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Exposure to bisphenol A, but not phthalates, increases spontaneous diabetes type 1 development in NOD mice

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ABSTRACT

Type 1 diabetes mellitus (T1DM) is an autoimmune destruction of insulin producing pancreatic beta-cells due to a genetic predisposition and can be triggered by environmental factors. We have previously shown that bisphenol A (BPA) accelerates the spontaneous development of diabetes in non-obese diabetic (NOD) mice. Here, we hypothesized that oral exposure to a mixture of the endocrine disruptors BPA and phthalates, relevant for human exposure, would accelerate diabetes development compared to BPA alone. NOD mice were exposed to BPA (1 mg/l), a mixture of phthalates (DEHP 1 mg/l, DBP 0.2 mg/l, BBP 10 mg/l and DiBP 20 mg/l) or a combination of BPA and the phthalate mixture through drinking water from conception and throughout life. Previous observations that BPA exposure increased the prevalence of diabetes and insulitis and decreased the number of tissue resident macrophages in pancreas were confirmed, and extended by demonstrating that BPA exposure also impaired the phagocytic activity of peritoneal macrophages. None of these effects were observed after phthalate exposure alone. The phthalate exposure in combination with BPA seemed to dampen the BPA effects on macrophage number and function as well as diabetes development, but not insulitis development. Exposure to BPA alone or in combination with phthalates decreased cytokine release (TNFα, IL-6, IL-10, IFNγ, IL-4) from *in vitro* stimulated splenocytes and lymph node cells, indicating systemic changes in immune function. In conclusion, exposure to BPA, but not to phthalates or mixed exposure to BPA and phthalates, accelerated diabetes development in NOD mice, apparently in part *via* systemic immune alterations including decreased macrophage function.

1. Introduction

Diabetes mellitus type 1 (T1DM) is an autoimmune disease where pancreatic beta-cells are destroyed by autoreactive immune cells, resulting in insulin deficiency. During the last decades, the incidence has increased in many European countries [1]. T1DM develops on a predisposing genetic background, and can be triggered by

Abbreviations: BPA, bisphenol A; DEHP, bis(2-ethylhexyl) benzene-1,2-dicarboxylate; DBP, dibutyl phthalate; BBP, butylbenzyl phthalate; DiBP, diisobutyl phthalate.

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several environmental factors. Examples of triggering environmental factors are intestinal viruses and vitamin D deficiency or possible disturbances in the development of the foetal immune system [2]. Some evidence of associations between T1DM and environmental pollutants like PCBs, ozone and sulphate, has been reported in epidemiological studies [35,3,4].

Noteworthy, an increasing human exposure burden of endocrine disruptors like bisphenol A (BPA) and phthalates has been reported [5] in the same time frame as the increased T1DM prevalence [6].

BPA is used in the production of polycarbonate plastic bottles and food containers as well as epoxy resins in the lining of metal cans. BPA leaking into food and beverages is the major human BPA exposure. BPA has been detected in over 90% of all analyzed human urine samples, indicating a widespread human exposure to BPA [7]. Ingested BPA is rapidly conjugated in the liver and is excreted in the urine within 24 h. BPA is only biologically active in its unconjugated form.

Phthalates are used as plasticizers and found in a variety of consumer products. Phthalates are also rapidly metabolized and secreted *via* urine. For many phthalates, including diethylhexyl phthalate (DEHP), dibutyl phthalate (DBP), butylbenzyl phthalate (BBP) and di-i-butyl phthalate (DiBP), ingestion seems to be the main exposure route in humans [8]. As for BPA, phthalate metabolites are widely detected in urine, confirming a widespread exposure [9,10]. However, both the phthalate parent form and the metabolites may be biologically active [11].

Exposure to endocrine disruptors like phthalates and BPA has not been studied in relation to development of T1DM in epidemiological studies, most likely because such studies are hampered by the low incidence in humans making prospective studies particularly difficult. However, T1DM is an immunological disease, and in epidemiological studies exposure to both phthalates and BPA has been associated with negative effects on or related to the immune system, such as asthma, allergies, altered levels of thyroid hormones, and inflammatory diseases like diabetes type 2 and cardiovascular disease [11–23].

BPA exposure has also been reported to modulate the immune system in animal studies, by promoting asthma and allergy development in experimental mouse models [24], as well as having a modulatory effect on macrophage activity [25–27,56]. Similarly, phthalates increased inflammation in a murine peritonitis model, inhibited alveolar macrophage killing of bacteria, and reduced phagocytic activity, suggesting that phthalates may impair macrophage functionality [28,29,32]. *In vitro* studies also report that phthalates can induce differentiation [30] and increased apoptosis in RAW264.7 macrophages [31,32].

Some experimental studies have assessed the impact of BPA on diabetes development and cellular endpoints relevant for diabetes, but type 2 diabetes has received most attention. Impaired mitochondrial function and altered morphology of the insulin producing beta-cells has been reported in BPA exposed rat primary pancreatic islets *in vitro* [33]. We have previously reported that both long term BPA exposure starting at 4 weeks of age continuing through-out life and exposure during gestation and lactation only, accelerated the spontaneous development of T1DM in NOD mice [34–36]. However, a possible impact of phthalates on T1DM development has not been studied previously.

In the present study, we examine the effects of BPA (1 mg/l), a mixture of phthalates (DEHP 1 mg/l, DBP 0.2 mg/l, BBP 10 mg/l and DiBP 20 mg/l) or a combination of BPA and the phthalate mixture on T1DM development in the NOD mice, after exposure through drinking water from conception and throughout life of the offspring. We hypothesized that oral exposure to a combination of BPA and phthalates, relevant for human mixed exposures, could give a more rapid acceleration of the diabetes development than BPA alone. In addition, we further explore the role of macrophages and systemic effects induced by environmental chemicals in the diabetes development in the NOD mouse model.

2. Materials and methods

2.1. Mice and exposure conditions

120 female (randomized into four groups) and 60 male non diabetic NOD/ShiLtJ mice from Jackson Laboratory (Maine, USA) were used for breeding at 8 and 10 weeks of age, respectively. The exposure to BPA and/or phthalates *via* drinking water started at the time of mating of the mice and continued throughout the life time of the female offspring. The 4 exposure groups included: (1) negative control (water only), (2) phthalates: DEHP 1 mg/l, DBP 0.2 mg/l, BBP 10 mg/l and DiBP 20 mg/l, (3) BPA 1 mg/l, and (4) BPA 1 mg/l + DEHP 1 mg/l, DBP 0.2 mg/l, BBP 10 mg/l and DiBP 20 mg/l. The exposure level of each chemical was chosen at a dose corresponding to 3 × tolerable daily intake (TDI) for the respective chemical, since BPA at this exposure level has previously been shown to affect T1DM development in NOD mice [36].

Only female offspring were selected at the time of weaning, since insulitis and diabetes development is most prevalent in female mice [37]. The endocrine disruptors (EDs) were dissolved in deionized autoclaved water heated to 60 °C. Controls received similar water without EDs. BPA-free water bottles, made of 100% polyethylene terephthalate (PET) that does not require the use of phthalates or other softening additives (Innovive, San Diego, USA) were used and the water was changed once every week. Also cages were BPA-free and with minimal leakage of phthalates (100% PET polyethylene terephthalate plastic). The diet contained minimal levels of phytoestrogens (2919X, Harlan Laboratories, Indianapolis, USA) and the mice had free access to feed and water. The mice were exposed to a 12-h light/12-h dark cycle and 35–75% humidity. To keep the dams as the statistical unit for all data, female siblings from each dam (approximately 3–4 females/dam) were separated into different cages, and divided into 4 sub-groups; (i) 25 female offspring were used to monitor diabetes development by weekly measurements of blood glucose from 6 to 35 weeks of age, and blood samples were collected at 6 and 10 weeks of age, (ii and iii) two separate subgroups, each containing 8 offspring, were used for histological examination of pancreas, *ex vivo* assessment

of phagocytic function of isolated peritoneal macrophages and isolation of splenocytes and pancreatic lymph node cells at 7 and 11 weeks of age, respectively, and (iv) a subgroup of 4 offspring was used for isolation of pancreatic islets at 8 weeks of age, for *ex vivo* assessment of cytokine induced apoptosis induction. All experiments were performed in conformity with the laws and regulations for experiments with live animals and were approved by the local representative of the Norwegian Animal Research Authority.

2.2. Blood glucose measurements

In the diabetes development sub-group, all mice were monitored for blood glucose levels every week from 6 to 35 weeks of age, whereas for the two histology sub-groups, blood glucose levels were only determined once before euthanizing at the age of 7 or 11 weeks of age. Blood glucose levels were determined in blood samples from the femoral vein using Accu-Check (Roche Diagnostics, GmbH Mannheim, Germany). Mice were considered diabetic after two consecutive measurements within 24 h with glucose levels above 13.9 mmol/l and were immediately euthanized.

2.3. Serum insulin and testosterone measurements

Since testosterone has been shown to protect against diabetes development in NOD mice [38] the serum testosterone levels were analyzed in blood samples from 10 weeks old NOD mice. Further, elevated insulin secretion may also be protective against diabetes in the NOD mouse and BPA has been shown to induce insulin secretion in beta cells [39,45]. Therefore, the non-fasting serum insulin levels were also determined at week 10. Serum insulin and testosterone concentrations were determined with ELISA-kits from Mercodia (Uppsala, Sweden) and DRG Instruments GmbH (Marburg, Germany), respectively, according to the manufacturer's recommendations.

2.4. Histological evaluation

For histological evaluation, pancreata were collected from 8 mice (at 7 and 11 weeks of age), fixed in formalin, embedded in paraffin and processed as described previously before haematoxylin and eosin staining [34,35]. For each mouse 6 sections at different depth of the pancreas were examined and all islets present in the sections (10–15 islets/section) were graded for insulitis according to the area of an islet infiltrated by lymphocytes. 0% infiltration = grade 0, periinsulitis and up to 10% infiltration = grade 1, 10–49% infiltration = grade 2, 50–74% infiltration = grade 3 and 75–100% infiltration = grade 4, as previously illustrated [36]. For each section, an overall grade was assigned which corresponded to the highest grade detected in at least 3 islets. Then, the final grade for a pancreas/mouse was set to the highest grade determined for the 6 analyzed sections. The mean insulitis grade for each exposure group corresponds to the mean of the final pancreas grade for each mouse.

Sections of the formalin fixed pancreas were also stained over-night with antibodies towards Foxp3 (regulatory T-cells, eBioscience, San Diego, USA, 1:50), F4/80 (tissue resident macrophages, AbD Serotec, Oxford, UK, 1:50) and CD68 (invasive macrophages, AbD Serotec, 1:300), and active caspase-3 (apoptotic cells, Cell Signalling Technology, Beverly, MA, USA, 1:400), as previously described [36]. For each antibody staining and insulitis grade, the number of positive cells per islet was counted in two pancreatic sections per mouse. The counts per islet were compared between the exposure groups within each insulitis grade with a particular focus on grade 0 islets to investigate the effects of exposure on initial events in diabetes development.

2.5. Phagocytic function of peritoneal macrophages

To investigate if the exposures affected the macrophage functionality, the phagocytic activity of peritoneal macrophages was investigated by flow cytometry. Peritoneal macrophages were isolated from mice at 7 and 11 weeks of age ($n = 8$) by peritoneal lavage. The cell suspension was centrifuged at $250 \times g$ for 10 min and erythrocytes were removed by dissolving the cell pellet in 0.2% NaCl on ice for 4 min. The cell concentration was determined and cells were seeded at 2×10^6 cells/ml in 48 wells plates with RPMI cell culture medium. After 1 h of culture 85–95% of the attached cells are assumed to be macrophages [40]. Then the medium with unattached cells was discarded, replaced with fresh medium, and the cells were incubated over night. To assess the phagocytic function, FITC-labelled Zymosan particles were added at a ratio 20 particles per cell (In vitrogen Life Technologies, Carlsbad, CA, United States) incubated for 30 min at 37 °C, washed twice in PBS and incubated with Accutase (In vitrogen Life Technologies) for at least 15 min to remove particles from cell surfaces and to detach cells. The cells were washed twice with PBS, fixed in 0.2% paraformaldehyde in cell culture medium and analyzed for phagocytosis (FITC intensity per cell) by flow cytometry (LSR II, BD Bioscience, Franklin Lakes, NJ, USA).

2.6. Insulin secretion and NO production in isolated pancreatic islets

Pancreatic islets were isolated from 8 weeks old NOD mice ($n = 5$) as previously described [41] using injection of collagenase in the porta vein of euthanized animals before excision of the pancreas. Further dispersion of whole islets was performed in 37 °C collagenase for 17 min, after which islets were washed and thereafter hand-picked with pipette from a petri dish using a microscope. Glucose-induced insulin secretion was determined for 10 islets of equal size per mice as described previously [42]. Briefly, islets were incubated in glucose free medium in 48 wells plates for 30 min before a 60 min stimulation with either 6 or 16 mM glucose. Supernatants were harvested for insulin determination according to manufacturer's recommendation (ELISA, Mercodia) and islets were frozen for analysis of protein content.

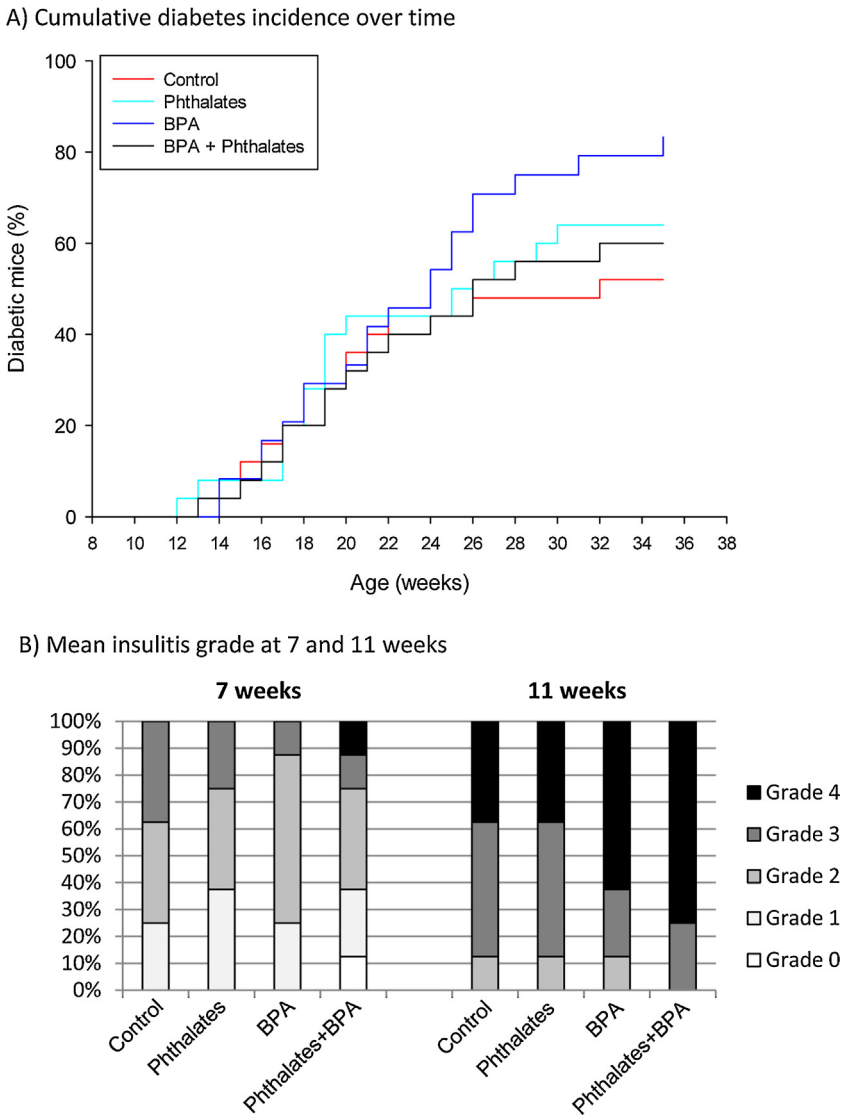

Fig. 1. (A) Spontaneous cumulative diabetes incidence *i.e.* a glucose level above threshold for diabetes in these mice (13.9 mmol/l), shown as percentage of diabetic female offspring of NOD mice exposed to BPA and phthalate in the drinking water from before birth and throughout life ($n = 25$). The blue line represents animals receiving 1 mg/l BPA exposure, the turquoise line the phthalate exposed mice (DEHP 1 mg/l, DBP 0.2 mg/l, BBP 10 mg/l and DiBP 20 mg/l corresponding to 3 times tolerable daily intake for each chemical), the black line the combination of BPA and phthalates and the red line resembles the control group. (B) Histological evaluation of insulitis grade in pancreatic islets from 7 and 11 weeks old female NOD offspring after continuous BPA and phthalate exposure. Formalin fixed pancreas were sectioned and stained with haematoxylin–eosin and evaluated for severity of lymphocyte infiltration using light microscopy ($n = 8$). Grade 0 = no infiltration, grade 1 = periinsulitis <10% infiltration, grade 2 = 10–49% infiltration, grade 3 = 50–74% infiltration, and grade 4 = 75–100% infiltration. The insulitis grade per pancreas was set to the highest grade observed in 3 or more islets after grading all islets in 3 sections from different depths (5–15 islets per section), and presented as percentage of mice within each insulitis grade.

NO production was measured using the Griess assay (Sigma–Aldrich) according to manufacturer's instructions.

2.7. Isolation of beta-cells and islet macrophages, analysis of cytokine induced apoptosis

To investigate the cellular sensitivity to cytokine-induced apoptosis [43], 60 isolated pancreatic islets from 4 offspring per exposure group were incubated for 48 h and stimulated with 50 U/ml rm-IL-1β, 1000 U/ml rm-INFγ and 1000 U/ml rm-TNFα. The islets were then moved to 1 ml calcium free HEPES-based beta-cell isolation solution and incubated for 12 min at 37 °C, before 2 ml RPMI medium (5% FCS) was added and the islets were dispersed by harsh pipetting. The cell suspension were washed and resuspended in binding buffer (BD Biosciences, Franklin Lakes, NJ, USA) stained for PE-annexin V (apoptotic cells, BD Biosciences), PI 7-AAD (necrotic cells, BD Biosciences) and APC-F4/80 (macrophages, eBioscience). Cells were fixed and permeabilized for intracellular pacific blue-insulin (beta cells, Cell Signalling Technology, Beverly, MA,

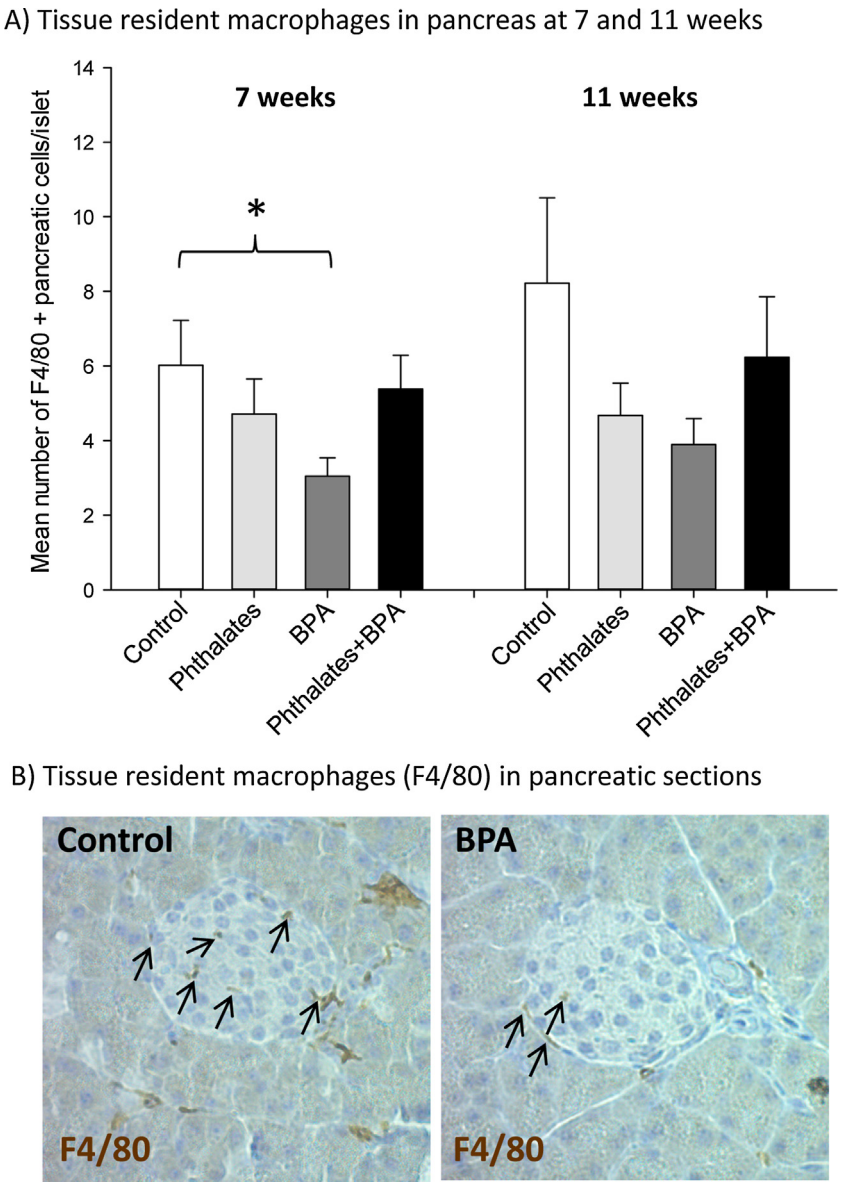

Fig. 2. Immunohistochemical staining of sections of formalin fixed pancreas in female NOD offspring after continuous BPA and phthalate exposure. (A) Mean number of tissue resident macrophages (F4/80 positive cells) in pancreatic islets of grade 0 from 7 and 11 weeks old mice. (B) Representative pictures of immunocytochemical staining of resident F4/80-macrophages (arrow indicating the brown colour of F4/80 positive cells) in pancreatic islets with insulitis grade 0, from 7 weeks old control and BPA exposed mice. All islets from 2 sections per pancreas were analyzed (group mean ± SEM, $n = 8$). *Significant differences from the control group, $p < 0.05$.

USA) staining. Flow cytometry was applied to determine the percentage of apoptotic and necrotic beta cells and apoptotic macrophages originating from the pancreatic islets challenged with cytokines *in vitro*.

2.8. Cytokine release in ex vivo stimulated splenocytes and pancreatic lymphocytes

Splenocytes and pancreatic lymph node cells were isolated at 7 and 11 weeks of age ($n = 8$) as previously described [34,35]. Subsequently, cytokine secretion upon *ex vivo* stimulation for 48 h with LPS or ConA was determined for all exposure groups in splenocyte cell cultures and with LPS in pancreatic lymph node cell and islet cultures, to examine possible effects on systemic immune functions, performed as previously described [34,35,44]. Stimulation with LPS activates B-cells and macrophages (10 μg/ml, Sigma–Aldrich, GmbH Steinheim, Germany), while ConA stimulation for 48 h activates T-cells (50 μg/ml, Sigma–Aldrich). The cytokine levels (INFγ, TNFα, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13 and IL-17) in the supernatants were determined by cytometric bead based array (FlexSet, BD Bioscience) analyzed on a LSRII flow cytometer (BD Bioscience).

2.9. Statistical analysis

Data are presented as group 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). The Holm–Sidiak *post hoc* test was performed to evaluate significant differences between the groups. For all analyses, p-values <0.05 were considered statistically significant.

3. Results

3.1. Diabetes development

In the evaluation of diabetes development, mice with sustained elevated blood glucose levels, at 13.9 mmol/l or above, at two measurements within 24 h were considered diabetic. The spontaneous diabetes development started from 12 to 14 weeks of age in NOD mice for all exposure groups (Fig. 1A). The diabetes incidence in the BPA treated group seemed to be increased from 25 weeks of age compared to the control group. However, cox regression analysis did not confirm statistical differences between the exposure groups. Phthalate exposure or the combination of BPA and phthalates did not affect diabetes development to the same extent as BPA alone. In the histological evaluation of insulitis, there were no significant differences in the mean insulitis grade between the exposure groups at 7 or 11 weeks of age (Fig. 1B). However, the percentage of mice with a final insulitis grade of 4 at 11 weeks of age were 62.5% and 75% in the BPA and the combined BPA and phthalate exposure groups, respectively, compared to 37.5% in the control group (Fig. 1B), suggesting an accelerated insulitis development in these two groups. The number of offspring (in average about 8 per dam), sex distribution (mean 50% females), body weight (mean 11, 23 and 29 g at age 3, 11 and 20 weeks respectively) or water intake at 10 and 20 weeks of age (typically 3 and 3.5 ml per animal per day, respectively) were not affected by any of the exposures. Likewise, the non-fasting serum levels of insulin or testosterone did not differ between the exposure groups (data not shown).

3.2. Cellular infiltration in the pancreatic islets

Prior to insulitis (*i.e.* in islets with insulitis grade 0), the number of F4/80+ tissue resident macrophages was significantly reduced in the BPA exposed group at 7 weeks of age compared to control (Fig. 2). The same trend was observed at 11 weeks of age, although not statistically significant. In contrast, exposure to phthalates or the combination of BPA and phthalates, did not reduce the number of tissue resident macrophages significantly compared to control prior to insulitis at 7 or at 11 weeks of age. That is, all of the exposure groups except BPA alone having about 5 tissue resident macrophages per section of islet at grade 0 at 7 weeks of age (Fig. 2A). However, the number of apoptotic cells, evaluated by the number of active caspase-3 positive cells, was increased both in the BPA and the combined BPA and phthalates exposure groups (Fig. 3) compared to the control at 11 weeks of age. A similar trend was observed after 7 weeks of age after BPA exposure, with increased

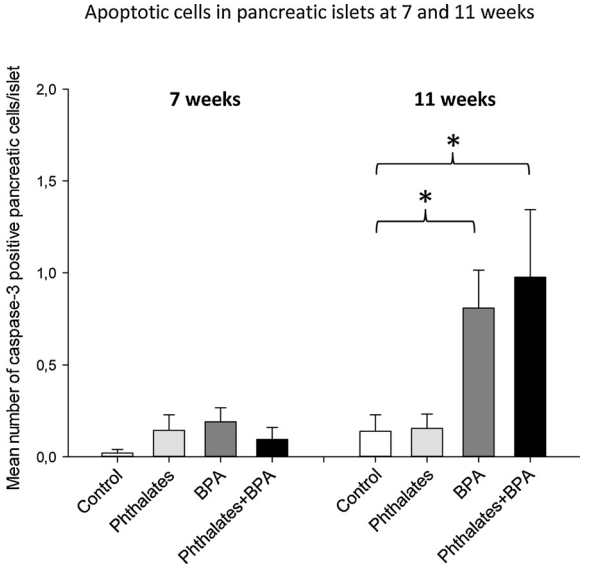

Fig. 3. Immunohistochemical results from sections of formalin fixed pancreas in female NOD offspring after continuous BPA and phthalate exposure. Mean number of apoptotic cells (active caspasae-3 positive cells) per pancreatic islet of grade 0 at 7 and 11 weeks of age. All islets from 2 sections per pancreas were analyzed (group mean ± SEM, $n = 8$). *Significant differences from the control group, $p < 0.05$.

apoptotic cells in pancreatic islets at grade 0, although not significantly different from control (Fig. 3). Phthalate exposure alone did not affect the number of apoptotic cells in pancreatic islets at any time point, nor was the number of regulatory T-cells and CD68+ macrophages affected by any of the environmental chemical exposures (data not shown). The apoptotic cells observed in the islets after BPA exposure were identified in an earlier study to be alpha cells, beta cells and tissue resident macrophages [34,35].

3.3. Phagocytic function of peritoneal macrophages

BPA exposure alone decreased the relative number of phagocytic macrophages with about 30% compared to control levels at 7 weeks of age (Fig. 4), suggesting impaired macrophage function. In contrast, exposure to phthalates alone or in combination with BPA did not affect the phagocytic function in peritoneal macrophages significantly (Fig. 4). There was a similar trend, however not statistically significant at 11 weeks of age (Fig. 4).

3.4. Insulin secretion and NO production in pancreatic islets

There was no difference between the exposure groups in the glucose induced insulin secretion or in the NO production in isolated pancreatic islets (data not shown).

3.5. Apoptosis sensitivity in isolated beta cells and macrophages

The percentage of apoptotic beta cells after *in vitro* cytokine stimulation was lower in mice from the phthalate exposure group at 8 weeks of age compared to the control

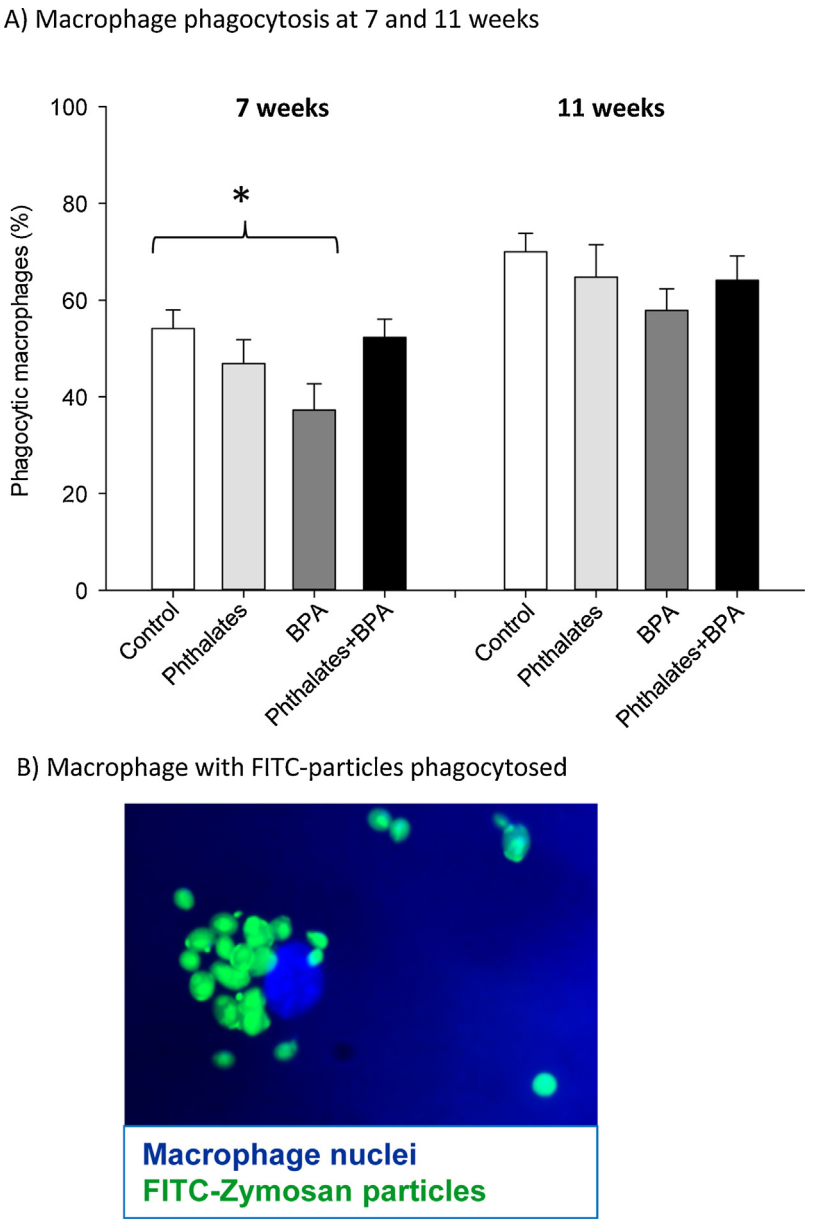

Fig. 4. Peritoneal macrophage phagocytosis of FITC-conjugated Zymosan beads at 7 and 11 weeks of age in female NOD offspring after continuous BPA and phthalate exposure, % phagocytic macrophages determined by flow cytometry (group mean ± SEM, $n = 8$). *Significant differences from the control group, $p < 0.05$. Panel B shows FITC-conjugated Zymosan particles (green) and macrophage nuclei (blue) in a cytospin sample after phagocytosis analysis.

group, suggesting a decreased sensitivity to cytokine-induced apoptosis in these cells (Fig. 5A). A similar but non-significant trend was observed for the combined BPA and phthalate exposures. For macrophages, the percentage of apoptotic cells was increased in the BPA exposure group compared to the control, suggesting increased sensitivity to cytokine-induced apoptosis in macrophages (Fig. 5B).

3.6. *Cytokine release from splenocytes, pancreatic lymph node cells and pancreatic islets*

Possible systemic immunomodulating effects of BPA and phthalate exposure in NOD mice were examined by *ex vivo* stimulation of splenocytes and pancreatic lymph node cells with LPS (activating B-cells and macrophages) and ConA (activating T-cells). ConA stimulation of splenocytes *ex vivo* resulted in increased release of IL-6 in the phthalate exposure group, and reduced release of IL-10, IL-4 and TNFα in both the BPA and the combined BPA and phthalate exposure groups compared to control (Fig. 6A). Exposure to BPA alone or in combination with phthalates generally decreased the LPS-induced *in vitro* secretion of TNFα, IL-6, IL-10 and IFNγ in splenocytes, with the largest effects for the combined exposure group (Fig. 6B). Levels of IL-4 were below the detection level after LPS stimulation in splenocytes.

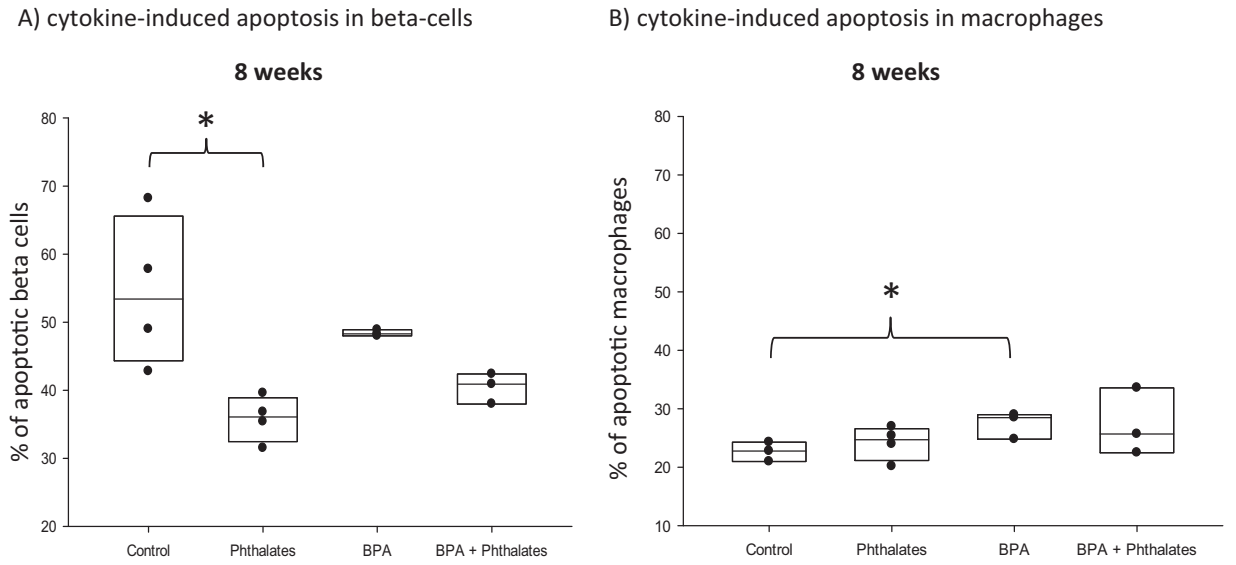

Fig. 5. Sensitivity of beta-cells and macrophages from pancreatic islets to *ex vivo* cytokine-induced apoptosis, after continuous BPA and phthalate exposure, isolated from 8 week old offspring. Apoptotic pancreatic beta cells (A) in % of total beta cells after 48 h cytokine induced apoptosis in beta cells isolated from 8 weeks old female NOD mice exposed to BPA and phthalates in drinking water ($n = 4$). Panel B shows % apoptotic macrophages out of total macrophage numbers in pancreatic islets isolated from exposed NOD mice at 8 weeks of age ($n = 4$).*Significant differences from the control group, $p < 0.05$.

Ex *vivo* LPS stimulated pancreatic lymph node cells also showed a non-significant trend for decreased levels of TNFα and IL-6 secretion in the BPA and combined BPA and phthalate exposure group (Fig. 6A), whereas IL-4 and IFNγ levels were below the detection limit. Overall, exposure to BPA alone or in combination with phthalates induced systemic effects in the NOD mice in terms of altered functionality of splenocytes and pancreatic lymph node cells. There were no significant differences in LPS-induced cytokine secretion from isolated pancreatic islets, except for a non-significant trend of decreased LPS-induced IL-10 secretion after combined BPA and phthalate exposure (Fig. 6D).

4. Discussion

This study confirms our previous findings that exposure to BPA through the drinking water increases diabetes development in NOD mice [34–36]. In contrast, phthalate exposure or mixed exposure to BPA and phthalates, did not affect diabetes development. The combined BPA and phthalate exposure did, however, induce systemic alterations in the immune system, seen as altered cytokine responses in splenocytes and pancreatic lymphocytes. Phthalate exposure alone only leads to increased IL-6 secretion after *ex vivo* T-cell stimulation of splenocytes and seemed to have a protective effect against *ex vivo*-induced apoptosis in beta-cells.

BPA exposure alone increased the number of diabetic mice at 25 weeks of age, whereas phthalate or the combined BPA and phthalate exposure did not affect the diabetes incidence. We even observed an apparent counteracting effect of phthalates on the BPA-induced increase in diabetes, *i.e.* a lack of effect of the mixture of BPA and phthalates on the diabetes incidence. This could be due to a non-monotonic dose response effect, since BPA and each phthalate was given at a 3 × tolerable daily intake level (TDI), and higher doses of estrogenic compounds have been reported to increase the insulin release in beta-cells and delay the spontaneous diabetes development in NOD mice [45–48]. In line with this, we previously showed that a higher exposure of BPA (100 mg/l in drinking water reduced the development of diabetes in NOD mice [36]).

Another explanation of the apparent opposing effect of phthalates could be linked to the observed increase in IL-6 secretion from immune cells in the phthalate exposure group. Since it has been shown that IL-6 enhances glucose induced insulin secretion in pancreatic beta-cells [49,50], this could contribute to a delay of the T1DM development by increasing serum insulin levels. In mice from the phthalate-exposed groups (with or without BPA) the sensitivity to cytokine induced apoptosis was reduced in isolated beta cells. Interestingly, IL-6 has also been reported to protect beta-cells from cytokine-induced apoptosis [49–51]. Accordingly, the reduced sensitivity with regard to beta-cell apoptosis, possibly through elevated IL-6 levels, may be another explanation of how the phthalates can counteract the BPA-induced diabetes development in NOD mice.

In our previous studies, the largest differences between the exposure groups in the insulitis development were observed at 11 weeks of age. However, in the present study, the tendency towards a higher insulitis grade per mouse and exposure group for BPA and combined BPA and phthalate exposure was not significantly different compared to the control group. In retrospect, however, 11 weeks of age might be before the time-point exhibiting the largest differences in insulitis development, since the mice in the present study developed diabetes later in life compared to our previous studies using the same strain and housing

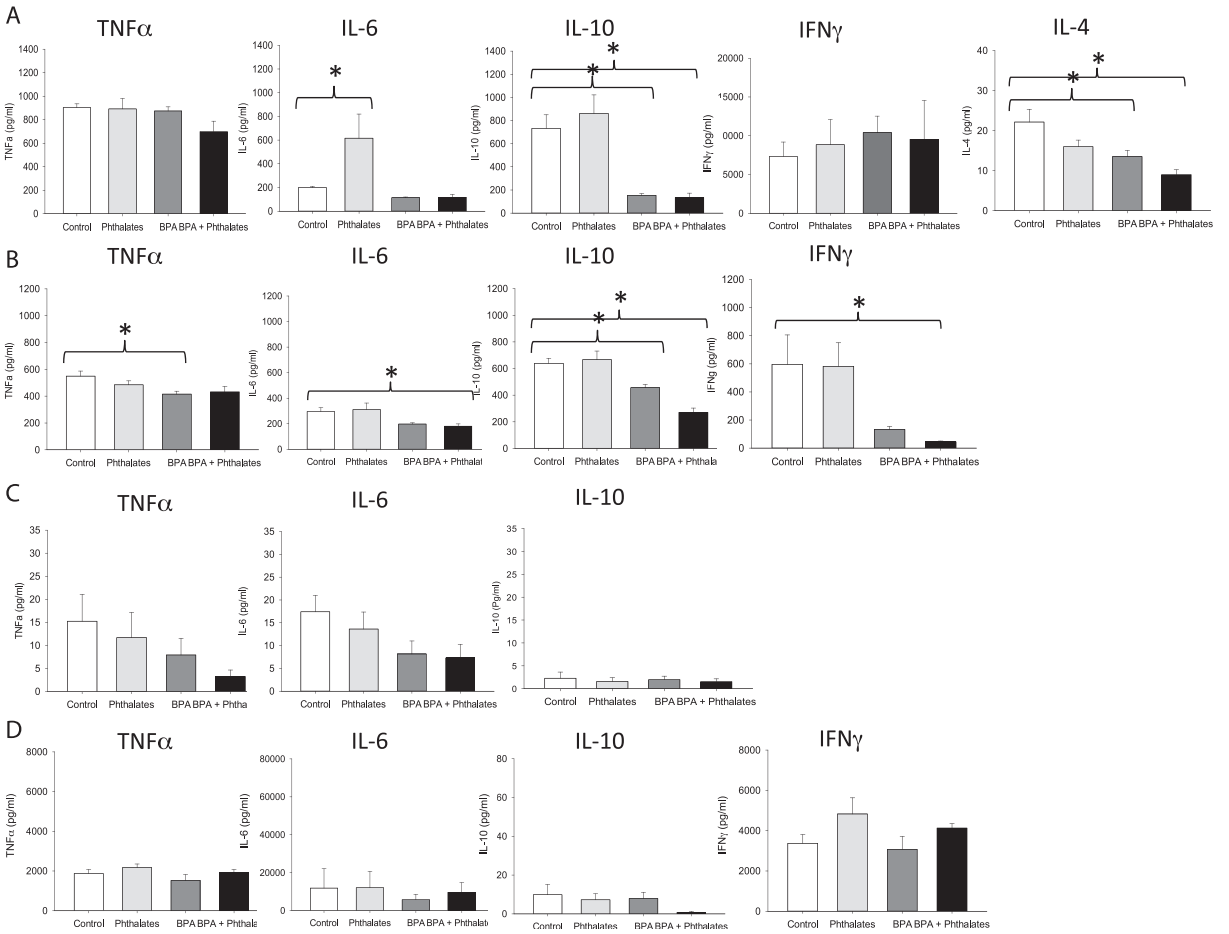

Fig. 6. TNFα, IL-6, IL-10, IFNγ and IL-4 cytokine release in cells isolated from 11 weeks old NOD offspring continuously exposed to BPA and phthalates (group mean ± SEM, $n = 8$). ConA-induced (A) and LPS-induced (B) cytokine release from splenocytes. (C) LPS-induced cytokine release from pancreatic draining lymph node cells. (D) LPS-induced cytokine release from pancreatic islets. All cytokines were measured for all cell types, but are not shown when the cytokine levels in general were below detection limits. *Significant differences from the control group, $p < 0.05$.

conditions [34–36]. Stress and infections are known to delay diabetes development in NOD mice [52–54]. However, a thorough pathological examination of 5 NOD mice in the population gave no indications of infection.

Fewer tissue resident macrophages and/or impaired macrophage function can potentially result in a decreased clearance of apoptotic cells and a more severe insulitis grade [55]. We observed an increased number of apoptotic cells in pancreatic islets prior to insulitis both for BPA and combined BPA and phthalates exposure, but not phthalate exposure alone, suggesting a possible direct toxic effect or an effect on the macrophage clearance function by BPA exposure. With regard to direct toxic effects, the BPA-induced increase in pancreatic apoptotic cells seemed to be evident in all populations analyzed, including beta cells, alpha cells and tissue specific macrophages [34,35]. However, several observations in the present study support the notion that BPA affects macrophage numbers and functionality. Firstly, the analysis of phagocytosis revealed that BPA exposure decreased the phagocytic function in peritoneal macrophages in NOD mice. Secondly, a reduced LPS-induced cytokine release from splenocytes, lymph node cells and possibly also pancreatic islets, suggested impaired release of inflammatory cytokines from macrophages in general after exposure to BPA alone or in combination with phthalates. Both these observations are in line with a previously reported lower peritoneal macrophage function due to BPA exposure, seen as decreased NO and TNFα production in BPA exposed murine peritoneal macrophages [56]. Lastly, BPA exposure increased the sensitivity of pancreatic macrophages to cytokine induced apoptosis and reduced the number of tissue resident macrophages in pancreatic islets. Thus, one may hypothesize that this increased apoptosis sensitivity could contribute to the reduced pancreatic macrophage numbers prior to insulitis. Why some of these endpoint effects appeared to be opposed by phthalates (number of tissue-resident macrophages, macrophage phagocytosis) while others were not (increased apoptosis sensitivity, LPS-induced cytokine secretion) cannot be determined from the present study. It is possible, however, that the observed phthalate induced effects (increased IL-6 from

lymphocytes), or other phthalate induced effects on the immune system that were not assessed presently, could affect the above endpoints differently and thereby explain why only some BPA-induced effects were counteracted by phthalates.

Presently, the combined BPA and phthalate exposure decreased LPS-induced IL-6, IL-10 and IFNγ secretion and ConA-induced IL-10 and TNFα secretion in splenocytes *in vitro*, while BPA exposure alone decreased the LPS-induced IL-10 and TNFα secretion. The anti-inflammatory cytokine IL-10 is necessary for maintenance of Foxp3 expression in regulatory T-cells [57], which are involved in the suppression of cytotoxic T-cell proliferation. Further, IL-10 acts as a potent immunosuppressant in itself, by reducing pro-inflammatory cytokine release in activated monocytes, polymorphonuclear leukocytes and eosinophils [58]. Thus, it can be speculated that the increased insulitis and diabetes development induced by BPA exposure in part could be related to reduced IL-10 production with subsequent reduced clearance of apoptotic cells and an increase in the peripheral pool of cytotoxic T-cells in the pancreas. This is in line with a study by Khanna and co-authors [59], reporting increased inflammation and reduced clearance of apoptotic cells in wounds of diabetic mice, possibly caused by a reduced level of the anti-inflammatory cytokine IL-10 secreted from wound-related macrophages.

Moreover, since lower levels of TNFα secreted by splenocytes and lymphocytes were observed after a mixed exposure to BPA and phthalates, this could reflect a reduced inflammation process and thereby potentially contribute to reduce the BPA-induced acceleration in diabetes development in NOD mice. The local insulitis in the pancreas was not decreased after the combined exposure, suggesting that systemic effects like cytokine and hormone levels were more essential than insulitis grade for the overall diabetes development in these mice.

In conclusion, BPA, but not phthalate exposure, increase the diabetes development in NOD mice, probably due to reduced macrophage number and function. The combination of BPA and phthalates resulted in systemic alterations in cytokine secretion from immune cells, but did not affect the overall diabetes development. The apparent counteracting effect of phthalates on the BPA-induced T1D development could be due to non-monotonic effects of endocrine disruptors in the NOD mouse model, where high doses of EDs may decrease the diabetes development, and/or altered cytokine levels affecting inflammation or beta-cell survival and insulin secretion. There are so far no epidemiological studies investigating associations between T1DM development and exposure to BPA and/or phthalates. Such studies are necessary to document the human relevance of these environmental chemicals as triggers for development of T1DM.

Conflict of interest

The authors have no conflict of interests. All applicable international and national institutional guidelines for the care and use of animals were followed.

Transparency document

The Transparency document associated with this article can be found in the online version.

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