into four groups) and 60 male non diabetic NOD/ShiLtJ mice from Jackson Laboratory (Maine) were used for breeding at the age of 10 weeks. The exposure to BPA via the drinking water started at the time of mating of the NOD mice and ended at weaning when the offspring were 3 weeks old. BPA (0.1, 1, or 10 mg/l) was dissolved in deionized autoclaved water heated to 60°C. Controls received similar water without BPA. BPA-free water bottles (Innovive, San Diego) were used and the water was changed once per week. Female offspring were kept on autoclaved deionized water from weaning until the end of the experiment and fed a diet with minimal content of phytoestrogens (2919X, Harlan Laboratories, Indianapolis). The mice had free access to food and water, and were exposed to a 12-h light/12-h dark cycle and 35%–75% humidity. Female siblings from each dam were separated into different cages. Blood glucose was continuously monitored from 7 to 28 weeks, serum auto-antibody analysis performed at 6, 9, and 13 weeks, and histological examination of pancreas performed in separate groups of mice after 7 and 11 weeks. The dams were kept as the statistical unit for all data.

Blood glucose measurements. For each BPA treatment and control group, 20 female offspring were monitored for blood glucose levels every week from 6 to 28 weeks of age. Blood glucose levels were determined in blood samples from the femoral vein using Accu-Chek (Roche Diagnostics, GmbH Mannheim, Germany). Mice were considered diabetic after two consecutive measurements within 24h with glucose levels above 13.9 mmol/l and euthanized.

Autoantibody detection by ELISA. Additional blood samples were collected from 20 offspring per group at 6, 9, and 13 weeks of age for analysis of autoantibodies in serum. Insulin autoantibodies were detected with Europolium-based ELISA as previously described (Bodin et al., 2013). In brief, each sample was analyzed before and after unspecific background binding to insulin (1 h incubation with insulin, 4 μg/ml I9278, Sigma–Aldrich, GmbH Steinheim, Germany) and the IgG1 insulin auto antibody was detected with rat anti-mouse IgG1-biotin diluted 1:5000 (Abcam, Cambridge, UK) followed by Europium labeled streptavidin. DELFIA enhancement solution was applied 10 min before reading the plates at a FLUOstar OPTIMA instrument (BMG LABTECH, Ortenberg, Germany). The unspecific insulin background binding was then subtracted for each sample.

GAD65 and HSP60 autoantibodies were detected with ELISA as described previously (Bodin et al., 2013), coated with 1 μg/ml recombinant human GAD65 (Merck, Uppsala, Sweden) or 2 μg/ml recombinant rat HSP60 (Enzo Life Sciences, Lausen, Switzerland) respectively over-night and subsequently blocked with 1% BSA for 2h. Serum samples were diluted 1:50 in 0.1% BSA, incubated on the coated plate over-night and detected with rat anti mouse IgG1-biotin (Abcam, Cambridge, UK, 1:3000) and streptavidin-HRP.

Serum thyroxine and testosterone measurements. Total thyroxine (T4) and testosterone in serum samples from 7 weeks old mice were determined with ELISA-kits from Calbiotech (Spring Valley, CA) and DRG Instruments GmbH (Marburg, Germany), respectively, according to the manufacturer’s recommendations.

Cytokine levels in serum. The concentrations of IP-10, MCP-1, IL-1β, TGF-β, IFNγ, and IL-10 in mouse serum of female offspring at 7 weeks of age were determined by Lumienx assay (duoPlex cytokine beads from Invitrogen on a BioPlex platform). The cytokines were detected with biotinylated secondary anti-mouse antibodies followed by streptavidin-RPE and analyzed on a Bio-Plex 200 system (Bio-Rad Laboratories, Hercules).

Histological evaluation of insulitis. Separate groups of offspring were used for the histological evaluation. At the age of 7 and 11 weeks, the pancreas was collected from 8 mice in each exposure group, fixed in formalin and embedded in paraffin. Pancreatic sections of 4 μm were stained with hematoxylin and eosin. For each mouse 6 sections of the pancreas were examined and all islets present in the sections (5–15 islets/section) were graded for insulitis according to the area of an islet infiltrated by lymphocytes. 0% infiltration = grade 0, perinsulitis and up to 10% infiltration = grade 1, 10%–49% infiltration = grade 2, 50%–74% infiltration = grade 3, and 75%–100% infiltration = grade 4. Since the NOD mice spontaneously develop insulitis, islets of all grades could be present in a section. For each section, an overall grade was assigned which corresponded to the highest grade detected in at least 2 islets.
Then, the final grade for a pancreas/mouse was set to the highest grade determined for the 6 analyzed sections.

**Immunohistochemistry.** Sections of the formalin fixed pancreas from 7 and 11 weeks old mice (n = 8) were stained over-night for foxp3 (regulatory T-cells, ebBioscience, San Diego, 1:50), F4/80 (macrophages, AbD Serotec, Oxford, UK, 1:50 and F4/80 from Abcam, Cambridge, UK, 1:10 for fluorescence and CD68 AbD Serotec, 1:300), active caspase-3 (apoptotic cells, Cell Signalling Technology, Beverly, MA, 1:800 for HRP detection and 1:100 for fluorescence), glucagon C-18 (alpha cells, Santa Cruz, Dallas, TX, 1:50) and insulin (beta-cells, Dako Glostrup, Denmark 1:6000) as previously described (Bodin et al., 2013). For each staining, the positive cells per islet and grade were counted in two pancreatic sections per mouse. The counts per islet were compared between the exposure groups according to the insulitis grade of each islet.

**Phenotyping of cells from pancreatic lymph node.** The draining lymph node of the pancreas was excised and dispersed into single cell suspensions as previously described (Hansen et al., 2011) prior to staining with CD4, CD19, CD25, foxp3, and CD49b (DX5) for NKT-cells (all from BD Bioscience, Franklin Lakes, NJ). The different T cell populations (T helper, regulatory T-cells, and NKT-cells) were determined by flow cytometric analyses (LSR II, BD Bioscience).

**Proliferation of and cytokine release from splenocytes.** To investigate possible systemic BPA-effects on immune functions, we measured cytokine secretion or proliferation in splenocytes isolated from exposed and control offspring. Splenocytes were prepared as described previously (Hansen et al., 2011). To investigate the functionality of the splenocytes, the cultures were stimulated for 48h with LPS to activate B-cells and macrophages (10 µg/ml, Sigma–Aldrich), ConA to activate T-cells (50 µg/ml, Sigma–Aldrich) or insulin to activate autoreactive T-cells (10 µg/ml, Sigma–Aldrich) for 48h. Proliferation was detected using incorporation of BrdU (10 µM, Roche Diagnostics, Indianapolis, IN) the last 24 hours of culture and detected with anti-BrdU-conjugate according to the manufacturer’s recommendations. Stimulations with LPS, ConA, or insulin are expected to reflect any alterations in immune function of the cells at the time of harvest (WHO, 1996).

In supernatants of splenocytes stimulated with LPS or ConA for 48h secreted cytokines (INFγ, TNFα, IL-1β), IL-2, IL-4, IL-6, IL-10, IL-13, and IL-17) were determined with cytometric bead array based assay (FlexSet, BD Bioscience, Franklin Lakes, NJ) and analyzed by flow cytometry.

**Statistical analysis.** Data are presented as means ± standard errors of the mean (SEM). Diabetes incidence data were analyzed by Cox regression analysis, while all other data sets were analyzed by one way analysis of variance (ANOVA). Post-hoc test using the Holm-Sidak method was performed to evaluate significant differences between the groups. For all analyses, p-values <.05 were considered statistically significant.

**RESULTS**

**BPA Exposure Levels**

The water intake of individually housed NOD dams had mean values of 6 ml/day during pregnancy and 9 ml/day during lactation, with similar levels for all treatment groups (data not shown). Thus, a pregnant mouse weighing 20g, drinking 6ml/day and exposed to 10 mg/l BPA in the drinking water had a BPA intake of 3000 µg/kg/day, while a lactating mouse of similar weight and dose level had an intake of 4500 µg/kg/day. Correspondingly, 1 and 0.1 mg/l BPA in drinking water gave a maternal intake level of 300 and 30 µg/kg/day, respectively.

**Diabetes Development**

At 11 weeks of age, transmaternal exposure to the highest BPA concentration of 10 mg/l caused a higher mean insulitis grade than in the control group, suggesting accelerated insulitis development (Fig. 1), whereas no effect of BPA treatment was seen on the insulitis grade at 7 weeks of age (data not shown). In agreement, a shift toward earlier time points for the diabetes onset was observed for the two highest exposed groups for the 28-week follow-up group, also indicating a tendency toward accelerated diabetes development. Finally, a significantly increased number of diabetic mice in the highest BPA exposure group was observed at ages above 20 weeks (Fig. 2). We did not observe any differences in the offspring number, sex distribution, or weight at 3 and 10 weeks of age due to transmaternal BPA exposure (data not shown).

**Cellular Infiltration in the Pancreas**

To study the mechanisms contributing to accelerated insulitis development, we analyzed the cell populations infiltrating the pancreas prior to insulitis or during early insulitis development, that is, in islets of grade 0 or 1. In female offspring of both 7 and 11 weeks of age, the numbers of F4/80-positive tissue resident macrophages were reduced in pancreatic islets prior to insulitis in the highest (BPA) exposure group (Fig. 3), while the numbers of regulatory T cells were increased in this exposure group during early insulitis development (Fig. 4). Furthermore, the number of activated caspase-3 positive apoptotic cells in pancreatic islets was increased prior to insulitis at both time points in a dose dependent manner (Fig. 5). Double staining with active caspase-3 and insulin, glucagon or F4/80 suggested that the apoptotic cell fraction consisted mostly of glucagon producing alpha-cells and to some extent also tissue resident macrophages and a few insulin producing beta-cells. Apoptotic (caspase-3 positive) pancreatic islet cells positive for insulin, glucagon or F4/80 were observed in all exposure groups. There were however, significantly higher numbers of double positive cells for all of the stainings in the higher BPA exposure groups (Fig. 6A), indicating overall more apoptotic cells in all populations analyzed (beta-cells, alpha-cells, and tissue specific macrophages) due to maternal BPA exposure. Figures 6B–D displays representative images of the different cell populations in a pancreatic islet of an animal exposed to the highest dose of BPA.

**Lymph Node Cell Composition and Splenocyte Activity**

Phenotyping of pancreatic lymph node cells and determination of functional changes in splenocytes were performed to identify possible immune modulating effects of BPA. Except for a reduced proportion of B cells in dams in the low BPA exposure group (0.1 mg/l), the phenotype analysis of the splenocytes and lymph node cells from dams and offspring revealed no differences in the proportion of B cells, T-cells, T helper cells, regulatory T cells, and NKT-cells at 7 and 11 weeks of
However, the LPS-induced secretion of IL-2, IL-10, and IL-17 from splenocytes was increased in offspring in the highest BPA exposure group (Figs. 7A–C). Furthermore at 7 weeks, the IL-2 secretion from ConA stimulated splenocytes was significantly reduced in offspring from the 1 and 10 mg/l BPA exposure groups compared to control (Fig. 7D). There were no differences in the LPS, ConA, or insulin-induced proliferation between any of the groups of isolated splenocytes (data not shown).

**Serum Levels of Autoantibodies, Cytokines, Thyroxine and Testosterone**

Transmaternal BPA exposure did not influence the cytokine or thyroxine levels in serum in offspring at 7 weeks of age (in any of the exposure groups). Likewise, the autoantibodies against insulin, GAD65, and HSP60 in serum did not differ between the groups in serum samples from 6, 9, and 13 weeks of age (data not shown). Serum levels of testosterone at 11 weeks of age were not significantly different between the treatment groups (data not shown).

**DISCUSSION**

In this study, transmaternal exposure to BPA, that is, exposure in utero and via milk, accelerated the insulitis and spontaneous diabetes type 1 development in adult female NOD mice. This was accompanied by increased numbers of apoptotic cells, decreased numbers of tissue resident macrophages and
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Increased numbers of regulatory T cells in pancreatic islets prior to or during early insulitis. The altered cytokine release from offspring splenocytes after ex vivo LPS or ConA stimulation also indicate that transmaternal BPA exposure induced immune modulatory effects in the offspring lasting to adulthood.

Local Immune Effects in Pancreas

Apoptosis in the endocrine pancreas is the underlying mechanism of insulitis, and apoptosis of both beta-cells and macrophages have been reported during insulitis development (O’Brien et al., 1996, 1997). The increase in the number of apoptotic cells in the pancreas observed in this study, including mostly alpha-cells, but also some beta-cells and macrophages, may possibly attract more immune cells thereby resulting in a higher insulitis grade. Regulation of alpha-cell secretion of glucagon is often impaired in T1DM, in both NOD mice and humans, leading to hyperglucagonemia (Ohneda et al., 1984; Taborsky et al., 2009; Welzen-Coppens et al., 2013). According to our results and previous studies, apoptotic beta-cells are difficult to detect in the pancreatic islets, most likely due to a very fast turn over (Bodin et al., 2013; Reddy et al., 2003).

Increased apoptosis of tissue resident macrophages may explain the decreased numbers of the macrophages in BPA-exposed groups. In NOD mice, the function of macrophages is genetically impaired (O’Brien et al., 2002, 2006), thus the BPA-induced reduction of this cell population is likely to worsen the already impaired clearance, thereby accelerating the insulitis development and increase the number of detectable apoptotic cells in the pancreas. The tissue resident macrophages also control the proliferation of T-cells (Fu et al., 2012; Parsa et al., 2012), thus another possible consequence of reduced macrophage numbers is impaired regulation of T-cell proliferation. This may indirectly influence the insulitis development, with a hypothetically higher amount of cytotoxic T-cells in the pancreatic islets.

The mechanism of BPA-induced apoptosis in pancreatic cells (beta-cells, alpha-cells, and tissue specific macrophages) could be either direct, or indirect via systemic alterations on the developing immune system. In a recent in vitro study, BPA exposure induced apoptosis via suppressed Bcl-2 (anti-apoptotic) nodule formation in RAW 264.7 murine macrophage cells (Hwang et al., 2013). Transmaternal BPA exposure could possibly have a similar direct effect on pancreatic cells in particular or on macrophages in general. BPA has also been shown to induce reactive oxygen species (ROS) production in hepatocytes, causing endoplasmatic reticulum (ER) stress-induced apoptosis (Asahi et al., 2010). With regard to indirect effects, alterations in the developing immune system such as impaired macrophage clearance of apoptotic cells in general would lead to higher number of apoptotic cells in the pancreatic sections, reflecting a possible systemic effect of BPA exposure. Indeed, this could explain the increased number of both apoptotic beta-cells, alpha-cells, and tissue specific macrophages present prior to insulitis in the higher BPA exposure groups.

In the group with the highest transmaternal BPA-exposure, the number of pancreatic regulatory T cells in early insulitis development was increased compared to control. We speculate that this cell population is either increased due to a higher lymphocyte infiltration grade due to insulitis progression or is not functioning satisfactory in regulating the auto-reactive T-cells.
and is therefore up-regulated in numbers. Unfortunately the functionality of the regulatory T cells could not be determined in our analyses. The increased number of apoptotic cells and regulatory T cells together with decreased numbers of tissue resident macrophages in islets prior to or during early insulitis, suggests that BPA caused immune modulatory effects and/or possibly toxic effects on alpha-cells and tissue resident macrophages rather than direct effects on the insulin producing beta-cells.

A deficiency in the NKT-cell population in local lymph node has been suggested to contribute to the diabetes development in NOD mice (Sharif et al., 2002; Wang et al., 2001). There was, however, no significant effect of BPA on the total T-cell population or specifically the regulatory T cells or NKT-cell population in the pancreatic lymph nodes in dams or female offspring at 7 or 11 weeks of age. This lack of effect may be due to the timing and we cannot exclude that a transient alteration of the cell populations might have occurred at an earlier time point as previously reported (Welzen-Coppens et al., 2013). Further studies in younger mice could reveal if maternal BPA exposure results in transient effects on local lymph node cells. One should also take into account that the cell proportion obtained by phenotyping is a relatively rough measure that might not detect subtle but biologically significant changes in cell sub-populations.

**Systemic Immune Effects**

The observed functional changes in splenocytes, with altered cytokine secretion after ex vivo stimulation with LPS or ConA, suggest that transmaternal BPA exposure may have a modulating effect on the developing immune system. An increased cytokine production could either be due to increased levels of the cytokine producing cells in the spleen, or a modulation of the cellular function. Increased numbers of the various cell types could reflect a higher proliferation rate, although increased proliferation was not detected upon ConA, LPS, or insulin stimulation of splenocytes from offspring transmaternally exposed to BPA. Overall, early-life BPA exposure appears to systemically modulate the function of immune cells.

**FIG. 3.** A, Mean number of tissue resident macrophages (F4/80 positive cells) in pancreatic islets of grade 0 from 7 and 11 weeks old female NOD mice, all islets from 2 sections per pancreas were analyzed (group mean ± SEM, n = 8). *Significant differences from the control group. B, Representative pictures of immunostaining of resident macrophages (F4/80, brown color) in pancreatic islets with insulitis grade 0 in sections of formalin fixed pancreas from 7 weeks old NOD mice. Scale bars = 40 µm.
In accordance with our previous study in NOD mice, the levels of autoantibodies and cytokines in serum did not differ between the exposure groups (Bodin et al., 2013). In our previous study, the BPA exposure at 4 weeks of age and we hypothesized that this was too late to influence the autoantibody levels. Similarly, the present finding of a lack of effect on autoantibody levels even after transmaternal BPA exposures, suggests that the already disputed presence of autoantibodies in the NOD mice (Bodin et al., 2013; Inoue et al., 2007) is not involved in the BPA induced effects on diabetes development.

Epidemiological studies have shown associations between elevated urinary and serum BPA levels and altered serum hormone levels, like decreased thyroxine and testosterone levels (Meeker and Ferguson, 2011; Zhou et al., 2013). Thyroxine has also been shown to reduce macrophage activity (De et al., 2011). In this study, we found no significant differences in serum thyroxine levels between the groups. Although BPA exposure showed a tendency to increase the thyroxine levels in our previous study, results in other studies of transmaternal BPA exposure in mice and serum thyroxine levels are diverging, showing increased levels as well as no difference (Bodin et al., 2013; Kobayashi et al., 2005; Zoeller et al., 2005). Interestingly, it has been shown that the testosterone level in the female NOD mouse is negatively correlated with the development of T1DM (Markle et al., 2013). We did not find any significant differences in serum testosterone levels after transmaternal BPA exposure. Thus with no effects on serum auto-antibodies or hormones, the observed systemic effects of transmaternal BPA exposure in our study suggest a mechanism of modulation of immune cell function.

**Implications of Early Life Exposures**

Alterations in immune cell populations in the pancreatic islets prior to or during early insulitis development together with altered ex vivo splenocyte cytokine secretion imply modulation of the immune response to transmaternal BPA exposure. This is in agreement with developmental immunomodulating effects of BPA in other immune disease models.
Bodin et al. (2013). Underlying immune dysfunctions may be manifested as different immune disease patterns (Dietert et al., 2010). In particular, T1DM has been suggested to be an entryway for other autoimmune-related diseases such as celiac disease, autoimmune thyroiditis, atherosclerosis, multiple sclerosis as well as hearing loss (Dietert and Zelikoff, 2010). Rapidly progressing hearing loss is another known phenotypic manifestation in NOD mice (Johnson and Zheng, 2002; Ohlemiller et al., 2008). Thus, our data support a call for further studies of BPA as a developmental immunotoxicant.

**Transmaternal Versus Long-term BPA Exposure**

In our previous study with continuous BPA exposure of mice from 4 weeks of age, accelerated T1DM development was observed at a concentration of 1 mg/l (Bodin et al., 2013). Presently, significant effects were only observed in offspring in the 10 mg/l exposure group; although some of the immune parameters also appeared to be affected in offspring from the 1 mg/l BPA exposure group. The foetal BPA exposure level has been reported to be similar to or lower than the blood level of unconjugated BPA in the dams, which is less than 1% of the maternally ingested BPA (Chou et al., 2011; Kosarac et al., 2012; Lee et al., 2008; Schönfelder et al., 2002; Unal et al., 2012; Zhang et al., 2013). The exposure through lactation is about one tenth of the unconjugated serum BPA levels (Ye et al., 2006). This implies that the total BPA exposure presently was considerably lower for offspring than for dams. One possible explanation for the lack of accelerated diabetes development at 1 mg/l could therefore be that the transmaternal exposure was lower than the direct BPA exposure from the drinking water applied in our previous long term study. However, we cannot exclude the possibility that a continuous low BPA intake after weaning, similar to a predicted human BPA exposure pattern.

**FIG. 5.**  A, Mean number of active caspase-3 positive cells in pancreatic islets with insulitis grade 0 from female NOD mice at 7 and 11 weeks of age, all islets from 2 sections per pancreas were analyzed (group mean ± SEM, n = 8). *Significant differences from the control group. B, Representative pictures of immunostaining of active caspase-3 positive cells (brown colour) in islets of Langerhans in sections of formalin fixed pancreas from 11 weeks old NOD mice. Scale bars = 40 μm.
FIG. 6.  A. Mean number of active caspase-3 and insulin, glucagon or F4/80 double positive cells in pancreatic islets with insulitis grade 0 from female NOD mice at 11 weeks of age. Representative immunostaining pictures of cells positive for active-caspase-3 (red color) and insulin (green color) panel (B), glucagon panel (C) and tissue resident macrophages (F4/80) panel (D) in NOD mouse pancreas islets, in sections from 11 weeks old mice transmaternally exposed to 10 mg/l BPA. Blue color represents Hoechst nuclear staining. Scale bars = 40 µm.
Throughout life, might have further effects on the diabetes development.

Relevance to Human BPA Exposures

Since the pharmacokinetics of BPA is similar in humans and mice, it is relevant to compare intake and serum levels between the species (Doerge et al., 2011; Fisher et al., 2011; Taylor et al., 2011). The predicted daily intake of BPA in humans through food is estimated to be 1.5 µg/kg/day for adults (EFSA—European Food Safety Authority, 2006), and unconjugated BPA levels between 0.3 and 4 ng/ml have been measured in human serum. However, it has been suggested that a relatively high oral dose of about 500 µg/kg body weight is required to obtain the reported human serum levels, implying that several additional sources such as smoking and dermal exposures may contribute to the total intake (Taylor et al., 2011; Vandenberg et al., 2007, 2010). Interestingly, rats exposed to 250 µg BPA/kg/day by a subcutaneous pump displayed serum levels of unconjugated BPA equivalent to previously reported human levels (Acevedo et al., 2013).

The 10 mg/l maternal BPA dose, causing effects without further exposure after weaning, corresponds to 3000 µg/kg/day in dams during pregnancy; a dose 60 times higher than the current tolerable daily intake of 50 µg/kg/day determined by EFSA (EFSA—European Food Safety Authority, 2006). However, one could hypothesize that exposure to BPA for an extended period of time after weaning could result in accelerated T1DM development at lower BPA concentrations. In this context, the effects observed in our previous study after direct exposure to 1 mg/l from 4 weeks of age (Bodin et al., 2013), could add to the trend observed presently in some parameters at 1 mg/l, corresponding to 6 times TDI for the lactating dams. Overall, the BPA concentrations showing effects in the present study are likely to be higher than those obtained through environmental...
exposure. There is however still a need for a better characterization of human BPA exposure, and also of the relationship to the measured serum levels of BPA.

In conclusion, we show in this study that foetal and early life exposure to BPA has a small but significant effect on the developing immune system, leading to an acceleration of the spontaneous T1DM development in adult NOD mice. Together with our previous study, the present data in NOD mice suggest associations between BPA exposure and T1DM development. It would therefore be interesting to investigate associations between BPA exposure and T1DM in epidemiological birth cohort studies.

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